UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

DETERMINAÇÃO DE ESTIMULANTES ANFETAMÍNICOS NO FLUIDO ORAL: ESPECIFICIDADE DOS MÉTODOS DE TRIAGEM E ANÁLISE CONFIRMATÓRIA

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PORTO ALEGRE, 2010

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Dissertação apresentada por **Daniele Zago Souza** para obtenção do GRAU DE MESTRE em Ciências Farmacêuticas.

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RESUMO

O Brasil destaca-se no cenário mundial em relação ao consumo de estimulantes anfetamínicos (ATS), e estudos nacionais têm evidenciado grande prevalência na utilização destas substâncias por motoristas profissionais. O fluido oral apresenta uma série de vantagens sobre as matrizes tradicionais para a monitorização do consumo de ATS no trânsito, e vem sendo empregada em diversos países. Este trabalho objetivou avaliar a especificidade de imunoensaios comerciais na detecção preliminar dos ATS comercializados no Brasil em amostras de fluído oral e desenvolver e validar um método para a confirmação e quantificação de anfetamina (AMP), metanfetamina (MET), anfepramona (DIE), fenproporex (FEN) e metilfenidato (MPH) no fluído oral por microextração em fase sólida (SPME) e cromatografia a gás com detector de massas (CG/EM). A especificidade analítica dos imunoensaios foi avaliada por meio do estudo das informações técnicas de diversos produtos e da realização de ensaios experimentais com três testes. O método confirmatório por SPME-CG/EM foi desenvolvido a partir da técnica de imersão (DI-SPME), sob agitação magnética, à temperatura ambiente, utilizando fibras revestida por polidimetilsiloxano (30 µm), propilcloroformato como agente derivatizante e adição de Na_2CO_3 e de Na_2SO_4 para aumentar o pH e a força iônica do meio, respectivamente. Os testes imunológicos atualmente disponíveis para a triagem de ATS em fluido oral são importados e não detectam, mesmo em altas concentrações, os principais ATS consumidos no Brasil: FEN, DIE e MPH. O método por SPME-CG/EM foi linear para os ATS estudados no intervalo de 2-256 $ng.mL^{-1}$, exceto para o FEN cujo intervalo foi de 4-256 $ng.mL^{-1}$. Os limites de detecção foram 0,5 $ng.mL^{-1}$ (MET), 1 $ng.mL^{-1}$ (MPH) e 2 $ng.mL^{-1}$ (DIE, AMP, FEN). A exatidão do método situou-se entre 98,2 – 111,9% e a precisão não excedeu 15% de desvio padrão relativo. O método foi aplicado com sucesso na estimação do perfil farmacocinético do FEN e da AMP no fluído oral de seis indivíduos do sexo masculino, após a administração de dose única de especialidade farmacêutica nacional contendo 25 mg de cloridrato de FEN.

Palavras-chave: anfetamina, metanfetamina, anfepramona, femproporex, metilfenidato, fluido oral.

ABSTRACT

The Brazil stands out on the world as a major consumer of amphetamine-type stimulants (ATS), and several national studies have shown high prevalence in the consumption of these substances by professional drivers. Oral fluid has many advantages over the conventional biological fluids for monitoring ATS use on roads, and has been employed with this purpose in several countries. The aim of this study is to assess the specificity/cross-reactivity of commercial oral fluid immunoassays in detecting the prescription ATS marketed in Brazil and to develop and validate a method for confirmation and quantification of amphetamine (AMP), methamphetamine (MET), amfepramone (DIE), fenproporex (FEN) and methylphenidate (MPH) in oral fluid by solid phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). Analytical specificity of immunoassays was evaluated through the study of the technical information of commercial products and through experimental testing of three kits. The confirmatory SPME-GC-MS method employed SPME immersion technique (DI-SPME), under magnetic stirring, at room temperature, using polydimethylsiloxane (30 µm) fibers, in-matrix propylchloroformate derivatization, Na_2CO_3 and Na_2SO_4 to increase both pH and ionic strength. Immunological tests currently available for ATS screening in oral fluid are imported and do not detect, even at high concentrations, the main ATS consumed in Brazil: FEN, MPH and DIE. The SPME-GC-MS method was linear for the studied ATS over the range of 2-256 $ng.mL^{-1}$, except for FEN where the linear range was 4-256 $ng.mL^{-1}$. The detection limits were 0.5 $ng.mL^{-1}$ (MET), 1 $ng.mL^{-1}$ (MPH) and 2 $ng.mL^{-1}$ (DIE, AMP, FEN). Accuracy was within 98.2 – 111.9% of the target concentrations and precision did not exceed 15% of relative standard deviation. The method was successfully applied to estimate the pharmacokinetic profile of FEN and AMP in oral fluid of six male subjects after administration of a single dose of 25 mg FEN hydrochloride.

Keywords: amphetamine, methamphetamine, diethylpropion, fenproporex, methylphenidate, oral fluid.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ALCA	Alquilcarbamatos
ALCL	Alquilcloroformatos
AMP	Anfetamina (da nomenclatura adotada nos Estados Unidos da América, *Amphetamine*)
ATS	Estimulantes anfetamínicos (do inglês, Amphetamine-type stimulants)
BUCL	Butilcloroformato
CG	Cromatografia a gás
CG/EM	Cromatografia a gás com detector de massas
DIE	Anfepramona (da nomenclatura adotada nos Estados Unidos da América, *Diethylpropion*)
DI-SPME	Microextração em fase sólida com imersão da fibra na amostra (do inglês, *Direct Immersion-Solid Phase Microextraction*)
ETCL	Etilcloroformato
FEN	Femproporex (da nomenclatura adotada nos Estados Unidos da América, *Fenproporex*)
HS-SPME	Microextração em fase sólida com exposição da fibra à fase gasosa sobre a amostra (do inglês, *Headspace-Solid Phase Microextraction*)
MET	Metanfetamina (da nomenclatura adotada nos Estados Unidos da América, *Methamphetamine*)
MPH	Metilfenidato (da nomenclatura adotada nos Estados Unidos da América, *Methylphenidate*)
OF	Fluido oral (do inglês, *Oral Fluid*)
PDMS	Polidimetilsiloxano
PHCL	Fenilcloroformato (do inglês, *Phenylchloroformate*)
PRCL	Propilcloroformato
SNC	Sistema Nervoso Central
SPME	Microextração em fase sólida (do inglês, *Solid Phase Microextraction)*

SUMÁRIO

1 INTRODUÇÃO

Apesar do mercado de drogas ilícitas como opióides e cocaína ter experimentado certa estabilização e até declínio nos últimos anos, o problema do abuso de estimulantes anfetamínicos (ATS) continua em expansão (UNODC, 2010). Mundialmente, o consumo de ATS só é superado pelo de *Cannabis* (UNODC, 2010), sendo que no último ano entre 13,7 e 52,9 milhões de pessoas entre 15 e 64 anos fizeram o uso de ATS. Desde 1999, as apreensões globais de ATS têm aumentado, acompanhadas da diversificação dos países, locais e técnicas de produção ilícita (UNODC, 2010). A síntese ilegal destes estimulantes já foi relatada em mais de 60 países, dentre eles a Argentina e o Brasil (UNODC, 2010).

Cerca de 92% do volume de ATS apreendidos no mundo correspondem a derivados não-metilenodioxi, sendo a anfetamina (AMP) e a metanfetamina (MET) as substâncias mais prevalentes (UNODC, 2010). Apesar de existirem poucos registros de apreensões de AMP e MET pela Polícia Federal brasileira, tal número vem aumentando desde 2007, o que pode refletir uma nova tendência no consumo nacional (BRASIL, 2010a).

Recentemente a *International Narcotics Control Board* (INCB, 2010) destacou o crescimento no abuso de medicamentos à base de ATS verificado nos últimos anos, de modo que em alguns países a utilização indevida de especialidades farmacêuticas psicoativas só é excedida pelo consumo de *Cannabis*. Em relação ao continente americano, o consumo per capita de anorexígenos supera em mais de três vezes o consumo verificado em outras regiões do mundo (INCB, 2010) e até 2007, o Brasil figurava, junto com a Argentina e os Estados Unidos, entre os países com maior taxa de utilização destes medicamentos (INCB, 2009). A anfepramona (DIE) e o femproporex (FEN) estão entre os três supressores de apetite anfetamínicos mais utilizados mundialmente (INCB, 2010), e o metilfenidato (MPH) constitui o psicoestimulante mais empregado no tratamento da narcolepsia e do transtorno do déficit de atenção e hiperatividade (INCB, 2010). Só entre os anos de 2004-2008, o consumo global de MPH cresceu 80% (INCB, 2010).

No Brasil, estes fármacos são amplamente utilizados (CARNEIRO; GUERRA JÚNIOR; ACURCIO, 2008; KOROLKOVAS; FRANÇA, 2009; NOTO et al., 2002;

PASTURA; MATTOS, 2004), havendo diversas especialidades farmacêuticas registradas no país à base de DIE, três contendo MPH e uma contendo FEN (BRASIL, 2010b). Tais substâncias também são comercializadas sob a forma de preparações magistrais, por vezes em dosagens muito elevadas e em associações medicamentosas proibidas (CARNEIRO; GUERRA JÚNIOR; ACURCIO, 2008; NOTO et al., 2002).

Devido às importantes alterações comportamentais e psicomotoras provocadas no organismo pelos ATS (LEYTON et al., 2002; PONCE; LEYTON, 2008; SILBER et al., 2005a), a utilização destes compostos por motoristas e suas conseqüências no trânsito têm sido objeto de constante preocupação. Diversos trabalhos têm mostrado alta prevalência no consumo de ATS por motoristas envolvidos em acidentes de trânsito (DRUMMER et al., 2003; HOLMGREN; HOLMGREN; AHLNER, 2005; LISA; LYNDAL; ELIAS, 2009), estando a culpabilidade destes condutores positivamente associada ao consumo destes estimulantes (DRUMMER et al., 2004).

O Código de Trânsito Brasileiro (BRASIL, 1997), recentemente alterado pela Lei nº 11.705 (BRASIL, 2008), determina que "conduzir veículo automotor, na via pública, estando com concentração de álcool por litro de sangue igual ou superior a 6 (seis) decigramas, ou sob a influência de qualquer outra substância psicoativa que determine dependência" constitui crime sujeito a pena de detenção. Apesar disso, diversos estudos nacionais têm evidenciado grande prevalência do consumo de substâncias psicoativas no trânsito, notadamente de ATS, os quais são utilizados por motoristas profissionais para aumentar o estado de alerta físico e mental durante as longas jornadas de trabalho. Um estudo realizado em 2002, com 318 motoristas de caminhão do Estado do Paraná, revelou que 96,88 % deles já fizeram uso de medicamentos anorexígenos para aumentar o tempo de vigília, sendo os produtos mais utilizados aqueles contendo FEN e DIE (WENDLER; BUSATO; MIYOSHI, 2003). Em 2005, Souza e colaboradores entrevistaram 260 motoristas de caminhão do Estado do Mato Grosso do Sul e identificaram uma prevalência de 11,1% no consumo de FEN e DIE. Em estudo posterior, realizado por Nascimento e colaboradores (2007) com 91 caminhoneiros do Estado de Minas Gerais, evidenciou que 66% dos condutores utilizavam ATS durante os percursos de viagem.

A introdução de critérios mais rígidos para a prescrição e comercialização de medicamentos anorexígenos no Brasil (BRASIL, 2007) parece não ter contido o abuso destes fármacos por motoristas profissionais. Em estudo recente conduzido por Moreira e Ganadi (2009) no Estado do Mato Grosso do Sul, 65% dos caminhoneiros relataram fazer uso medicamentos contendo FEN ou DIE, adquiridos, em sua maioria, em postos de gasolina (62%), além de restaurantes e nas próprias empresas transportadoras.

Em relação ao consumo de MPH por motoristas, apesar de subestimados, os resultados preliminares obtidos por Pechansky, Duarte e De Boni (2010) mostram que do total de amostras de fluido oral (OF) positivas para outras substâncias psicoativas que não o etanol, 3,4% apresentavam MPH. Considerando que a narcolepsia é um distúrbio raro e que o transtorno do déficit de atenção e hiperatividade tende a ser diagnosticado e tratado na fase infantil (LONGO et al., 2000; MELO, 2009; PASTURA; MATTOS, 2004), a presença de MPH em motoristas pode indicar o emprego não terapêutico desta substância.

A utilização de OF como matriz biológica para a monitorização do consumo de substâncias psicoativas no trânsito apresenta uma série de vantagens sobre as matrizes tradicionais (urina e sangue), e vem sendo empregada em diversos países do mundo (BERNHOFT et al., 2005; DRUMMER et al., 2007; SAMYN; DE BOECK; VERSTRAETE, 2002; TOENNES et al., 2005; WYLIE et al., 2005). Entre as vantagens do OF, destaca-se a coleta fácil e não invasiva, a qual pode ser realizada dentro do veículo do condutor e sob vigilância da autoridade de trânsito, e o fato de fornecer informação sobre o consumo recente de substâncias (CONE; HUESTIS, 2007).

Entre os testes de triagem empregados na detecção preliminar de ATS no fluido oral, destacam-se os imunoensaios, devido à praticidade, baixo custo e elevada sensibilidade (LIMBERGER et al., 2010). Os testes disponíveis no mercado, no entanto, possuem anticorpos direcionados a determinados ATS alvo e podem não apresentar reatividade cruzada com outros ATS de interesse (ALFA SCIENTIFIC DESIGNS, 2009; IMMUNALYSIS CORPORATION, 2005a, 2005b; SECURETEC, 2010).

Face ao exposto, este trabalho objetivou avaliar a especificidade de testes imunológicos comerciais na detecção preliminar de AMP, MET, FEN, DIE e MPH em amostras de OF de motoristas brasileiros, bem como desenvolver e validar um método analítico, por cromatografia a gás, para a confirmação da presença destes estimulantes nas amostras de OF. Estes estudos integram o projeto “Estudo do impacto do uso de bebidas alcoólicas e outras substâncias psicoativas no trânsito brasileiro” (PECHANSKY; DE BONI, 2007), já parcialmente publicado (PECHANSKY; DUARTE; DE BONI, 2010), o qual visa avaliar o consumo de substâncias psicoativas por motoristas profissionais e privados do Brasil, através da coleta de amostras de fluído oral.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a especificidade de ensaios imunológicos para a detecção preliminar de estimulantes anfetamínicos (ATS) em fluido oral (OF) e desenvolver e validar um método para a confirmação e quantificação de anfetamina (AMP), metanfetamina (MET), anfepramona (DIE), fenproporex (FEN) e metilfenidato (MPH) no OF por microextração em fase sólida (SPME) e cromatografia a gás com detector de massas (CG/EM).

2.2 Objetivos específicos

- Testar a especificidade de imunoensaios comerciais na detecção preliminar dos ATS comercializados no Brasil em amostras de OF;
- Determinar o pré-tratamento ideal para as amostras de OF submetidas ao método confirmatório por SPME-CG/EM, incluindo a modificação do pH e da força iônica do meio, além da derivatização dos ATS com diferentes alquilcloroformatos;
- Estabelecer as condições de sorção e dessorção mais adequadas para a extração e pré-concentração dos ATS por SPME, visando à maior sensibilidade no menor tempo de análise;
- Avaliar a recuperação dos ATS pelo dispositivo coletor de OF;
- Determinar a estabilidade dos ATS no tampão de transporte e armazenamento do dispositivo coletor de OF;
- Estudar a influência de possíveis substâncias interferentes na identificação e quantificação de AMP, MET, FEN, DIE e MPH em OF pelo método proposto;
- Aplicar o método confirmatório validado para estimar o perfil farmacocinético do FEN e da AMP no OF de indivíduos do sexo masculino, após a administração de dose única de especialidade farmacêutica contendo FEN, comercializada no mercado nacional.

3 REVISÃO DA LITERATURA

3.1 Estimulantes anfetamínicos

Os estimulantes anfetamínicos (ATS) constituem um grupo de substâncias, a maioria de origem sintética, com potente ação central e periférica, cujo protótipo é a AMP (FENTON, 2002; LA TORRE et al., 2004). Quimicamente, apresentam o esqueleto básico da β-fenetilamina (figura 1) e, farmacologicamente, atuam como aminas simpatomiméticas de ação agonista indireta, estimulando a liberação de dopamina e noradrenalina nas fendas sinápticas e impedindo sua recaptação neuronal (CHASIN e SILVA, 2003; LA TORRE et al., 2004; RANG et al., 2007; UNODC, 2006). Além disso, os ATS são inibidores da Monoamina Oxidase (MAO), enzima responsável pela oxidação das catecolaminas (RANG et al., 2007). Os ATS também aumentam os níveis pós-sinápticos de serotonina (SILBER et al., 2005a), apesar de em menor extensão quando comparada à dopamina e noradrenalina (GARCIA-MIJARES; BERNARDES; SILVA, 2009). Estudos mais recentes sugerem que os ATS também possam atuar como agonistas diretos, em receptores situados nos vasos sanguíneos (BROADLEY, 2010).

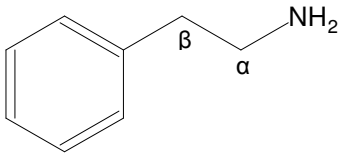


Figura 1: β-Fenetilamina.

Os principais efeitos dos ATS no organismo são redução do sono e do apetite, euforia, diminuição da fadiga, estimulação locomotora, midríase, taquicardia e elevação da pressão arterial (MABBOTT; HARTLEY, 1999; MOREIRA; GANADI, 2009; RANG et al., 2007). Além disso, os ATS diminuem a habilidade dos motoristas em focar a atenção durante múltiplas tarefas e aumentam o comportamento de risco (COUPER et al., 2002). A midríase prejudica a atividade de condução e, à noite, os faróis dos veículos em sentido contrário podem ofuscar a visão do motorista (LEYTON et al., 2002). Após os efeitos excitatórios, em geral advém depressão psicomotora e sonolência (LA TORRE et al., 2004), fase esta responsável por grande parte dos acidentes de trânsito envolvendo caminhoneiros que dormem ao volante (WILLIANSON, 2007; LISA; LYNDAL; ELIAS, 2009).

Quando utilizados em altas doses ou por longos períodos (uso crônico), os ATS podem causar agitação, irritabilidade, tremores, hipertensão, hipertermia, perda da memória, comportamentos agressivos, paranóia, pânico, surtos psicóticos e alucinações (KRASNOVA; CADET, 2009; MABBOTT; HARTLEY, 1999; UNODC, 2006). Os ATS apresentam elevado potencial abusivo, propiciando o desenvolvimento de tolerância e dependência (LEYTON et al., 2002; RANG et al. 2007; USP, 2005).

A farmacocinética dos ATS em geral se assemelha bastante, apresentando rápida absorção e boa biodisponibilidade oral, pico plasmático em cerca de 1-4 horas, baixa taxa de ligação a proteínas plasmáticas (menor que 20%) e grande volume de distribuição (LA TORRE et al., 2004). A biotransformação ocorre principalmente no fígado, sendo que muitos ATS podem ser convertidos a AMP e/ou MET nesta etapa (CONE; HUESTIS, 2007; KRAEMER; MAURER, 2002).

A maioria dos ATS possui ao menos um carbono quiral, podendo ser encontrados como misturas racêmicas ou enatiômeros puros (UNODC, 2006; LA TORRE et al., 2004).

3.1.1 Anfetamina e Metanfetamina

Em países como os Estados Unidos da América, Canadá e Chile, a anfetamina (AMP) e a metanfetamina (MET) (figuras 2 e 3) estão disponíveis na forma de medicamentos psicoestimulantes indicados no tratamento da narcolepsia e do transtorno do déficit de atenção e hiperatividade (FDA, 2010; HEALTH CANADA, 2010; ISPC, 2010). Atualmente não são comercializadas no Brasil especialidades farmacêuticas contendo AMP ou MET (BRASIL, 2010b) e, portanto, o uso abusivo destas substâncias no país provém fundamentalmente de fontes ilícitas.

A MET e a AMP são fortes estimulantes do Sistema Nervoso Central (SNC), sendo o primeiro ATS mais ativo que o segundo a nível central (SILBER et al., 2005a). Os isômeros dextrógiros da AMP e da MET possuem atividade psicoestimulante 5 vezes maior que os respectivos levógiros (KRAEMER; MAURER, 2002). Apesar dos estereoisômeros serem absorvidos de forma semelhante, *d*-AMP e *d*-MET são metabolizados mais rapidamente do que *l*-AMP e *l*-MET, devido à estereoseletividade enzimática (MUSSHOFF, 2000; KRAEMER; MAURER, 2002).

Cerca de 20-46% de uma dose de AMP é eliminada na urina na forma inalterada, podendo variar entre 1-5% e 54-74% se a urina estiver alcalina ou ácida, respectivamente (CODY; VALTIER; NELSON, 2003; CONE; HUESTIS, 2007; UNIDCP, 1995; VERSTRAETE, 2004). O restante é metabolizado a *p*-hidroxi-anfetamina (2-4%), norefedrina (2%), *p*-hidroxi-norefedrina (< 0,5%), fenilacetona (< 1%), ácido benzóico (4%) e ácido hipúrico (16-28%), sendo os metabólitos hidroxilados em parte conjugados antes da eliminação (UNIDCP, 1995; CONE; HUESTIS, 2007; MUSSHOFF, 2000; JENKINS; CONE, 1998).

A MET é eliminada na urina 43-44% como fármaco inalterado (variando de 15 a 55% de acordo com o pH urinário), 10-15% como *p*-hidroximetanfetamina e cerca de 4-20% como AMP, a qual é subseqüentemente metabolizada (JIROVSKÝ, 1998; LA TORRE et al., 2004; UNIDCP, 1995).

Através da biotransformação hepática, fármacos disponíveis no Brasil como a selegilina e o FEM, ou comercializados em outros países como a MET, o clobenzorex e a benzofetamina originam AMP no organismo (CONE; HUESTIS, 2007). Destes, a benzofetamina e a selegilina são primeiramente metabolizadas a MET (KRAEMER; MAURER, 2002).

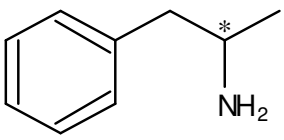


Figura 2: Anfetamina (*centro quiral).

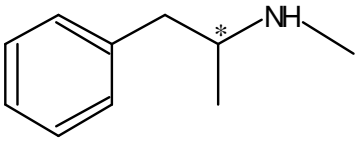


Figura 3: Metanfetamina (*centro quiral).

3.1.2 Femproporex e Anfepramona

O fenproporex (FEN) e a anfepramona (DIE) (figuras 4 e 5) são fármacos empregados no Brasil como anorexígenos e são controlados pela Portaria nº 344/98 da Secretaria de Vigilância Sanitária (BRASIL, 1999). A dispensação em farmácias e drogarias de medicamentos contendo tais substâncias está condicionada à apresentação, e retenção pelo estabelecimento farmacêutico, de “Notificação de Receita B2”, documento preenchido pelo médico que autoriza o paciente a adquirir produtos contendo FEN ou DIE no país (BRASIL, 1999, 2007).

Apenas 5-9% de uma dose de FEN é eliminada inalterada na urina ácida, e 27-56% biotransformada à AMP (CODY; VALTIER,1996; MUSSHOFF, 2000). Além da AMP, já foram descritos outros 13 metabólitos urinários do FEN (KRAEMER et al., 2000).

A DIE é rápida e extensamente metabolizada a 2-etilamino-1-fenil-propan-1-ona, 2-amino-1-fenil-propan-1-ona, N,N-dietilnorefedrina, N-etilnorefedrina e norefedrina, os quais são excretados quase que exclusivamente pela via renal (TESTA; BECKETT, 1972; YU et al., 2000). De acordo com os estudos realizados por Yu e colaboradores (2000), a 2-etilamino-1-fenil-propan-1-ona pode ser a responsável pelos efeitos da DIE no organismo.

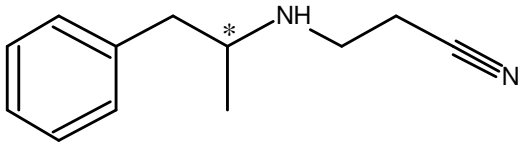


Figura 4: Femproporex (*centro quiral).

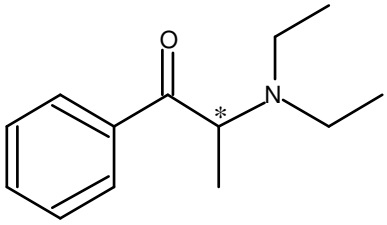


Figura 5: Anfepramona (*centro quiral).

3.1.3 Metilfenidato

O MPH (figura 6) é um fraco estimulante do SNC, com efeitos mais evidentes sobre as atividades mentais do que motoras (MELO, 2009). Seu mecanismo de ação no homem ainda não foi completamente elucidado, mas acredita-se que o seu efeito estimulante seja devido a uma inibição da recaptação de dopamina sem, no entanto, disparar a liberação deste neurotransmissor (MELO, 2009).

O MPH é largamente empregado no tratamento da narcolepsia e do transtorno do déficit de atenção e hiperatividade (INCB, 2010; MARCHEI et al., 2010; PASTURA; MATTOS, 2004). No Brasil, sua comercialização está controlada pela Portaria nº 344/98 da Secretaria de Vigilância Sanitária, e ocorre mediante a apresentação e retenção de “Notificação de Receita A” (BRASIL, 1999).

O MPH possui extenso metabolismo de primeira passagem no fígado, sendo sua biodisponibilidade sistêmica em geral menor que 30% (MELO, 2009). O MPH é

eliminado inalterado na urina apenas em pequenas quantidades (<1%), sendo a maior parte da dose (60-86%) excretada como ácido ritalínico (ácido alfa-fenil-2-piperidino acético), o qual corresponde ao MPH desesterificado (MELO, 2009; KOROLKOVAS; FRANÇA, 2009; MARCHEI et al., 2010).

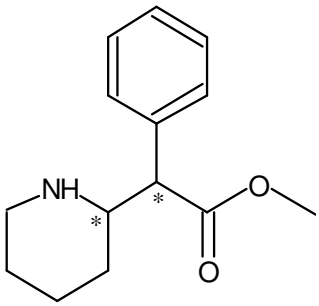


Figura 6: Metilfenidato (*centros quirais).

3.2 Fluido oral

3.2.1 Produção e composição

O fluido oral (OF) é constituído principalmente por saliva, misturada ao fluido crevicular gengival (exsudato gengival), ao transudato da mucosa oral, a fragmentos celulares, a microorganismos e a resíduos de alimentos (CONE; HUESTIS, 2007; SAMYN; LALOUP; DE BOECK, 2007).

A saliva é um fluido incolor, viscoso, formado pelas secreções de três pares de glândulas principais, as submandibulares, as parótidas e as sublinguais, além de outras glândulas menores (SAMYN; LALOUP; DE BOECK, 2007). A secreção submandibular contribui com cerca de 70% do volume total da saliva, a secreção parótida com 25% e a sublingual e as glândulas menores com os 5% restantes (MOFFAT; OSSELTON; WIDDOP, 2004). O fluxo diário de saliva em um indivíduo adulto varia de 500 a 1500 mL (UNIDCP, 2001). A saliva é composta por 99% de água, 0,3% de enzimas (principalmente amilase), 0,3% de glicoproteínas (mucina) e o restante por eletrólitos, imunoglobulinas, albumina e outras proteínas e peptídeos (KIDWELL; HOLLAND; ATHANASELIS, 1998; LIMA et al., 2010). O pH da saliva situa-se na faixa de 5,8 a 7,4 (CROUCH, 2005; FAGIOLINO, 1999), podendo aumentar até 8,0 quando sua produção é estimulada (MOORE, 2009).

As glândulas salivares recebem alto aporte sanguíneo das artérias carótidas (CONE; HUESTIS, 2007; UNIDCP, 2001) e uma fina camada de células epiteliais separa os ductos salivares da circulação sistêmica. Portanto a barreira existente entre a saliva e o sangue é constituída apenas pela parede capilar, membrana basal e membrana das células epiteliais glandulares (SCHEPERS et al., 2003; UNIDCP, 2001). Desta forma, o principal mecanismo de transporte do sangue para a saliva de substâncias com massa molecular entre 100 e 500 Da (maioria dos fármacos) é a difusão passiva (figura 7) pelas membranas celulares (CONE; HUESTIS, 2007). Este tipo de transporte só ocorre com moléculas não-ionizadas, lipossolúveis e não ligadas a proteínas plasmáticas, as quais conseguem atravessar livremente as membranas a favor de um gradiente de concentração (KIDWELL; HOLLAND; ATHANASELIS, 1998; SAMYN; LALOUP; DE BOECK, 2007). O transporte transmembrana ainda depende do pKa do fármaco e do pH da saliva (CONE; HUESTIS, 2007).

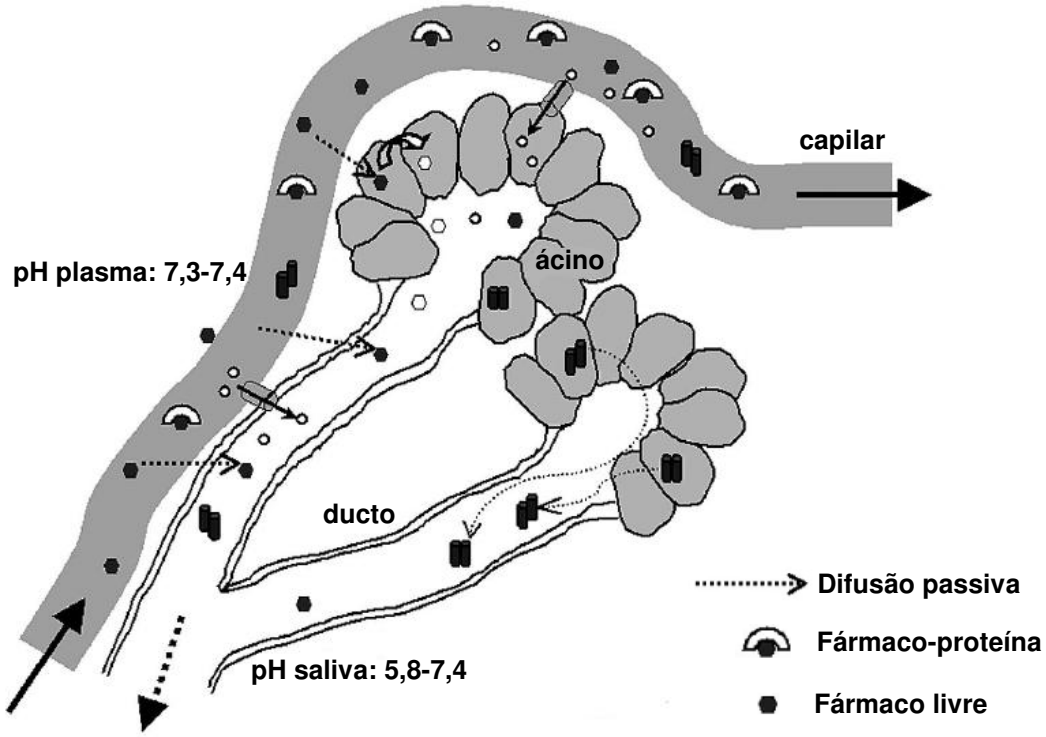


Figura 7: Difusão passiva de fármacos do sangue para a saliva. Modificado de Gröschl, 2008.

3.2.2 Estimulantes anfetamínicos no fluído oral

A AMP, a MET, o MPH, o FEN e a DIE são bases fracas, com pKa em torno de 10,1 (AMP e MET), 8,9, 7,7 e 7,6, respectivamente (MOFFAT; OSSELTON; WIDDOP, 2004; VCCLAB, 2005), massa molecular relativamente baixa (entre 135 e 233 Da) e escassa ligação a proteínas plasmáticas (LA TORRE et al., 2004). Devido a estas características, os ATS se difundem facilmente pelas membranas celulares, vindo a se acumular em locais com pH mais baixo do que o do sangue (como a saliva), onde a ionização dos grupamentos amínicos impede o retorno dos fármacos à corrente sanguínea (CONE; HUESTIS, 2007). De fato, as concentrações de AMP, MET e MPH encontradas no OF superam em 2 a 13 vezes aquelas presentes no sangue (CONE; HUESTIS, 2007; SILBER et al., 2005b; MARCHEI et al., 2010), sendo analiticamente vantajoso a utilização do OF como matriz biológica para a detecção do consumo de ATS.

Para bases fracas, a razão teórica entre a concentração no OF e no plasma (OF/P) pode ser calculada pela equação de Henderson-Hasselbach modificada (UNIDCP, 2001; CROUCH, 2005; FAGIOLINO, 1999):

$$\frac{OF}{P} = \frac{1 + 10^{(pKa-pHof)} \times f_p}{1 + 10^{(pKa-pHp)} \times f_{of}}$$

onde pKa é o pKa do fármaco, pH_{of} é o pH do fluído oral, f_p é a fração do fármaco livre no plasma, pH_p é o pH do plasma e f_{of} é a fração do fármaco livre no OF. Na equação acima, o pH plasmático pode ser considerado constante em 7,4 e a ligação do fármaco a proteínas no fluído oral é próxima de zero (f_{of} =1).

Segundo Fagiolino (1999), em geral a razão OF/P apresenta-se mais elevada e variável durante a fase de absorção do fármaco, tendendo à estabilização durante a fase de eliminação. Na prática, verifica-se que a razão OF/P para ATS apresenta grande variabilidade intra (MARCHEI et al., 2010; SCHEPERS et al., 2003) e interindividual (GJERDE; VERSTRAETE, 2010; MOORE, 2009; SCHEPERS et al., 2003; WILLE et al., 2009), conservando-se acima da unidade na maioria dos casos e com perfil farmacocinético semelhante ao do sangue (DRUMMER, 2005; HUESTIS; CONE, 2007; MARCHEI et al, 2010).

3.2.3 Dispositivos de coleta

O OF pode ser coletado diretamente em recipientes apropriados, por aspiração ou através de dispositivos coletores comerciais, a maioria importados (GRÖSCHL et al. 2008; CROUCH, 2005). A vantagem destes dispositivos está em propiciar uma coleta limpa, fácil, rápida e sem constrangimentos (SAMYN; LALOUP; DE BOECK, 2007).

A maioria dos dispositivos (figura 8) utiliza suabes absorventes (em geral de celulose ou polietileno), presos (Quantisal®, Cozart®, Intercept®, Omni-sal®) ou não (Salivette®, Finger Collector®) a hastes plásticas, podendo estar impregnados com estimulantes da salivação como ácido cítrico (Intercept®) e/ou acompanhados de frascos contendo volumes fixos de soluções tampões conservantes (Quantisal®, Cozart®), utilizadas para inibir o crescimento microbiano e a degradação dos analitos (GRÖSCHL et al. 2008; CROUCH, 2005; ORASURE TECHNOLOGIES, 2008; COOPER et al., 2006; MOORE, 2009). Há varios problemas associados com a estimulação da produção de OF (RAGGAM et al., 2008; SAMYN; LALOUP; DE BOECK, 2007), entre eles o aumento do pH e da concentração de íons bicarbonato, cloro e sódio, resultando na diminuição da concentração de fármacos básicos no fluido (DRUMMER, 2006; FAGIOLINO, 1999). Na tentativa de controlar o volume de OF coletado, alguns dispositivos (Quantisal®, Cozart®) possuem indicadores de volume que assinalam quando cerca de 1 mL de amostra foi coletado (COOPER et al., 2006; QUINTELA et al., 2006). Outros dispositivos (Saliva Collection System-SCS®), utilizam uma solução extratora contendo um corante como padrão interno, que após bochechada por alguns minutos, é analizada em fotômetro para a determinação do volume exato de fluído oral coletado (RAGGAM et al., 2008).

Figura 8: Alguns dispositivos comerciais de coleta de fluído oral. Fonte: SciMart (2010), OraSure Technologies (2009), Greiner Bio-One (2007), Immunalysis Corporation (2010).

O dispositivo de coleta Quantisal® (Immunalysis Corporation, Pomona, California) foi o escolhido pelo nosso grupo de pesquisas para a coleta das amostras de OF de motoristas brasileiros por possuir indicador de volume e tampão conservante para as amostras de OF. Na figura 9 está ilustrada a metodologia de coleta utilizando o dispositivo Quantisal®:

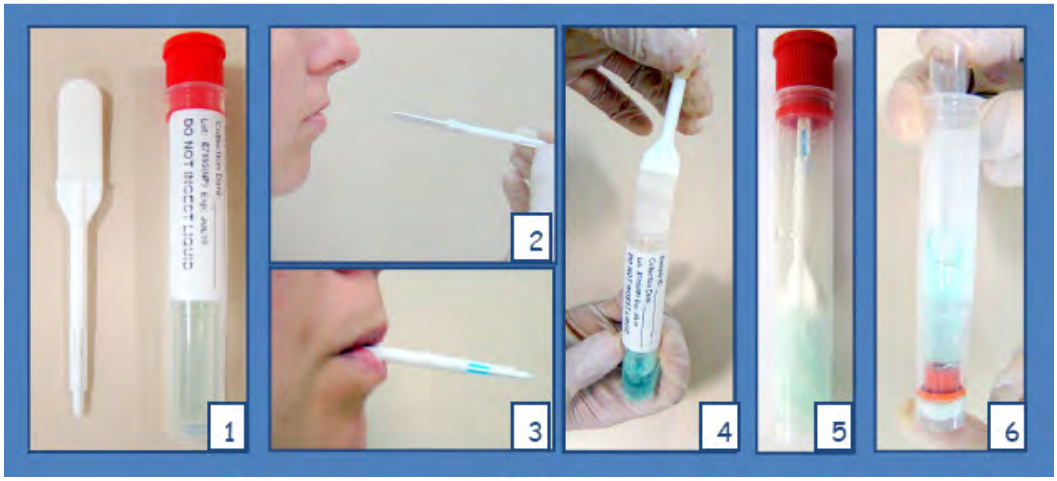


Figura 9: Coleta de Fluido Oral com Quantisal®: 1- Dispositivo de coleta e frasco com tampão conservante; 2 – Insira o dispositivo coletor sob a língua; 3 – Aguarde até o indicador de volume tornar-se azul; 4,5 – Insira o dispositivo no frasco com tampão conservante e feche a tampa; 6 – Após remover a haste plástica do dispositivo, insira o filtro plático Quantisal® para separar a solução do suabe coletor.

3.2.4 Métodos de triagem

Dentre as alternativas disponíveis para a etapa de triagem da presença de ATS em fluído oral, os imunoensaios têm sido os mais empregados, devido à elevada sensibilidade, reduzido tempo entre a coleta e obtenção dos resultados, utilização de pequeno volume de amostra, simplicidade e praticidade operacional (LIMBERGER et al., 2010). As técnicas imunológicas mais utilizadas com esta finalidade são o ELISA (*enzyme linked immunosorbent assay*) e a imunocromatografia.

O ELISA utiliza um suporte sólido (placa plástica com pequenos poços) no qual estão imobilizados anticorpos (figura 10). Na técnica mais utilizada (ELISA competitivo), ocorre uma competição entre os ATS presentes no OF e àqueles marcados enzimaticamente (adicionados a uma concentração fixa) pela ligação com os anticorpos da placa (FLANAGAN et al., 2007). Após a lavagem dos poços, é adicionado um substrato que, sob ação da enzima que permaneceu ligada à placa, origina um produto corado. A determinação da intensidade da coloração é realizada por um fotômetro, e está inversamente relacionada à concentração de ATS no fluído oral, por meio de uma curva de concentração-resposta (IMMUNALYSIS CORPORATION, 2005a, 2005b; LIMBERGER et al., 2010). Entre as vantagens da técnica está a possibilidade de automação e a capacidade de realizar várias análises ao mesmo tempo.

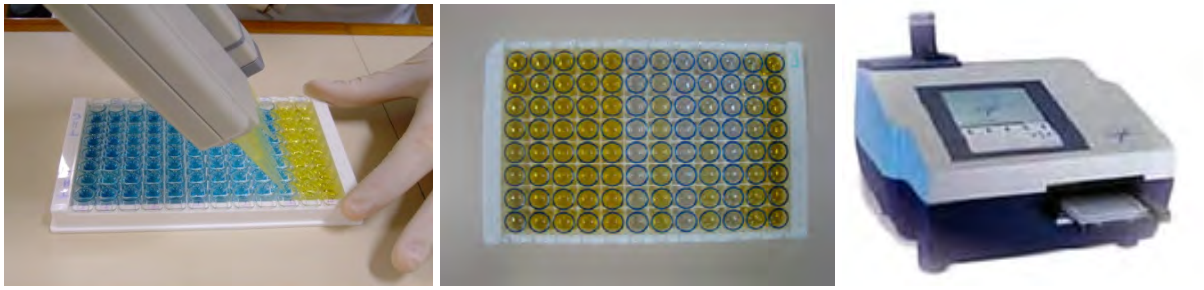


Figura 10: Amphetamine Direct ELISA® (*enzyme linked immuno sorbent assay*) fabricado pela empresa Immunalysis Corporation (CA, EUA): placas de ELISA e fotômetro para a leitura dos resultados.

Na imunocromatografia, o ensaio mais empregado também é o competitivo. O dispositivo básico (figura 11) consiste em uma fita de membrana porosa (nitrocelulose), armazenada em um suporte plástico, e ligada a uma porção coletora,

que é introduzida na boca do indivíduo para absorver o fluído oral (ALFA SCIENTIFIC DESIGNS, 2009). Quando o volume adequado é coletado, o fluído oral migra, por capilaridade, através da fita, passando por uma região onde estão depositados anticorpos marcados com partículas coloridas (HAND; BALDWIN, 2004). Na ausência ou em quantidades muito baixas de ATS no fluído oral, os anticorpos marcados migram através da fita e se ligam aos ATS imobilizados em uma pequena faixa na região de leitura do teste, originando uma linha colorida (resultado negativo). Ao contrário, quando os ATS no fluído oral estão presentes em concentração acima do limite de detecção do teste, os sítios de ligação dos anticorpos são ocupados pelos ATS da amostra antes de atingirem a região de leitura do teste, não havendo a formação da linha colorida (teste positivo). A técnica imunocromatográfica é atualmente a mais utilizada para monitorar o consumo de substâncias psicoativas no trânsito (tabela 1), devido à possibilidade de realização do teste *in loco*, com o motorista ainda dentro do veículo, e originar resultados em poucos minutos, subsidiando ações policiais imediatas. Com este escopo, testes imunocromatográficos já estão sendo utilizados por agentes de trânsito em alguns países como Portugal (PORTUGAL, 2007) e Austrália (VICTORIA, 2010a, 2010b). Um fator importante a ser considerado é a questão ética de aplicação destes testes, uma vez que o resultado é gerado na presença do condutor, aspecto relevante se considerarmos a probabilidade de resultados falso-positivos (LIMBERGER et al., 2010).

Figura 11: Teste imunocromatográfico Oral-View® da empresa Alfa Scientific Designs (ALFA SCIENTIFIC DESIGNS, 2009).

A triagem de ATS no fluído oral também pode ser realizada por técnicas de cromatografia gasosa e líquida (GJERDE et al., 2008; PÉREZ et al., 2009). Comparativamente às técnicas cromatográficas, os imunoensaios requerem mão de obra menos especializada, não exigem estrutura laboratorial complexa, nem padrões analíticos de elevada pureza, além de permitem a análise de um elevado número de amostras em um curto espaço de tempo e a um menor custo.

Tabela 1
Alguns estudos utilizando técnicas de triagem de estimulantes anfetamínicos no fluido oral de motoristas.

Autor do estudo	País	Técnica de Triagem
GRÖNHOLM e LILLSUNDE, 2001	Finlândia	Imunocromatografia
BIERMANN et al., 2004	Alemanha	Imunocromatografia
BERNHOFTA et al., 2005	Dinamarca	ELISA[a]
TOENNES et al., 2005	Alemanha	Imunocromatografia
NHTSA, 2007	Estados Unidos da América	ELISA[a]
DRUMMER et al., 2007	Austrália	Imunocromatografia
CONCHEIRO et al., 2007	Espanha	Imunocromatografia
GJERDE et al., 2008	Noruega	CLAE/EM[b]
PEHRSSON et al., 2008	Finlândia	Imunocromatografia
PÉREZ et al., 2009	Espanha	CG/EM[c]
DAVEY, FREEMANA e LAVELLE, 2009	Austrália	Imunocromatografia
PECHANSKY, DUARTE e DE BONI, 2010	Brasil	ELISA[a]

[a] ELISA (*enzyme linked immunosorbent assay*); [b] CLAE/EM (Cromatografia Líquida de Alta Eficiência com Detector de Massas); [c] CG/EM (Cromatografia a Gás com Detector de Massas).

3.3 Microextração em fase sólida

A microextração em fase sólida (SPME) é uma técnica de extração e concentração de compostos orgânicos presentes em matrizes aquosas (QUEIROZ; LANÇAS, 2005). Foi introduzida em 1990 (PAWLISZYN, 1997) sendo posteriormente otimizada, automatizada e ampliada para análises por cromatografia líquida (OUYANG; PAWLISZYN, 2008; SNOW, 2000; VAS; VÉKEY, 2004). Desde a sua invenção, a SPME já foi utilizada em métodos voltados a diversas áreas do

conhecimento, incluindo análises ambientais, forenses, toxicológicas, clínicas, de alimentos, medicamentos, e até aplicações *in vivo* (CHEN et al., 2008; MUSTEATA; PAWLISZYN, 2007; PRAGST, 2007; SNOW, 2000; VAS; VÉKEY, 2004).

O dispositivo clássico da SPME consiste de uma fibra de sílica fundida recoberta por uma fina camada de material polimérico e acondicionada dentro da agulha de uma microseringa (PRAGST, 2007). A extração pode ser realizada de duas formas principais (figura 12): mergulhando a fibra diretamente na amostra (*direct-immersion* SPME ou DI-SPME), ou através da técnica de *headspace* (*headspace* SPME ou HS-SPME), na qual a fibra é exposta na fase vapor situada sobre a amostra (THEODORIDIS; KOSTER; JONG, 2000; ULRICH, 2000). Após a extração, no caso da análise ser realizada por CG, a fibra é dessorvida termicamente no injetor do equipamento, liberando os analitos para o sistema cromatográfico (STASHENKO; MARTÍNEZ, 2004). A quantidade extraída pela fibra é diretamente proporcional à concentração dos analitos na amostra, e esta é a base para a quantificação utilizando SPME (PAWLISZYN, 1997).

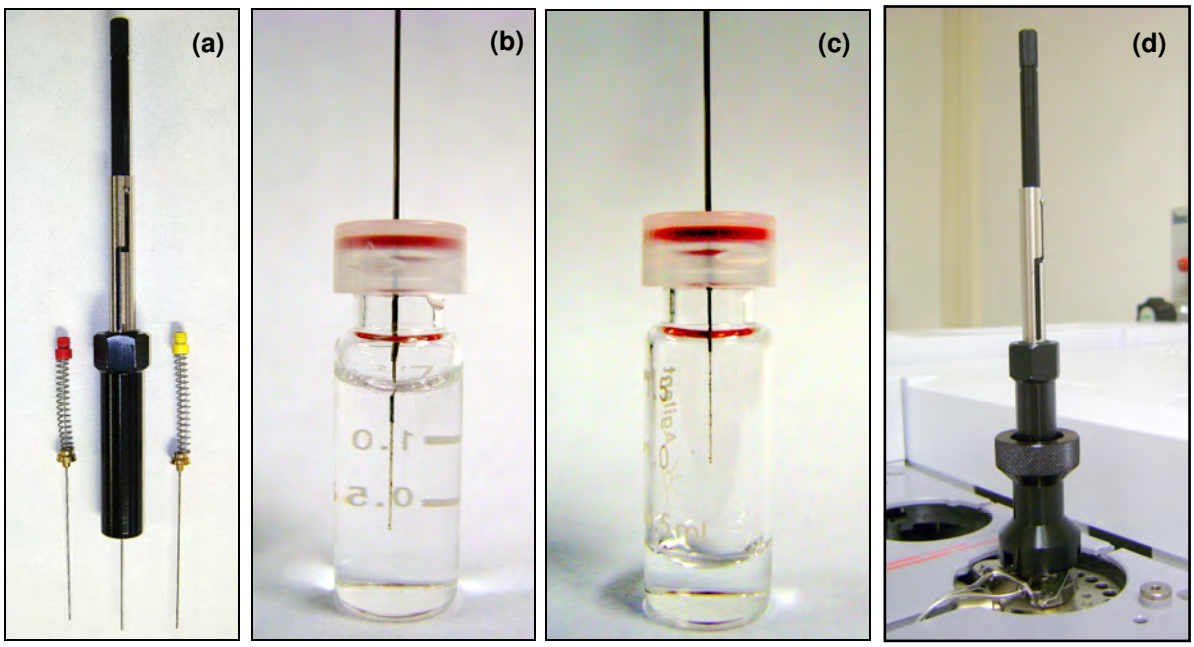


Figura 12: Dispositivo de microextração em fase sólida (SPME) montado, ladeado por fibras recobertas por 100 µm (vermelha) e 30 µm (amarela) de polidimetilsiloxano (a); extração por imersão (DI-SPME) (b); extração por exposição ao *headspace* (HS-SPME) (c); dessorção da fibra em injetor de cromatógrafo a gás (d).

Atualmente, estão disponíveis no mercado fibras de SPME com revestimentos poliméricos variados (SIGMA-ALDRICH, 2010), constituídos por material único

(polidimetilsiloxano, poliacrilato) ou por combinações de materiais (polietilenoglicol-Carbowax™; polidimetilsiloxano-divinilbenzeno; Carboxen™-polidimetilsiloxano, etc.), dispostos em camadas com espessuras entre 7 µm a 100 µm. As fibras recobertas por polidimetilsiloxano (PDMS), polímero resistente, não seletivo e de baixa polaridade, são as mais amplamente utilizadas (LANÇAS, 2004; VAS; VÉKEY, 2004).

O princípio da técnica baseia-se na partição dos analitos entre a matriz aquosa (ou seu *headspace*) e o filme polimérico da fibra. A extração é máxima quando o analito atingir o equilíbrio de distribuição entre a matriz e a fibra, ou entre a matriz, o *headspace* e a fibra (PAWLISZYN, 1997). No entanto, a extração pode ser interrompida antes que o equilíbrio seja alcançado, desde que se mantenham constantes o tempo de extração e as demais variáveis experimentais, a fim de garantir a reprodutibilidade da técnica (STALIKAS; FIAMEGOS, 2008). Ao contrário dos métodos clássicos de preparação de amostras, como as extrações líquido-líquido e sólido-líquido, a SPME é uma técnica de extração não exaustiva, na qual somente uma pequena quantidade de analito é removida da amostra (OUYANG; PAWLISZYN, 2008).

Entre os parâmetros que afetam a sensibilidade e o tempo de extração por SPME, destacam-se o tipo e a espessura do revestimento polimérico da fibra, a agitação, a temperatura, o pH, a quantidade de sais e de solventes orgânicos presentes na matriz (PAWLISZYN, 1997). A polaridade do recobrimento da fibra deve ser semelhante à das substâncias que se deseja extrair. Fibras com maior volume de filme polimérico tendem a extrair maior quantidade de analitos, porém demoram mais para alcançar o equilíbrio de sorção (VAS; VÉKEY, 2004). A agitação aumenta a taxa de extração e acelera a chegada ao equilíbrio (ULRICH, 2000). Elevadas temperaturas aceleram a obtenção do equilíbrio, diminuindo, no entanto, a quantidade de analito extraída (LORD; PAWLISZYN, 2000). A adição de sais para aumentar a força iônica do meio e o ajuste do pH para duas unidades acima (bases fracas) ou abaixo (ácido fracos) do pKa da substância tendem a favorecer a extração (PRAGST, 2007; SNOW, 2000). Solventes orgânicos miscíveis em água podem atuar como co-solventes, no entanto quando em concentração superior a 1%, podem alterar as constantes de distribuição dos analitos e ocasionar o

entumescimento da fibra (PAWLISZYN, 1997). Tais variáveis experimentais devem ser testadas e otimizadas a cada novo método de SPME desenvolvido.

A SPME foi escolhida como técnica de extração a ser empregada na análise de ATS no OF por apresentar diversas vantagens sobre outras técnicas de extração, tais como maior rapidez e simplicidade, menor manipulação da amostra, extração sem a necessidade de solventes orgânicos e adequabilidade a reduzidos volumes de amostra (MUSTEATA; PAWLISZIN, 2007; ULRICH, 2000; VAS; VÉKEY, 2004).

3.4 Derivatização de estimulantes anfetamínicos com alquilcloroformatos

Na análise de ATS por cromatografia a gás, a utilização de agentes derivatizantes é bastante recomendada, já que melhora a resolução e a simetria dos picos cromatográficos, reduz os limites de detecção e aumenta a precisão e a especificidade do método (GROB; BARRY, 2004). A substituição dos hidrogênios ativos dos grupamentos amínicos torna os ATS menos polares e reativos, melhorando suas características cromatográficas. Ainda, em técnicas confirmatórias por cromatografia a gás com detector de massas (CG/EM) voltadas à rotina forense, a derivatização de ATS de pequena massa molecular, como a AMP e a MET, é fundamental para confirmar a identidade das substâncias, já que as respectivas bases livres não geram fragmentos de alto peso molecular, resultando em espectros de massas pouco discriminativos (BROWN et al., 2003; GROB; BARRY, 2004).

Dentre os agentes derivatizantes já empregados com sucesso na reação com ATS presentes em matrizes aquosas, destacam-se os alquilcloroformatos (ALCL). Ao contrário da maioria dos agentes derivatizantes, a reação com ALCL não necessita de solventes orgânicos para se processar, podendo realizar-se diretamente nos fluídos biológicos em estudo (HUSEK, 1998; UGLAND; KROGH; RASMUSSEN, 1997). Não há necessidade de extração prévia dos ATS, nem de secagem do extrato contendo as respectivas bases livres, reduzindo o número de etapas analíticas e evitando perdas por evaporação ou adsorção dos ATS na vidraria (MEATHERALL, 1995). Como resultado, são minimizadas as fontes de erro, o tempo de preparação das amostras e os recursos materiais empregados. Ainda, os ALCL são reagentes de baixo custo (HUSEK, 1998; QIU et al., 2007) e estão

disponíveis no mercado com cadeias carbonadas de variados tamanhos e substituições (BROWN et al., 2003), ofertando diferentes possibilidade ao analista.

Os ALCL são usados há longa dada na proteção dos grupamentos amina de aminoácidos durante a síntese de peptídeos e proteínas (GREENE; WUTS, 1999), porém só a partir da década de 90 é que começaram a ser amplamente utilizados em química analítica como agentes derivatizantes (HUSEK, 1998). Diversos ALCL já foram testados para a acilação de anfetaminas em soluções aquosas visando análise por CG (tabela 2).

Apesar dos ALCL serem insolúveis em água, a agitação vigorosa provê a superfície de contato necessária à reação com as aminas em meio aquoso (MEATHERALL, 1995). A insolubilidade em água dos cloroformatos previne sua rápida hidrólise, permitindo que a reação se processe na interface entre as duas fases por maior tempo (MAURINO et al.,1999). Mesmo assim, em todas as reações em meio aquoso são detectados produtos de degradação dos ALCL, em especial os dialquilcarbonatos (ANGELINO et al., 1998).

A reação entre ALCL e aminas se dá por um mecanismo de substituição nucleofílica que ocorre em duas etapas (STREITWIESER; HEATHCOCK; KOSOWER, 1992): adição nucleofílica do nitrogênio da amina ao carbono da carbonila (etapa 1; figura 13), com formação de um intermediário tetraédrico; seguida pela eliminação de ácido clorídrico (etapa 2; figura 13). Os derivados alquilcarbamatos (ALCA) formados são estáveis em meio aquoso alcalino e possuem boas características cromatográficas (KATAOKA, 1996; UGLAND; KROGH; RASMUSSEN, 1997).

Para que a reação se processe adequadamente, há necessidade da constante remoção do HCl formado, o que ocorre quando a reação é realizada em meio alcalino (MEATHERALL,1995). A neutralização do HCl pode ser realizada com aminas terciárias baratas e amplamente disponíveis no mercado como a piridina, ou com soluções aquosas de álcalis como NaOH e Na_2SO_4 (ALLINGER at al., 1976). Na ausência de álcali, apenas parte das aminas do meio se converte à ALCA, uma vez que os prótons formados na reação convertem as aminas às suas formas ionizadas, não nucleofílicas (MEAKINS, 1996). Ainda, segundo Meatherall (1998), o

HCl formado na reação pode combinar-se com os derivados ALCA, produzindo sais insolúveis.

Tabela 2
Alguns alquilcloroformatos já utilizados na derivatização de estimulantes anfetamínicos em matrizes biológicas e processos de extração dos derivados para análise por cromatografia a gás.

Autor	**ATS** [1]	**Matriz**	**Conc. (ng/mL)**	**Volume Pré-extração (mL)**	**Métodos de Extração** [2]	**Tipo/volume (µL) de ALCL**[3]
Meatherall (1995)	AMP MET PHT EPH	Urina	100-10.000	0,26	LLE - clorofórmio:n-hexano (1:3 v/v)	PRCL / 1
Ugland, Krogh e Rasmussen (1997)	AMP MET	Urina	100-10.000	1,50	LLE - clorofórmio:n-hexano (1:3 v/v) DI-SPME – PDMS 100 µm	MECL / 2, 4, 16 **PRCL** / **2**, **4**, **16** **BUCL** / **2**, **4**, **16**
Ugland, Krogh e Rasmussen (1999)	AMP MET MDA MDMA MDEA	Urina	100-10.000	1,50	LLE - clorofórmio:n-hexano (1:3 v/v) DI-SPME – PDMS 100 µm	PRCL / 4 PRCL / 8
Namera, Yashiki e Kojima (2002)	AMP MET FNF PHT PHA MDA MDMA 2C-B	Urina	1000 1-1000	1,00 1,53	LLE – acetato de etila HS-SPME – PDMS 100 µm	**ETCL**, PRCL, BUCL ETCL / 5, 10, **15**, **20**
Yonamine e col. (2003)	AMP MET	Fluído Oral	5-100	2,00	DI-SPME – PDMS 100 µm	BUCL / 2
Yahata e col. (2006)	AMP MET MDA MDMA	Cabelo	0,1-100	1,03	HS-SPME – PDMS 100 µm	ETCL / 5, 10, **20**, 30, 40
Nishida e col. (2006)	AMP MET	Cabelo	0,1-40	0,5	HS-SPME – PDMS 100 µm	PRCL / 10
Brown, Rhodes e Pritchard (2007)	MET MDMA	Urina	50-20.000	1,00	HS-SPME – PDMS 100 µm	HECL / 10

[1] EPH- efedrina; FNF-fenfluramina; MDA- 3,4-metilenodioxianfetamina; MDMA- 3,4-metilenodioximetanfetamina; MDEA- 3,4-metilenodioxietilanfetamina; PHT- fentermina; PHA- fenetilamina; 2C-B- 4-bromo-2,5-dimetoxifenetilamina.
[2] LLE– extração líquido-líquido.
[3] MECL- metilcloroformato; ETCL- etilcloroformato; PRCL- propilcloroformato; BUCL- butilcloroformato; HECL-hexilcloroformato; **em negrito** o tipo e o volume de ALCL que apresentou(aram) melhores resultados.

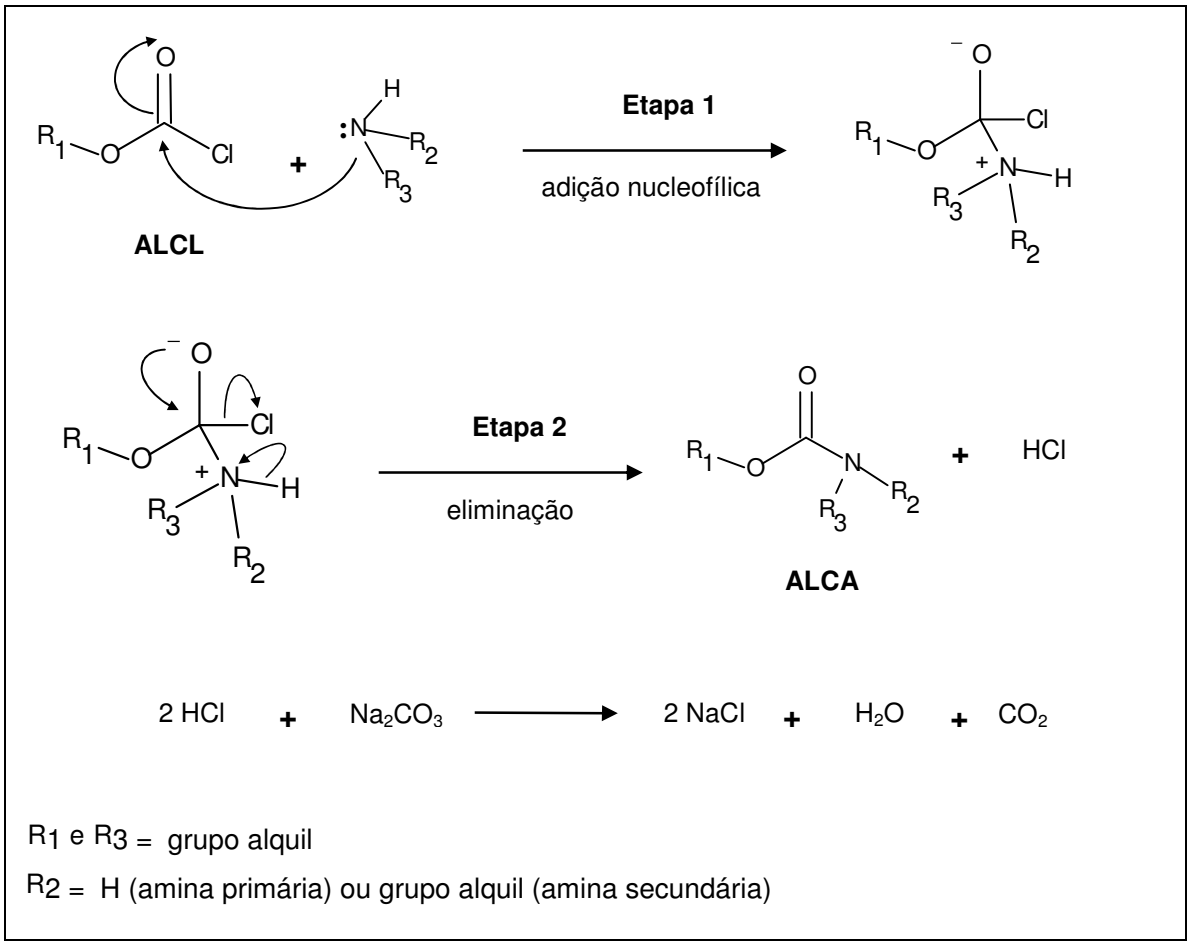


Figura 13: Representação geral da reação de substituição nucleofílica (mecanismo de adição-eliminação) entre alquilcloroformatos (ALCL) e aminas primárias e secundárias, em meio aquoso alcalinizado com Na_2CO_3, originando alquilcarbamatos (ALCA). Adaptado de Allinger et al. (1976), Streitwieser, Heathcock e Kosower (1992), Meakins (1996), Jones (1997), Kataoka (1996), Meatherall (1995) e Ugland, Krogh e Rasmussen (1997).

A reação entre aminas primárias e secundárias e ALCL ocorre similarmente à reação daquelas com halogenetos de acila para a formação de amidas. Ambas as reações processam-se em poucos minutos em meio aquoso alcalino, a frio, sob agitação, na presença de excesso do reagente carbonilado (ALLINGER et al., 1976; HUSEK, 1998). Para a reação completa com aminas terciárias, no entanto, são necessárias condições reacionais mais agressivas, já que deve ocorrer a dealquilação da amina (KNAPP, 1979; BLAU; KING, 1978; MILLAN; PRAGER, 1998).

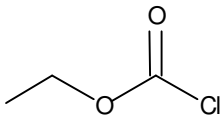
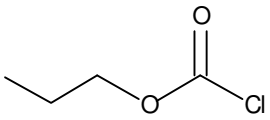
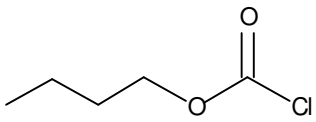
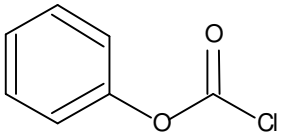
Nas condições usuais da reação com aminas primárias e secundárias, apenas uma acilação pelo ALCL ocorre, pois o ALCA formado é muito menos básico que a amina correspondente (ALLINGER et al., 1976). Tal fato agrega uma enorme vantagem na utilização de ALCL como agentes derivatizantes, já que as reações

empregadas em química analítica devem ser o mais completas e regioespecíficas possíveis, convertendo a substância de interesse, idealmente, em um único derivado (SEGURA; VENTURA; JURADO, 1998).

No desenvolvimento do presente trabalho, foram testados quatro ALCL (tabela 3) para a derivatização de AMP (amina primária) e MET, FEN e MPH (aminas secundárias). A DIE (amina terciária) foi analisada não derivatizada.

Tabela 3

Alquilcloroformatos testados como derivatizantes da anfetamina, metanfetamina, femproporex e metilfenidato.

Reagente	Massa Molar (g)	Fórmula Estrutural
Etilcloroformato (ETCL)	108,52	
Propilcloroformato (PRCL)	122,55	
Butilcloroformato (BUCL)	136,58	
Fenilcloroformato (PHCL)	156,57	

4 ARTIGO 1:

“What amphetamines the oral fluid screening tests really detect?”

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What amphetamines the oral fluid screening tests really detect?

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Abstract

We reviewed some of the immunological screening tests used around the world to detect amphetamine-type stimulants (ATS) in oral fluid and evaluated the suitability of three commercial tests in detecting the Brazilian drivers most abused ATS. The analytical specificity of Amphetamine and Methamphetamine Direct ELISA^{TM} (enzyme linked immuno sorbent assay) from Immunalysis Corporation and the immunochromatographic test from Alfa Scientific Designs (Oral-View^{TM}) was accessed. We also discussed the adequacy of the immunological kits used by some countries in detecting ATS abuse in traffic, by comparing the kits specificity data with the controlled ATS currently marketed or illicitly produced/consumed in these countries. The tested assays were not able to detect, even in high concentrations, methylphenidate, fenproporex or diethylpropion, the latter two substances widely consumed by Brazilian truck drivers. It's evident the necessity of developing new kits to enable the control of ATS abuse in Brazilian roads through oral fluid tests and to allow other countries to detect misuse of prescription ATS.

Keywords: amphetamine-type stimulants, oral fluid, screening tests.

1. Introduction

Drug driving is one of the major contributors to road fatalities in the world. Over the last years, several studies [1-6] have shown high prevalence in the consumption of psychoactive substances among drivers, especially amphetamine-type stimulants (ATS), which are widely used by professional conductors to increase their alertness during the long journeys [7-11]. The use of oral fluid for monitoring drug consumption on roads has many advantages over the conventional biological fluids (urine and blood) [12,13], including a good correlation with serum analytical data and impairment symptoms [14-18], non-invasive collection and difficulty to adulterate samples [19].

Over the last years, several immunoassays have been developed to screen psychoactive drugs in drivers' oral fluid and they have been extensively employed in both research [1,4,15-18,20-24] and traffic police routine [25-35].

This paper reviews published articles and legislation reporting the use of immunological screening tests to detect ATS in driver's oral fluid around the world, and shows a Brazilian experience in evaluating the specificity/cross-reactivity of three commercial tests. By compiling information about controlled ATS legally marketed or illicitly produced/consumed nowadays in several countries, we discuss the adequacy of the world used immunological tests in detecting ATS abuse on roads.

2. Materials and methods

2.1 Data compilation

The latest published researches, available at ScienceDirect, PubMed and Scirus databases, reporting the use of immunological screening tests to detect ATS consumption in conductors and the legislation of countries officially employing oral fluid tests for drugged drivers' control were reviewed [1,4,15-18,20-35]. We also overviewed leaflets and technical information provided by oral fluid kits manufacturers [36-43], as well as project reports and articles [44,45] which had evaluated these kits. With these data, the authors calculated ATS cross-reactivity values according to the formula showed in section 2.2.

In addition, it was accessed databases provided by drug regulatory agencies and specialized medicine information websites and books [46-68] in order to establish what pharmaceuticals containing controlled ATS are currently licensed for use in each country. Information on illegal consumption of ATS, either prescription medicines or illicitly manufactured drugs, was obtained mainly from the United Nations reports [69,70], Brazilian Federal Police [71] and recent articles.

2.2 Experimental specificity evaluation

The analytical specificity of three commercial oral fluid immunoassays for ATS detection was evaluated: Amphetamine Direct ELISA™ (enzyme linked immunosorbent assay) and Methamphetamine Direct ELISA™ kits, purchased from Immunalysis Corporation (Pomona, CA, USA) and Oral-View™ Saliva Multi-Drug of Abuse Test, manufactured by Alfa Scientific Designs (Poway, CA, USA) and kindly donated by Grimextur Brasil Diagnósticos (São Paulo, SP, Brazil).

Both Amphetamine and Methamphetamine Immunalysis ELISA kits consist of classic ELISA assays, performed in the laboratory with buffered oral fluid, ideally collected with the device from the same company (Quantisal™ oral fluid collection device) [42,43]. They are based on the competition between the drug present in the sample and the same drug labeled with enzyme (added to the system), for the binding sites of antibodies immobilized in the wells of a plate [42, 43]. The intensity of the developed color, measured in a photometer, is inversely proportional to the amount of the drug present in the sample [72].

On the other hand, Oral-View™ is an on-site immunochromatographic multi-analyte assay, designed to simultaneously detect up to five drug classes in the same cartridge, generally amphetamine, benzodiazepines, cocaine, morphine and tetrahydrocannabinol [73]. The device consists of a collection pad, attached to a porous membrane strip, which is inserted into the subject's mouth. From the pad, oral fluid migrates by capillary action, mobilizing a reservoir of colored antibodies, which flow with oral fluid along the strip, until reach lines with immobilized drugs. If a drug is present in the sample at or above the kit cutoff concentration, the binding sites of the respective colored antibody will saturate and not attach to the immobilized drug in the strip, resulting in the absence of a colored band in the results window [73].

The Methamphetamine Direct ELISA™ kit, Amphetamine Direct ELISA™ Kit and Oral-View™ have 50 ng mL^{-1} cutoff concentrations, being *d*-methamphetamine the target drug for the first kit and *d*-amphetamine for the others [42,43,73].

ELISA kits were tested for *d*,*l*-methamphetamine (*d*,*l*-MET) and *d*,*l*-amphetamine (*d*,*l*-AMP), purchased from Cerilliant (Round Rock, TX, USA); *d*,*l*-fenproporex (*d*,*l*-FEN), *d*,*l*-diethylpropion (*d*,*l*-DIE) and *d*,*l*-threo-methylphenidate (*d*,*l*-threo-MPH) hydrochlorides gently donated by Aché (Guarulhos, SP, Brazil) and Novartis (Resende, RJ, Brazil); *d*,*l*-3,4-methylenedioxymethamphetamine (*d*,*l*-MDMA), *d*,*l*-3,4-methylenedioxy-ethylamphetamine (*d*,*l*-MDEA), *d*,*l*-3,4-methylenedioxyphenyl-2-butanamine (*d*,*l*-MBDB) and *d*,*l*-3,4-methylenedioxyamphetamine (*d*,*l*-MDA) obtained from Lipomed (Arlesheim, Switzerland) as hydrochloride salts; *d*,*l*-synephrine (*d*,*l*-SYN) purchased from MP Biomedicals (São Paulo, SP, Brazil); *l*-phenylephrine (*l*-PHY) and *d*-pseudoephedrine (*d*-PSE) obtained from Sigma-Aldrich Corporation (São Paulo, SP, Brazil); and *l*-

ephedrine (*l*-EPH) from Merck (Darmstadt, Germany). Oral-View™ was tested only for *d*,*l*-AMP, *d*,*l*-DIE, *d*,*l*-FEN and *d*,*l*-threo-MPD.

Methanol stock solutions of all mentioned ATS were prepared at 500 µg mL^{-1} and added to blank oral fluid collected from eight volunteers, directly in polypropylene tubes, that were previously mixed, centrifuged, and pH adjusted to 6.5 with HCl. For Immunalisys ELISA, ATS-spiked oral fluids was tested at the concentrations of 50, 100, 150, 250, 500, 1,000, 10,000, 40,000 and 100,000 ng mL^{-1}, and for Oral-View™, only the concentrations of 100 and 500 ng mL^{-1} were evaluated.

Oral-View™ and Immunalysis ELISA were performed exactly as manufacturer's instructions [42,43,73], in duplicate for each concentration, employing ATS-spiked oral fluid in the neat form for the first immunoassay and diluted in Quantisal™ preservative buffer (1 mL of oral fluid in 3 mL of buffer) for the others. For Oral-View™ assay, the spiked oral fluid was slowly pipetted in the pad (about 0.6 mL), keeping the opposite end of the device angled downward, until a pink color began to appear in the result window. In all the experiments blank oral fluid was assayed as negative control.

The cross-reactivity profile of the immunoassays was calculated according to the formula [74]:

$$\text{Cross reactivity (\%)} = \frac{\text{Apparent concentration of the target ATS}}{\text{ATS concentration of spiked oral fluid}} \times 100$$

For the qualitative immunochromatographic assay Oral-View™ the apparent concentration of the target ATS is the kit cutoff concentration of *d*-AMP. For the ELISA semi-quantitative kits, the apparent concentration is the *d*-AMP or *d*-MET equivalent concentration calculated from the spiked sample response (sample absorbance divided by zero calibrator absorbance, multiplied by 100).

3. Results and Discussion

3.1 Oral fluid screening tests for ATS detection

The use of oral fluid to test drivers for drugs consumption have many advantages over the conventional biological fluids, since the collection does not

require medical experience, it is less intrusive to the donor's privacy, it can be easily observed by the police officer, it informs about recent drug use (within hours) and it correlates well with impairment symptoms of drivers under the influence of drugs [12,13,18,75].

Immunoassays are the most convenient technique for initial oral fluid drug testing, and can presents as on-site (e. g., immunochromatographic assays) or laboratory based tests (e.g., ELISA). The techniques available for testing of oral fluid are similar to urine testing, but the target analytes have to be different, since in oral fluid we are interested in found non-metabolized substances, which demonstrate recent drug use [75,76].

The screening immunoassays applied to drug driving detection mostly consist of immunocromatographic tests, produced by USA, German and British companies (table 1). In spite of not being reported by international studies, the Oral-View™ immunochromatographic multi-analyte assay was chosen to be tested by the authors because it is regularly imported by a national company, so it is freely available in Brazil.

Imunoassays have been used in many research studies and pilot tests around Europe, Oceania and North America (table 1). In some countries, however, they are currently being employed in traffic police routine to detect drugged drivers.

Table 1

Since 2007 [30,31], Portuguese legislation regulates the use of oral fluid screening tests by traffic agents, setting the types and models of immunoassays approved in the country. At the present time [32-35], five immunochromatographics assays are granted to be used by traffic agents in Portugal (table 1). In case of a positive result, the driver is taken to a public health establishment to collect blood for confirmatory tests [30].

In Australia, roadside drug testing have been conducted routinely and randomly by police enforcement of several states, employing on-site oral fluid immunoassays. [25,29]. In the State of Victoria, for example, it has been going on for almost 6 years [25,29], with the use of two consecutive immunochromatographics

assays (table 1) to detect THC, methamphetamine and MDMA in driver's oral fluid [26]. In case of positive results, oral fluid or blood sample can be used for laboratory confirmation [27, 28].

The analytical specificity or cross-reactivity of an immunoassay provides an indication of how the assay responds to other drugs relative to the drug used as a calibrator or standard [74]. In other words, relative to the kit target drug response.

Through the cross-reactivity information provided by the manufacturer (table 1), it was found that oral fluid screening tests current available on world market are directed to the detection of illicitly produced ATS, mainly AMP, MET and MDMA [36-44]. Prescription-only ATS like MPH and phentermine (PHT) generally do not cross-react or poorly react with AMP or MET kits. Information about DIE and FEN cross-reactivity was not found. Most ATS immunoassays employ only *d*-AMP or *d*-MET antibodies, which means that they will detect this target drugs with much better efficiency than other ATS, including the AMP and MET levo isomers and racemates. The manufacturers' data report cross-reactivity of less than 10% for *l*-AMP or *l*-MET and about 50% for *d,l*-AMP.

3.2 Experimental specificity evaluation

Table 2 shows the results of the performed cross-reactivity tests and the comparison with the manufacturers' technical information.

Table 2

In general, the experimental cross-reactivity agreed with the information of manufacturers. None of the kits detected FEN, DIE and MPH, neither in high concentrations. As the immunoassays from table 1, Oral-View™ showed a 50% cross-reactivity with *d,l*-AMP.

3.3 Controlled ATS used around the world

ATS rank as the second most commonly used drug in the world (*Cannabis* is the first) and in 2008 between 13.7 and 52.9 million people aged 15 to 64 had used ATS [69].

Oceania, East and South-East Asia, North America, and West and Central Europe are the regions with the highest prevalence rates of ATS use [69]. However, different ATS pose different problems for different world regions. In Oceania, illicit manufactured MET is the most consumed ATS, followed by AMP and MDMA; in Asia and North America, illegal MET and MDMA are more prevalent; in Europe, illicit AMP and MDMA (with few exceptions like Czech Republic and Slovakia, where MET abuse is higher); in Middle East, illegal AMP; in Africa, illicit MET and ATS containing medicines are the major problem; and in South America, the consumption of ATS is mainly through diverted pharmaceuticals (anorectics and psychostimulants) [2, 29, 69, 77-79].

According to the latest United Nations Office on Drugs and Crime World Drug Report [69], the global number of people using ATS is growing, as well as the problem of prescription ATS misuse, obtained via black markets and used non-medically. Diversion of ATS medicines from licit domestic distribution channels is the main source used to supply illicit markets [70], and includes the use of falsified prescriptions, supplying of substances by pharmacies without the required prescriptions, obtaining from persons to whom they were prescribed by physicians, smuggling from other countries, theft from pharmacies, wholesalers or factories and marketing via illegal internet sites [70,80,81].

Prescription ATS used as appetite suppressants, like FEN, DIE, PHT, clobenzorex (CLO), benzphetamine (BEZ), and phendimetrazine (PHD), or to treat Narcolepsy and Attention Deficit Hyperactivity Disorder (ADHD), as AMP, MET and MPH, are under international control since the United Nations Convention on Psychotropic Substances of 1971 [82], making part of schedules II and IV of this convention. ATS was officially recognized to be liable to abuse and object of illicit traffic, being necessary rigorous measures to restrict the use of such substances to legitimate medical and scientific purposes [83]. In spite that, ATS medicines misuse and diversion remains a major problem worldwide [70,84]. In the United States of America (USA), where the problem is well documented, the abuse of prescription medicines is more prevalent than the abuse of cocaine, heroin or methamphetamine [70].

Table 3 shows that among the countries assessed, USA is the one with the largest number of ATS registered for medical purposes, followed by Mexico and Chile. In 2008, USA was the country with the highest per capita consumption of appetite suppressants, accounting for 58% of the global consumption, and it was responsible for about 75% of world use of MPH [70]. Compared to Europe, the Americas (North, Middle, South) have much more approved ATS medicines, a tendency already observed by the United Nations [70].

MPH is currently marketed in all the studied countries, DIE in 29% of then, PHT in 21% and FEN and AMP in 12% (table 3). According to the International Narcotics Control Board [70], MPH is the most used stimulant to treat Narcolepsy and ADHD in the world, and PHT, FEN and DIE are the most frequently used amphetamine-based anorectics. Over the last years, the consumption of anorectics has increased in some countries, like Australia, Chile, Switzerland, United Kingdom and USA, and the highest average per capita rates of MPH consumption have been observed in Iceland, USA, Canada, Norway, Israel, Netherlands and Switzerland [70].

Table 3

Nowadays, AMP and MET are available for therapeutic use in few countries, such as USA, Canada and Chile (table 3), and so the world's misuse of AMP and MET are mainly associated with illegal production sources (clandestine laboratories) [69]. In fact, AMP and MET can be produced in virtually anywhere at relatively low cost [69].

Unlike the countries where it is of concern the use of illegally produced ATS, such as AMP, MET or methylenedioxy ring-substituted ATS, in Brazil the major problem is the consumption of prescription ATS, specially DIE and FEN.

Brazilian Federal Police has only few records of seizures of illegal AMP and MET, and the registers of clandestine laboratories are restricted to MDMA manufacture [71]. So, AMP and MET are not common in the Brazilian illicit market. In fact, studies conducted in urine and oral fluid of Brazilian workers, including truck drivers, have shown no positive samples for MET [7, 90, 91] and positive AMP results was mainly due to FEN consumption, since FEN metabolizes to AMP [7]. Otherwise,

the number of Federal Police seizures of medicines containing FEN and DIE are raising over the years, being observed in 2009 an increase of 61% and 183%, respectively, compared to 2008. There are also several national studies showing high prevalence in the consumption of medicines containing DIE and FEN [92,93] specially by professional truck drivers [94-96] to avoid fatigue and to keep them driving for longer periods. In general, this pharmaceuticals are irrationally prescribed and used by Brazilians [92,93] existing many reports of illegal sales at gas stations, tire repair shops, restaurants, snack bars, markets and even at the commercial trucking companies [94,95,96]. There are also reports about anorectics smuggled from Paraguay [95], probably FEN, since it's the only anorectic legally marketed in that country (table 3).

3.4 Important Issues

In Brazil, the national traffic code [97] states that driving on public roads with alcohol blood concentration equal or higher than 0.6 g L^{-1} or under the influence of any other psychoactive substance is crime subject to arrest, fine and driver's license suspension. The law also establishes that every driver stopped by traffic patrol on suspicion of driving under the influence of drugs will be subjected to clinical examinations, alcohol and drug tests to certify their condition. However, the only apparatus available for roadside police enforcement are breathalyzers to evaluate alcohol consumption. The Brazilian officers do not dispose of any on-site screening kit to attest preliminarily drug consumption, neither oral fluid collection devices to take a biological sample representative of the driver's condition at the moment of the police approach. In case of suspected impairment due to drugs other than alcohol, the conductor must be taken to the nearest forensic unit to collect blood and urine samples, what may take more than an hour. Thus, in spite of legal provisions, Brazil has not yet regulated on-site tests or oral fluid collection devices to be employed by roadside police enforcement.

As already pointed out, several national studies have demonstrated high prevalence of ATS consumption among Brazilians truck drivers. These researches, conducted though questionnaires, have shown a self-reported prevalence of anorectics use (at least once) of 11% [8], 65% [96], 66% [94] and 97% [95]. However, some studies employing oral fluid and urine samples to determinate ATS

trucker's consumption through laboratory tests have shown prevalences ranging from 0,7% to 4,8% [7,90]. This great lack of concurrence between self-reported ATS-use and positive laboratory tests, suggests that the tests used in the laboratories were not adequate to detect the appetite suppressants abused by Brazilian truck drivers. The studies mentioned have employed immunoassays to screen presumably positive urine samples or gas chromatograph-mass spectrometry (GC-MS) with selected-ion monitoring (SIM) mode directed only to AMP and MET m/z fragments, to analyze oral fluid samples.

Aware of the possible problems of the commercial immunoassays and thinking on tools to assist Brazilian police in identifying drugged drivers, our research group, with governmental financial support, have decided to evaluate the adequacy of some available oral fluid immunoassays.

Our results, either from experimental assays or from the study of kits technical information, have confirmed our initial thoughts. The current AMP and MET oral fluid immunoassays are not able to detect the appetite suppressants misused in Brazil, not even in very high concentrations, and the same occurring with MPH. Therefore, an initial screening of oral fluid using these kits will produce a lot of false negative results.

Considering that most of the roadside studies (or routine police applications) employ immunoassays as preliminary drug screening tests, and that only positive results in these assays are subsequently submitted to confirmatory techniques, then it depends on the antibodies' specificity the drugs that can be detected in the evaluated population.

According to Walsh [98], over the years the immunological screening assays have been designed to be more and more specific and no longer cross-react with other similar compounds, and this can be one of the explanations for the lack of concurrence between the number of laboratory positive tests (66% decrease) and self-reported drug-use (30% increase) also observed in USA in the period of 1988-2004. Another explanation gave by Walsh is that immunoassays could not be testing for the right drugs. In fact, most of the current AMP or MET kits (tables 1 an 2) probably will not detect the USA controlled ATS (table 3) like MPH, PHD, DIE and

BEZ, neither new appearing drugs as FEN. Despite never having been approved for marketing in USA, imported FEN pills from Brazil have been detected in USA residents over the last years [99-101].

Recent international recommendations for research in drugged driving [102] suggest that epidemiological studies should monitor the broadest spectrum of licit and illicit drugs as possible, with the inclusion of psychostimulants like MPH. Walsh and coworkers [102] pointed that oral fluid and urine immunoassays evaluate a narrow spectrum of drugs, limiting drug detectability.

The current available oral fluid assays restrict not only the detection of prescription ATS, but also the clandestinely manufactured ones. According to the United Nations Office on Drugs and Crime [103] illicit AMP are typically encountered as *d,l*-AMP, for which oral fluid immunoassays (table 1 and 2) have shown low to regular cross-reactivity (37-62%). So, immunological kits based on *d*-AMP antibodies seems not to be adequate for illegal AMP detection. The same does not occur with MET, since it is frequently seen in the illicit market either as the dextro enantiomer or the racemic mixture [77, 103], and both cross-react well with some available kits (table 1 and 2).

The issue of *d*,*l*-AMP low cross-reactivity of oral fluid imunoassays also hinder the indirect detection of FEN, since it is marketed as racemate [104] and metabolizes to *d*,*l*-AMP [19,105,106]. Considering that *d*-AMP cutoff concentration in oral fluid immunoassays usually ranges from 25-50 ng mL^{-1} (table 1) and the average cross-reactivity of *d*,*l*-AMP is around 50% (tables 1 and 2), *d*,*l*-AMP generated from FEN biotransformation will be detected in oral fluid only in concentrations above 50-100 ng ml^{-1}, restricting the detection window for FEN consumption. The same will not occur with BEZ and CLO, since they metabolizes to *d*-AMP [19,87,107]. However, AMP and MET oral fluid immunoassay will fail to detect very recent use of FEN, CLO or BENZ and stimulants not biotransformed to AMP or MET, as DIE, PHD and MPH. The only appetite suppressant that seems to be detected by some kits is PHT, due to its high molecular similarity with AMP, but the cross-reactivity in general is not high (<0.05 to 25% according table 1). This goes against the fact that PHT is the most frequently consumed amphetamine-based anorectic in the world [70], and thus its detectability should be much better.

In spite of the USA alleged medicines diversion problem [70, 84], a recent pilot study [22] conduced to evaluate procedures that would be used in the USA national roadside surveys, determined psychostimulants consumption among drivers using the same oral fluid AMP and MET ELISA kits tested by the authors (Immunalysis Corporation), plus an additional MPH ELISA kit. It means that drivers' use of DIE could not be detected in this study, the same probably occurring with PHD and BENZ (table 3).

It seems that the lack of detection of the world's most common prescription ATS is not a problem restricted to oral fluid kits, happening also with urine. In an earlier study performed in USA [9], truck drivers underwent field sobriety tests conducted by drug recognition expert (DRE) officers, and urine samples were taken and screened for drugs of abuse with EMIT (Enzyme multiplied immunoassay technique). Fifty percent of the drivers arrested for driving under the influence of central nervous system (CNS) stimulants, according to DRE officers' evaluation, had urine positive for these compounds. The authors concluded that it probably occurred due to a very early drug consumption, low immunoassay sensitivity or presence of stimulants undetected by the kit, suggesting that the screening methodology was inappropriate. In fact, the study casually revealed a variety of ATS in truckers' urine, ranging from illicit to over-the-counter (OTCs), including AMP, MET, PHT, phenmetrazine (metabolite of PHD), ephedrine and pseudoephedrine. In another research employing urine immunological kits to detect ATS use among truck drivers, conducted in Brazil [7], the authors also realized that the AMP and MET fluorescence polarization immunoassay (FPIA) used in the screening phase was not appropriate to detect the appetite suppressants abused by truckers. According to their results, in 52,7% of the positive AMP samples FEN was also identified in the confirmation step by GC-MS, showing that AMP was in fact a metabolite of the ingested FEN.

Although recent publications report the use of ATS by Australian drivers as mainly related to illicit manufactured drugs, as MET, AMP and MDMA [4,108], previous studies have shown that prescription appetite suppressants as DIE and PHT are also widely used among conductors, specially by truck drivers as a countermeasure to combat fatigue [11]. Through a questionnaire applied to truckers in Western Australia, Mabbott and Hartley found a prevalence of 20.8% in the consumption of prescription and/or illicit ATS, being AMP and MET the most abused,

followed by prescription DIE and PHT, illegally obtained. An investigation of the incidence of drugs in drivers killed in Australian road accidents between 1990 and 1999 [109], through the analysis of blood samples taken at autopsy or in the hospital, found PHT as the third most prevalent CNS stimulant. In a later study, fatally injured truck drivers cases occurred in Victoria between 1999 and 2007 were reviewed and 13.1% of them were positive for stimulants including PHT. As DIE and PHT are still marketed in Australia (table 1), and the current oral fluid (and probably urine) immunoassays can fail to detect these ATS (at least DIE), the drivers' consumption of such substances may be being underestimated in more recent studies.

In evaluating a survey conducted with car drivers in a Danish rural area, Behrensdorff and Steentoft [21] have discussed the limitations of the two oral fluid screening immunoassays employed, highlighting that some frequently used medicines in Denmark could not be detected by these kits. As shown in table 3, DIE is an anorectic currently marketed in Denmark and probably the kits used in the study did not detect this substance (table 1).

The issue of ATS imunoassays lack of specificity, detecting OTCs like ephedrine, pseudoephedrine and phenylephrine, and causing a high number of false positive results are well known [21,24]. However, the problem of non-detectability (false-negatives) of prescription ATS, like MPH, DIE, FEN, CLO, PHT, PHD, is very little discussed. Since in toxicological analysis negative screened results are not subsequently assayed thought confirmatory methods [110,111], the employed preliminary kits should at least detect the controlled ATS marketed in a country, failing or not on detecting OTCs. Many companies claim that their immunassays detect amphetamines and derivatives, that is not true.

No matter what drug detection immunoassay is chosen, it is imperative the evaluation of the specificity and sensibility of the kit used before application in real samples, at risk of underestimating the real problem of drug's misuse.

5. Conclusion and perspectives

Ordinary ATS immunoassays can fail to cross react with DIE, FEN, CLO, BEZ, PHD, PHT and MPH, since the kit's antibodies are mainly directed to AMP or MET molecules. Considering that DIE, FEN and MPH are the most used controlled

ATS in Brazil, it is evident the necessity of the development of new kits to enable the correct control of ATS abuse in Brazilian roads through oral fluid tests. Due to the large structural variability of the ATS, ideally immunoassays must consist of a pool of antibodies, mono or polyclonal, directed to each different ATS molecule.

We agree with some recent studies [102,112-114] that, in spite of being a promising technique, oral fluid drug detection immunoassays still need great development and performance improvement, before they can be introduced massively on worldwide police routine. Beyond problems like low sensitivity and high number of false-positive results, already extensively discussed for ATS immunoassays [21,24,111,112,114], we consider a major cause of concern the non-detection of controlled ATS legally market in some countries, what can lead to an erroneous estimative of the stimulants being consumed on roads. Indeed, the latest International Narcotics Control Board report [70] recommended authorities to give increased attention to the problem of prescription drug abuse and to include, as far as possible, the monitoring of controlled medicines' abuse in their national surveys.

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Table 1

Oral fluid screening immunoassays used around the world for the detection of amphetamine-type stimulants (ATS) in drivers.

Screening test	Assay Type[a]	Target Drug (cutoff in ng/mL)	Cross-reactivity (%)[b] reported by the manufacturer[c] [36- 45]	Country/Year of kit use
Cozart DDS™ / RapiScan™ (Cozart Biosciences LTD.,UK)	IMMU	AMP (45 ng mL^{-1}) MET (50 ng mL^{-1})	**AMP test:** AMP (100); MDA (45); MDMA (0.9); MDEA (0.1); MBDB (0.1); MET and *d*-EPH (<0.04). **MET test:** MET (100); MBDB (50); MDMA (17); MDEA (2); ANF, MDA and *d*-EPH (<0.05).	Victoria State-Australia / Used since 2004 [25-28] Finland / 2001 [20] Queensland State-Austrália / 2006-2007 [4] Portugal / Approved since 2008 [33] Denmark / 2000 [21]
Cozart™ Microplate EIA (Cozart Biosciences LTD.,UK)	ELISA	*d*-AMP (45 ng mL^{-1}) ---[d]	**AMP test:** *d*-AMP (100); MDA (188-216); MET (0.9-1.7); MDMA (1.0-1.8); MDEA (0.1-0.2); MBDB (0.1-0.2); *d*-EPH, *l*-EPH, *d*-PSE and *l*-PSE (<0.03); MPD (<0.002).	Denmark / 2002-2004 [1] Denmark / 2000 [21]
Immunalysis Direct ELISA™ kits (Immunalysis Corporation, USA)	ELISA	*d*-AMP (50 ng mL^{-1}) *d*-MET (50 ng mL^{-1})	**AMP test:** *d,l*-MDA (178); *d*-AMP (100); *l*-AMP (10); *d*-PSE (1-4); *l*-EPH (0.4-1); *d*-MET (<0.1); *l*-MET (<0.02); *d,l*-MET, *d,l*-MDMA and *d,l*-MDEA (not detected). **MET test:** *d*-MET (100); *d,l*-MDMA (78-98); *d,l*-MDEA (6); *l*-MET (2-3); *d*-PSE (1-4); *l*-EPH (1-2); *d*-AMP (100); *d,l*-AMP (0.2); *l*-AMP (0.2) and *d,l*-MDA (<1).	USA / 2007 [22]
Dräger DrugCheck™ (Dräger Safety AG & Co. KGaA, Germany)	IMMU	*d*-AMP (50 ng mL^{-1}) *d*-MET (50 ng mL^{-1})	**AMP test:** *d*-AMP (100); *d,l*-AMP (50); *d,l*-MDA (50); PHT (17); *l*-AMP (5); *d,l*-MDEA (0.5); *d,l*-MDMA (0.2); *d,l*-MET (0.05); EPH and MPH (< 0.05). **MET test:** *d*-MET (100); *d,l*-MET (100); *d,l*-MDMA (50); *d,l*-MDEA (1); *d,l*-AMP (0.2); *l*-AMP (0.2); PSE (0.1); *d,l*-MDA (0.05); EPH and MPH (< 0.05).	Germany / 2001 [18] Portugal / Approved since 2008 [32]
Drager DrugTest™ 5000 (Dräger Safety AG & Co. KGaA, Germany)	IMMU	*d*-AMP (50 ng mL^{-1}) *d*-MET (35 ng mL^{-1})	**AMP test:** *d*-AMP (100); *d,l*-MDA (25); PHT, MPH, *d*-EPH, *d*-PSE and *l*-PSE (< 0.05). **MET test:** *d*-MET (100); MBDB (87); MDMA (44); MDEA (5.8); MDA (0.5); *d*-EPH (0.07); PHT, MPH, *d*-PSE and *l*-PSE (< 0.05).	Portugal / Approved since 2009 [35] Belgium / 2009 [23] Spain / 2004-2005 [24]
Drugwipe™ (Securetec Detektions-Systeme AG, Germany)	IMMU	*d*-AMP (50-200 ng mL^{-1}) *d*-MET / MDMA (25-100 ng mL^{-1})	---	Victoria State-Australia / Used since 2004 [25-28] Finland / 2001 [20] Finland / 2004-2005 [17] Belgium / 1999-2000 [15] Portugal / Approved since 2008 [34] Belgium / 2009 [23]
Oratec™ III (Branan Medical Corporation, USA)	IMMU	*d*-AMP (25 ng mL^{-1}) *d*-MET / MDMA (25 ng mL^{-1})	**AMP test:** *d*-AMP (100); *d,l*-AMP and MDA (62); MDA; MDEA (25); PHT (25); *l*-AMP (3.2); *d,l*-MET, *l*-MET, *d*-MET and PHY (< 0.25). **MET / MDMA test:** *d*- MET and MDMA (100); *d,l*-MET (83); MDEA (8.3); *l*-MET (5); *d,l*-EPH and *l*-EPH (2.5); *d*-PSE and PHY (0.5), *d,l*-AMP, *l*-AMP, *d*-AMP and PHT (< 0.25).	Portugal / Approved since 2008 [32]
RapidSTAT™ (Mavand Solutions, Germany)	IMMU	*d*-AMP (25 ng mL^{-1}) MET (25 ng mL^{-1}) MDMA (50 ng mL^{-1})	---	Belgium / 2009 [23]
Toxiquick™ (Biomar Systems, Germany)	IMMU	AMP (500 ng mL^{-1}) MET (500 ng mL^{-1})	---	Germany /2000-2002 [16]
Varian Oralab™ test (Varian, USA)	IMMU	*d*-AMP (50 ng mL^{-1}) *d*-MET (50 ng mL^{-1})	---	Spain / 2004-2005 [24]

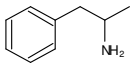
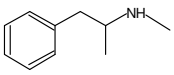
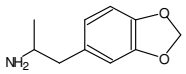
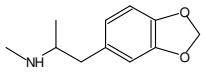
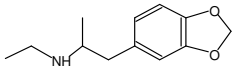
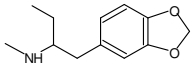
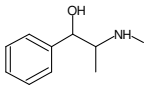
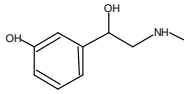
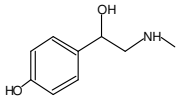
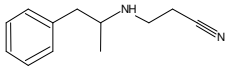
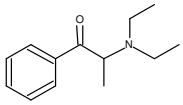
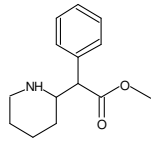
[a] IMMU (immunochromatographic assay); ELISA (enzyme linked immunosorbent assay).

[b] Cross-reactivity (%) was calculated as follows: (Apparent concentration of the target ATS/ ATS concentration of spiked oral fluid) x 100. For immunochromatographic tests, apparent concentration was kit cutoff concentration; for ELISA, it was the equivalent target drug concentration calculated from the spiked sample response (absorbance).

[c] AMP (Amphetamine); EPH (Ephedrine); MBDB (3,4-methylenedioxyphenyl-2-butanamine); MDA (3,4-Methylenedioxyamphetamine); MDEA (3,4-Methylenedioxyethylamphetamine); MDMA (3,4-Methylene-dioxymethamphetamine); MET (Methamphetamine); MPH (Methylphenidate); PHT (Phentermine); PHY (Phenylephrine); PSE (Pseudoephedrine).

[d] Information not found on the Internet and not sent by the manufacturer on request.

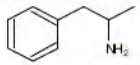
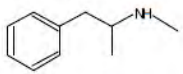
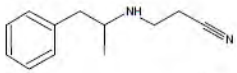
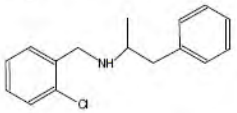
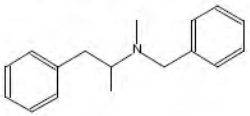
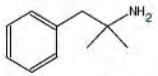
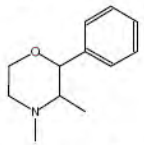
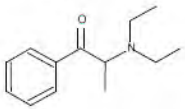
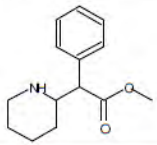
Table 2
Experimental (EXP) and manufacturer (MAN) [42,43,73] analytical specificity of Immunalysis Amphetamine (AMP ELISA) and Methamphetamine (MET ELISA) Direct ELISA™ kits and Oral-View™.

ATS[a]		Cross-reactivity (%)[b]					
		AMP ELISA		MET ELISA		Oral-View	
		EXP	MAN	EXP	MAN	EXP	MAN
***d*-AMP**		-[d]	100	-	< 1	-	100
***d,l*-AMP**		37	-	0.14	< 1	50	50
***l*-AMP**		-	10	-	< 1	-	-
***d*-MET**		-	< 0.1	-	100	-	-
***d,l*-MET**		0.05	n.d.[c]	51	-	-	-
***l*-MET**		-	< 0.02	-	2-3	-	0.1
***d,l*-MDA**		347	178	0.17	< 1	-	50
***d,l*-MDMA**		0.2	n.d.	95	78-98	-	< 5
***d,l*-MDEA**		0.2	n.d.	28	6	-	< 0.5
***d,l*-MBDB**		0.2	-	19	-	-	< 0.05
***d*-PSE**		< 0.007	1-4	0.6	1-4	-	-
***l*-EPH**		< 0.006	0.4-1	0.9	1-2	-	-
***l*-PHY**		< 0.006	-	< 0.02	-	-	-
***d,l*-SYN**		< 0.007	-	< 0.02	-	-	-
***d,l*-FEN**		< 0.01	-	< 0.02	-	< 10	-
***d,l*-DIE**		< 0.006	-	< 0.02	-	< 10	-
***d,l*-threo-MPH**		< 0.006	-	< 0.02	-	< 10	-

[a] ATS (amphetamine-type stimulant); AMP (Amphetamine); DIE (Diethylpropion); EPH (Ephedrine); FEN (Fenproporex); MBDB (3,4-methylenedioxyphenyl-2-butanamine); MDA (3,4-Methylenedioxyamphetamine); MDEA (3,4-methylenedioxyethylamphetamine); MDMA (3,4-Methylene-dioxymethamphetamine); MET (Methamphetamine); MPH (Methylphenidate); PHY (Phenylephrine); PSE (Pseudoephedrine); SYN (Synephrine).
[b] Cross-reactivity (%) was calculated as follows: (Apparent concentration of the target ATS/ ATS concentration of spiked oral fluid) x 100. For the immunochromatographic assay Oral-View™, apparent concentration was kit cutoff concentration; for Immunalysis ELISA, it was the equivalent target drug concentration calculated from the spiked sample response (absorbance).
[c] n.d. (not detected); [d] – (not tested)

Table 3

Some controlled amphetamine-type stimulants (ATS) currently marketed around the world [19,46-68,85-89][a,b,c]

ATS	Molecular structure	Biotransformed to AMP or MET	BRA	ARG	URY	PRY	COL	VEN	CHL	PER	MEX	USA	CAN	PRT	ESP	ITA	FRA	DEU	GBR	NLD	BEL	DNK	AUT	FIN	NOR	AUS
AMP		——	X	X	X	X	X	X	√	X	X	√	√	X	X	X	X	X	X	X	X	X	X	X	X	X
MET		AMP	X	X	X	X	X	X	X	X	X	√	X	X	X	X	X	X	X	X	X	X	X	X	X	X
FEN		AMP	√	X	X	√	X	X	√	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CLO		AMP	X	X	X	X	X	X	X	X	√	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
BEZ		MET, AMP	X	X	X	X	X	X	X	X	X	√	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PHT		UNB	X	X	X	X	X	√	X	X	√	√	X	X	X	X	X	X	X	X	X	X	√	X	X	√
PHD		UNB	X	X	X	X	X	X	X	X	X	√	X	X	X	X	X	X	X	X	X	X	X	X	X	X
DIE		UNB	√	X	X	X	X	X	√	X	√	√	X	X	X	X	X	√	X	X	X	√	X	X	X	√
MPH		UNB	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√

[a] AMP (*d,l*-Amphetamine or d-Amphetamine); MET (*d*-Methamphetamine); FEN (Fenproporex); CLO (Clobenzorex); BEZ (Benzphetamine); PHT (Phentermine); PHD (Phendimetrazine); DIE (Diethylpropion); MPH (Methylphenidate); UNB (not biotranformed to AMP or MET); X (not marketed); √ (marketed).

[b] BRA (Brazil); ARG (Argentina); URY (Uruguay); PRY (Paraguay); COL (Colombia); VEN (Venezuela); CHL (Chile); PER (PE); MEX (México); USA (United States of America); CAN (Canada); PRT (Portugal); ESP (Spain); ITA (Italy); FRA (France); DEU (Germany); GBR (United Kingdom); NLD (Netherlands); BEL (Belgium); DNK (Denmark); AUT (Austria); FIN (Finland); NOR (Norway); AUS (Australia).

[c] Ethylamphetamine, Amfetaminil, Mefenorex, Prenylamine, Fenethylline, Mesocarb, Fencamfamine, Dimethylamphetamine, Furfenorex, Famprofazone, Fencamine, Fenfluramine and Aminorex are not currently marketed in the countries surveyed.

5 ARTIGO 2:

"Determination of amphetamine–type stimulants in oral fluid by solid-phase microextraction and gas chromatography-mass spectrometry"

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Determination of amphetamine–type stimulants in oral fluid by solid-phase microextraction and gas chromatography-mass spectrometry

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Abstract

A method for the simultaneous identification and quantification of amphetamine (AMP), methamphetamine (MET), fenproporex (FEN), diethylpropion (DIE) and methylphenidate (MPH) in oral fluid collected with Quantisal™ device was developed and validated. In-matrix propylchloroformate derivatization, followed by direct immersion solid-phase microextraction and gas chromatography-mass spectrometry were employed. Deuterium labeled AMP was used as internal standard for all the stimulants and the analysis was performed in the selected ion monitoring mode. The detector response was linear for the studied drugs over the range of 2-256 ng mL^{-1} in neat oral fluid, except for FEN where the linear range was 4-256 ng mL^{-1}. The detection limits were 0.5 ng mL^{-1} (MET), 1 ng mL^{-1} (MPH) and 2 ng mL^{-1} (DIE, AMP, FEN). Accuracy of quality control samples were within 98.2 – 111.9% of the target concentrations and precision did not exceed 15% of relative standard deviation. Recoveries with Quantisal™ device ranged from 77.2 to 112.1%. The goodness-of–fit of the ordinary least squares model in the statistical inference of data was tested through residuals plotting and ANOVA. The validated method can be easily automated and then used for screening and confirmation of amphetamine-type stimulants in drivers' oral fluid.

Keywords: amphetamine, methamphetamine, diethylpropion, fenproporex, methylphenidate, oral fluid

1. Introduction

Drug driving is one of the major contributors to road fatalities in the world. Over the last years, several studies [1-6] have shown high prevalence in the consumption of psychoactive substances among drivers, especially amphetamine-type stimulants (ATS), which are widely used by professional drivers to increase their alertness during long journeys [7-11].

The use of oral fluid for monitoring drug consumption on roads has many advantages over the conventional biological fluids (urine and blood), since the collection does not require medical experience, it is less intrusive to the donor's privacy, it can be easily observed by the police officer, thus avoiding sample substitution or adulteration, and it informs about recent drug use [12,13]. Due to

these advantages, oral fluid tests have been applied successfully by some traffic polices around the world for drugged driver's control [14-16].

Amphetamine (AMP), methamphetamine (MET), fenproporex (FEN), diethylpropion (DIE) and methylphenidate (MPH) are some of the most abused ATS worldwide (fig. 1) [17,18], and the immunoassays applied as oral fluid screening tests can fail to detect some of these substances and, when detected, the results are not unambiguously guaranteed. Thus, it is important to develop tools for sensitive, specific and reliable detection of ATS in oral fluid [19-21], especially by gas chromatography-mass spectrometry (GC-MS), a technique widely available in forensics laboratories [22].

Fig. 1

Several GC-MS methods have been developed to analyze ATS in oral fluid [23-28], but none of them have been validated for simultaneous detection and quantification of AMP, MET, FEN, DIE e MPH. This one run low cost identification of five prescription ATS is particularly important in South America countries, where the consumption of ATS is mainly through diverted pharmaceuticals [9,10,18].

Among the derivatizing reagents that have been used with ATS prior to GC-MS analysis, alkylchloroformates (ALCL) stand out as the most suitable for biological fluids, since they react directly and quickly in alkaline aqueous samples, at room temperature, under agitation, resulting in water-stable alkylcarbamates [29-32]. Combined with solid-phase microextraction (SPME), this became a very practical and environmentally friendly technique, due to the small number of procedures, little sample handling, complete absence of organic solvents, no need for drying steps and suitability for small sample volumes [24,30,33-37].

The present work reports the development and validation of a simultaneous in-matrix propylchloroformate derivatization of AMP, MET, FEN, DIE and MPH in buffered oral fluid, followed by direct immersion SPME (DI-SPME) and GC-MS analysis.

2. Experimental

2.1 Materials

MET, AMP and AMP-D_5 (internal standard, IS) were purchased from Cerilliant (Round Rock, TX, USA) as 1mg mL^{-1} methanol solutions. FEN, DIE and MPH hydrochlorides were gently donated by Aché (Guarulhos, SP, Brazil) and Novartis (Resende, RJ, Brazil). Lidocaine, procaine, cocaine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA) and 3,4-methylenedioxyphenyl-2-butanamine (MBDB) were obtained from Lipomed (Arlesheim, Switzerland), as hydrochloride salts. Synephrine was purchased from MP Biomedicals (São Paulo, SP, Brazil), sibutramine hydrochloride monohydrate was obtained from Galena (Campinas, SP, Brazil) and atropine, homatropine, scopolamine and mazindol from Henripharma (Cambuci, SP, Brazil). SPME manual holder and polydimethylsiloxane (PDMS) coated fibers (30 µm and 100 µm), as well ethylchloroformate (ETCL), propylchloroformate (PRCL), butylchloroformate (BUCL), phenylchloroformate (PHCL), nicotine, caffeine, phenylephrine, pseudoephedrine, benzocaine, phenacetin, paracetamol and dipyrone were obtained from Sigma-Aldrich Corporation (São Paulo, SP, Brazil). Methanol, NaOH, KOH and ephedrine were purchased from Merck (Darmstadt, Germany), and Na_2CO_3, K_2CO_3, Na_2SO_4, NaCl and Na_3PO_4 from Synth (São Paulo, SP, Brazil). Quantisal™ oral fluid collection devices, filters and preservative buffer solution were purchased from Immunalysis Corporation (Pomona, CA, USA). Each device contained a collection pad with an indicator that turns blue when 1 mL of oral fluid has been collected, and a plastic transport tube with 3 mL of preservative buffer (final specimen volume of 4 mL).

2.2 Blank oral fluid

The blank oral fluid used in the validation experiments consisted of drug-free specimens collected from eight volunteers directly in polypropylene tubes. The specimens were pooled, centrifuged, and frozen. Each day of analysis an aliquot was thawed, and pH adjusted to 6.5 with HCl.

2.3 Standard solutions and work samples

Separate stock solutions of all substances tested were prepared in methanol at 50 µg mL^{-1}, aliquoted in 2 mL vials, and stored at -18 ± 4°C. AMP-D_5 working solution was made in methanol at the final concentration of 2.4 µg mL^{-1} and stored at 4 ± 2°C.

Standard curves were obtained by spiking blank oral fluid with AMP, MET, FEN, DIE and MPH stock solutions at final concentrations ranging from 2 to 256 ng mL^{-1}. Low, middle and high quality controls (QC) were obtained in a similar way, at 6 ng mL^{-1}, 100 ng mL^{-1} and 200 ng mL^{-1}, respectively, for all ATS.

All oral fluid work samples were prepared in the day of the analysis, with aliquots of freshly thawed vials. The final solutions were diluted with Quantisal™ preservative buffer (3 mL of buffer to each 1 mL of sample), in order to mimic a collection procedure with Quantisal™ device. When the device was used, the dilution ocurred directly in the transport tube.

2.3 Extraction and derivatization procedure

An aliquot of 1.5 mL of diluted oral fluid was transferred to a 2 mL vial containing a magnetic stirring bar, 75 mg of Na_2CO_3, 150 mg of Na_2SO_4, 5 µL of PRCL and 10 µL of a 2.4 µg mL^{-1} IS solution (final pH 10.1). After vortex for 1 min, a SPME fiber (PDMS 30 µm) was immersed into the solution and left under magnetic stirring (1200 rpm) for 20 min, at room temperature (20 ± 2 °C). Elapsed the extraction time, the fiber was desorbed in a GC-MS injector port (260 °C) for 15 min, and then rinsed with deionized water to remove excess of salts.

The method was established by testing four different ALCL, two SPME techniques (immersion and headspace SPME), several salts to increase pH and matrix ionic strength, two PDMS fiber coatings (30 µm and 100 µm) and many sorption and desorption times.

2.4 GC-MS analysis

The analysis were carried out on a 6890N gas chromatograph coupled to 5973 inert mass selective detector (Agilent Technologies, Palo Alto, CA, USA),

equipped with a 900 µL deactivated glass liner and a 30 m x 0.25 mm x 0.25 µm 5% phenyl-methylpolysiloxane column (HP-5MS, Agilent J&W Scientific, Folsom, CA, USA). The oven temperature was programmed at 100 ºC (1 min), increased at 15 ºC min^{-1} to 250 ºC (3 min), and then at 15 ºC min^{-1} to 280 ºC (4 min). Helium (99.9999% purity) was used as carrier gas at flow rate of 1 mL min^{-1}. The injector was maintained at 260 ºC and operated for 1 min in splitless mode. The temperatures of the interface, ion source and quadrupole were 280 ºC, 230 ºC and 150 ºC, respectively. The MS system was operated in electron impact ionization mode at 70 eV and in selected-ion monitoring (SIM). The following ions were monitored (underlined ions used for quantification): 100, 105, 72 (DIE); 130, 162, 91 (AMP propylcarbamate, AMP PROPYL); 134, 167, 92 (AMP-D_5 propylcarbamate, AMP-D_5 PROPYL); 144, 176, 102 (MET propylcarbamate, MET PROPYL); 183, 97, 91 (FEN propylcarbamate, FEN PROPYL) and 170, 128, 91(MPH propylcarbamate, MPH PROPYL). For stability evaluation of ATS stock solutions, GC-MS was operated in scan mode (range 30-350 m/z), with the injection of 0.2 µL of methanol stock solutions in split mode (ratio 5:1).

2.5 Method validation

2.5.1 Specificity

The method specificity was evaluated by analyzing oral fluid samples collected with Quantisal™ device from eight non-ATS-using volunteers. In addition, it was tested blank oral fluid spiked with each ATS separately, as well as samples fortified with 1000 ng mL^{-1} of potential interfering substances, including licit and illicit ATS, anorectics, tropane alkaloids, analgesics and local anesthetics used as cutting agents to adulterate drugs [38].

The substances tested were ephedrine, pseudoephedrine, phenylephrine, synephrine, MDA, MDMA, MDEA, MBDB, mazindol, sibutramine, lidocaine, benzocaine, procaine, phenacetin, paracetamol, dipyrone, cocaine, atropine, homatropine, scopolamine, caffeine and nicotine.

It was looked for interfering peaks at the retention times of DIE, AMP PROPYL, MET PROPYL, FEN PROPYL and MPH PROPYL.

2.5.2 Linearity

The linearity of the method was accessed through six standard curves, run at different days and prepared by spiking blank oral fluid with AMP, MET, FEN, DIE and MPH at the concentrations of 2, 4, 8, 16, 32, 64, 128 and 256 ng mL^{-1}.

2.5.3 Intra and Inter-day accuracy and precision

Intra-day accuracy and precision were evaluated during a single run, analyzing five replicates of low, middle and high QC samples. Inter-day assay were performed over five non-consecutive days, analyzing five (day 1), two (days 2 to 4) and three (day 5) replicates of each CQ sample (n=14). Precision was expressed as relative standard deviation (RSD) and accuracy as percent of theoretical concentration.

2.5.4 Detection and quantification Limits

The limits of detection (LOD) were determined by analyzing blank oral fluid spiked with low and decreasing ATS concentrations until a signal-to-noise ratio (peak height) of about three was achieved. The lower limits of quantification (LLOQ) were estimated in the same way, considering a minimum signal-to-noise ratio of five. LLOQ were tested for accuracy (between 80-120%) and precision (up to 20%) by analyzing six independent fortified oral fluid samples.

2.5.5 Recovery

We evaluated the efficiency of the Quantisal™ device for the recovery of ATS from oral fluid, taking into consideration the possible loss of analytes retained in the collection pad, filter and transport tube. Triplicates of low, middle and high QC oral fluid samples were pipetted (1 mL) on Quantisal™ collector pads and waited until the volume indicator turns blue. Then, the pads were inserted into the transport tubes, containing exactly pipetted 3 mL of preservative buffer, and left five hours at room temperature (21 ± 2°C). After passing through Quantisal™ filters, samples were extracted and analyzed by the proposed method. The results were compared with QC samples (n=3) directly diluted with preservative buffer (1:3 v/v), without using the collection device (100% recovery).

2.5.6 Stability

The stability of ATS methanol stock solutions were evaluated at room temperature (21 ± 2°C; 12 and 24 hours) and at -18 ± 4°C (1 and 3 months).

The stability of ATS in oral fluid diluted with Quantisal™ preservative buffer (1:3 v/v) were evaluated in low and high QC samples, in triplicate, after short-term (48 hours at 21 ± 2°C and at 4 ± 2°C) and long-term (3 months at -18 ± 4°C) storage, as well as after three freeze and thaw cycles. For short-term stability assays, the samples were kept inside the Quantisal™ plastic transport tubes (without collection pads), while for long-term and freeze-thaw cycles assays samples were transfered to glass tubes. The analytical results for stored and freezed-thawed QC samples were compared with those quantified just after the preparation.

2.6 Statistical analysis

It was assessed the suitability of ordinary least squares model in the statistical inference of data, by applying the model to the six standards curves generated for each ATS (n=48). Data were evaluated with and without mathematical transformations, through residuals and variance (ANOVA) analysis [39-41]. Before the establishment of the appropriate treatment for the data, we proceeded the identification and deletion of outliers in the replicates with Box-Plot and single Grubbs test at 95% of confidence [39,40].

3. Results and discussion

3.1 Derivatization

In ATS analysis by GC-MS, derivatization is necessary to improve resolution, sensitivity and selectivity, the last one essential for low molecular weight ATS, like AMP and MET, which show little discriminative mass spectra [22,42]. The performances of ETCL, PRCL, BUCL and PHCL in derivatizing the studied ATS were evaluated. According to some previously published papers [30,43,44], pH 10 or higher is the best for the reaction of ALCL and ATS in aqueous media. Thus pH 10 was chosen for method development, in order to achieve good yields of derivatives without damaging SPME fibers.

In the assays, PHCL quickly proved to be inappropriate for this issue, since it hydrolyzed in alkaline aqueous medium, originating a white precipitate, identified by GC/MS mass spectral library [45] as diphenylcarbonate.

The derivatization with ETCL, PRCL and BUCL was fast (less than 1 minute, under vigorous agitation) and complete, generating single derivatives of AMP, MET, FEN and MPH. The reaction conditions employed (ambient temperature, alkaline aqueous media) were too mild to derivatize DIE, a tertiary amine, no matter which ALCL concentration evaluated. ETCL, PRCL and BUCL derivatization increased ATS molecular weight in 72, 86 and 100 Da, respectively, and improved peak abundances by 18-58 times for AMP, 18-29 times for MET, 7-15 times for FEN and 3-7 times for MPH. Among the assessed reagents, PRCL proved to be the most suitable for the simultaneous derivatization of AMP, MET, FEN and MPH, since produced the highest peak areas/heights derivatives for MET and FEN, and the second highest for AMP and MPH. The largest peak areas/heights for AMP and MPH were achieved with BUCL and ETCL, respectively.

3.2 Optimization of extraction parameters

After some initial testes with headspace SPME (HS-SPME), we found DI-SPME more practical for ATS derivatives extraction, since there was no need to heat the samples to promote the volatilization of the analytes. Moreover, it's difficult to obtain homogeneous and constant heating on ordinary magnetic stirring plates, thus requiring more sophisticated apparatus.

Considering that Quantisal™ preservative solution is already buffered at pH 6.5 with Na_2HPO_4/NaH_2PO_4, it wasn't necessary to test buffer systems to keep oral fluid samples at the same pH, a very important issue to avoid differences in the SPME extraction efficiency [46]. Instead, we only tested simple alkalis to increase sample pH to around 10, as NaOH and KOH solutions, Na_2CO_3, K_2CO_3 and Na_3PO_4. Among these, Na_2CO_3 was the more appropriate because it does not dilute the samples, it is also cheap and widely available, as well as easy handling and less hygroscopic than the corresponding potassium salt.

The addition of NaCl or Na_2SO_4 to increase matrix ionic strength substantially improved ATS recovery, in a similar way for both salts in the quantities of 10 or 20% (w/v), so we chose Na_2SO_4 at the lower concentration.

Regarding the influence of SPME fiber coating thickness in sorption and desorption of ATS derivatives, we evaluated 30 µm and 100 µm PDMS fibers. In the sorption experiments (fig. 2), both coatings showed similar ATS recovery across time, except for MPH, with which 30 µm PDMS was greatly superior. Considering the sorption time, 20 min was appropriate to reach the desired method sensibility with both fibers, without increasing too much the desorption time.

When analyzing the desorption efficiency of the fibers, carryover was observed with 100 µm PDMS even after 16 min of desorption in the GC injection port (260 ºC). Since the 30 µm PDMS fiber desorbs easier than the thicker one, and the recovery results for MPH were better with this fiber, it was selected for method development.

Fig. 2

3.3 Goodness-of-fit and method validation

According to FDA bioanalytical guideline [47], the simplest mathematical model that adequately describes the concentration-response relationship should be used in a method validation. So, we began with the goodness-of-fit evaluation of ordinary least squares model for linear regression of the raw data. The visual inspection of the xy scatter plot of concentration *versus* peak area ratios between ATS and IS (fig. 3, chart A) showed that the analytical method was linear, since the responses were proportional to the concentration for all analytes. However, when we looked at the plots of residuals *versus* concentration and normal probability of residuals (fig. 3, chart B and C) we realized that there was a lack of homoscedasticity and normality in the residuals' distribution. The variance of errors were dependent to ATS concentration, increasing with it (fig. 3, chart B).

It is well known that under residuals heteroscedasticity and non-normality, the method of ordinary least squares does not produce the best estimators, and the components of variance and F test (ANOVA lack-of-fit test) can greatly be affected,

compromising the statistical conclusions [41]. In fact, ordinary least squares model for linear regression and ANOVA are based on the assumptions of normality, homoscedasticity and independency of residuals [41,48].

Ordinary least squares method (unweighted linear regression) assumes that the dispersion of y-residuals are nearly the same for all standards in the calibration range, and then each standard contributes equally in estimating the slope and intercept [41,49,50]. In bioanalysis, ranges have to span two or three orders of magnitude to cover at least part of *in vivo* expected concentrations, what is usually associated with significant heteroscedasticity [51,52]. If data RSD is more or less constant across the range, standard deviation and residuals size proportionally increases with analyte concentration, which means that absolute errors associated with high concentrations will be higher [50]. As unweighted regression treats all points equally, lower concentration points are more likely not to be fitted to the line [51], impairing the accuracy at the lower end of the range and raising the LLOD.

In agreement with previous findings [41,49,51-53], we observed that many publications in the field of analytical toxicology report the use of ordinary least squares without mention if the basic assumptions associated with the model were previous checked. A linear regression analysis should never be accepted without evaluating the validity of the model to the data, and the coefficients of correlation (r) or determination (r^2) do not inform about that.

When the basic assumptions of ordinary least squares model are not met, data should mathematically be transformed or weighted least squares model should be applied [40, 53].

In our opinion, it is easier to transform data then change model, since the simplest commercial spreadsheet softwares are able to calculate ordinary least squares parameters (line slope and intercept). That does not occur with more complicated models. Thus, we applied a mathematical transformation of data in order to stabilize the variance and normalize the residuals, and the function that showed the best results was natural logarithm (Ln) for both concentrations (x) and peak area ratios (y). The concentrations needed to be transformed in order to let then evenly spaced across the range, since we originally worked with concentrations multiples of

two (fig. 3, charts A and D). With respect to peak area ratios, Ln transformation homogenized and normalized the residuals along the regression line (fig. 3, charts D-F), satisfying the ordinary least squares and ANOVA requirements. In applying ANOVA lack-of-fit test to transformed data, it was verified no deviation from linearity (p>0.05) and a correct adjust to the model, being observed a high significance (p<0.0001) of the regression for all ATS curves.

Fig. 3

With transformed data to natural logarithm (Ln x, Ln y), we were able to achieve better correlation coefficients (table 1) and improve accuracy at the lower concentrations, reducing 2-8 times the LLOQ for all the studied ATS. The final linear ranges were from 2-4 ng mL^{-1} to 257 ng mL^{-1}.

Table 1

The method proved to be sensitive, with LOD between 0.5 and 2 ng mL^{-1} (table 1) and accuracy within 98.2 – 111.9% of the target concentrations (Table 2). The intra and inter-day precision did not exceed 15% of RSD for the ATS tested, thus it was considered acceptable for the intended purpose (Table 2).

Table 2

Among the eight sources of blank oral fluid and twenty two substances tested, none of them showed interference with the identification and quantification of the studied ATS, the same occurring when each ATS was analyzed separately. To increase method's specificity and sensibility we selected m/z fragments trying to cover the entire molecular structure of analytes (fig. 4) and used base peaks as quantification ions (fig. 5). In the case of FEN, we choose m/z 183 as the quantification ion instead of the base peak (m/z 97), because the blank matrix noise was filled with the last ion.

Fig. 4

Fig. 5

Recoveries from oral fluid collected with Quantisal™ device ranged from 77.2 to 112.1%, with the major losses being observed for MPH (table 3), but still considered adequate for the intended purpose.

Table 3

The stability assays were intended to reproduce a common routine of oral fluid collection and transport to laboratory for analysis, which can last up to 48 h refrigerated or at room temperature. In the same way, samples are usually frozen until the chromatographic analysis and may be thawed and frozen again if screened with immunological tests. All these conditions were evaluated in the proposed assays and no relevant degradation was observed, with deviations of less than 15% of the initial concentrations. ATS methanol stock solutions remained stable after 24 h at room temperature (21 ± 2°C) and after 3 months frozen (-18 ± 4°C).

4. Conclusion

The proposed method employs few sample preparation steps and does not use organic solvents, either in the extraction or derivatization. The validation showed that the method is linear, specific and highly sensitive, with recovery, precision and accuracy within the accepted limits for bioanalytical method validation [47]. The method can be easily automated with CombiPal autosampler (Varian, Palo Alto, USA) and then be used for screening, confirmation and quantification of AMP, MET, FEN, DIE e MPH in drivers' oral fluid in a single quick and inexpensive run.

We highlight the importance of residuals' evaluation in checking the model goodness-of-fit during the validation of a new analytical procedure. In our case, with a simple mathematical transformation that correctly fit data to the model, LLOQ for all ATS were reduced from 2 to 8 times.

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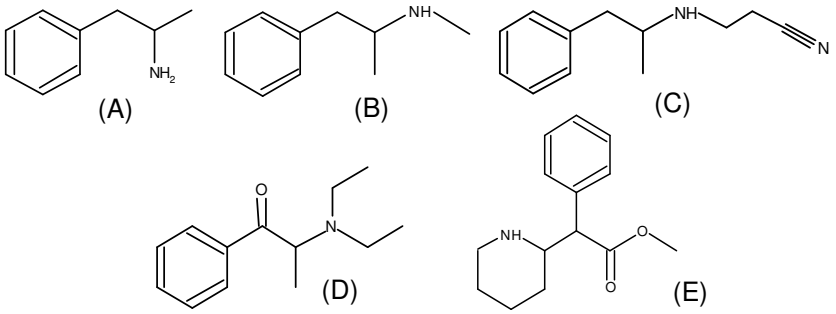


Fig. 1. Chemical structures of Amphetamine (A), Methamphetamine (B), Fenproporex (C), Diethylpropion (D) and Methylphenidate (E).

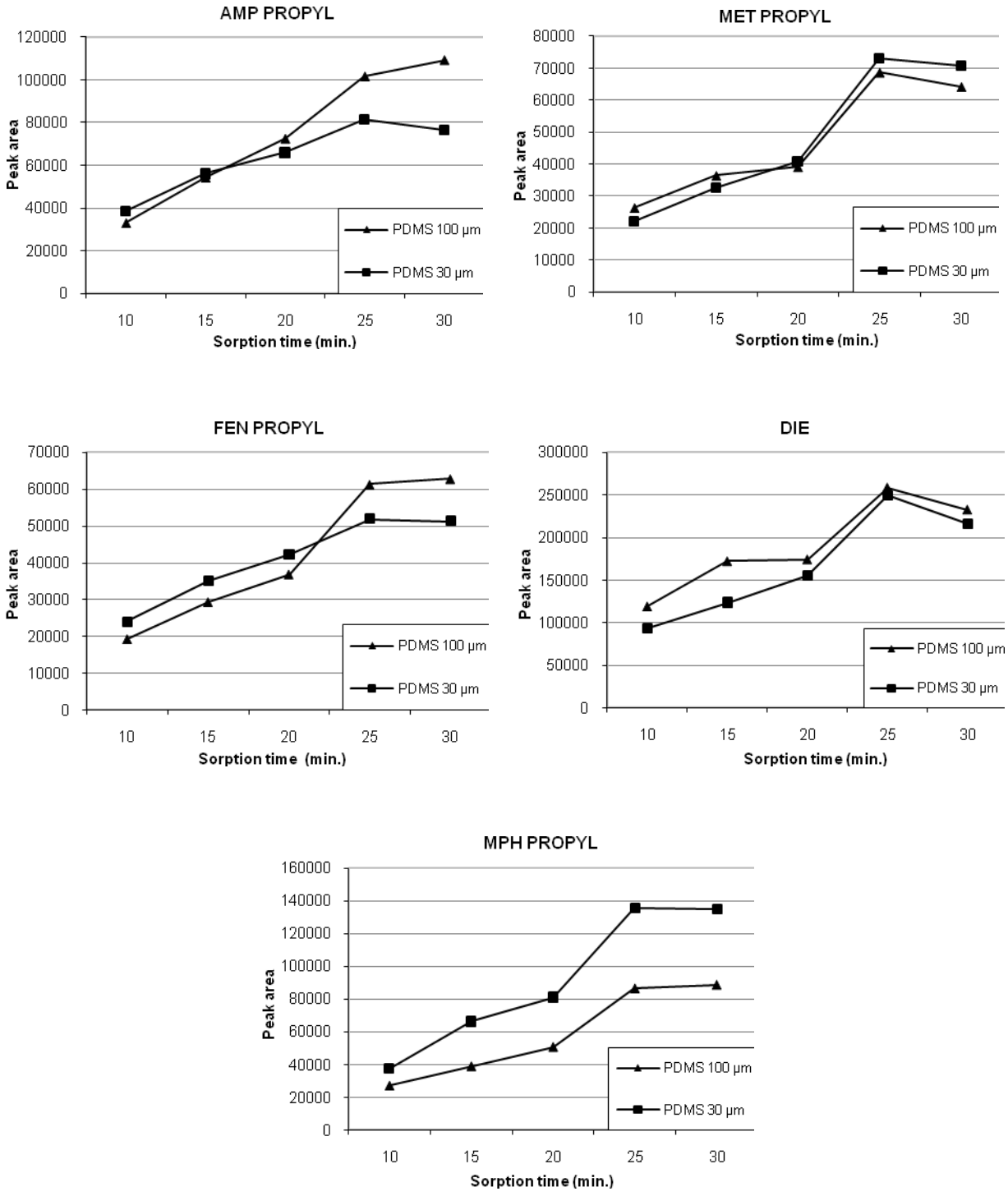


Fig. 2. SPME sorption curves (n=2) for AMP PROPYL (amphetamine propylcarbamate), MET PROPYL (metamphetamine propylcarbamate), FEN PROPYL (fenproporex propylcarbamate), DIE (diethylpropion) and MPH PROPYL (methylphenidate propylcarbamate), extracted with 100 µm and 30 µm PDMS coated fiber. PDMS, polydimethylsiloxane.

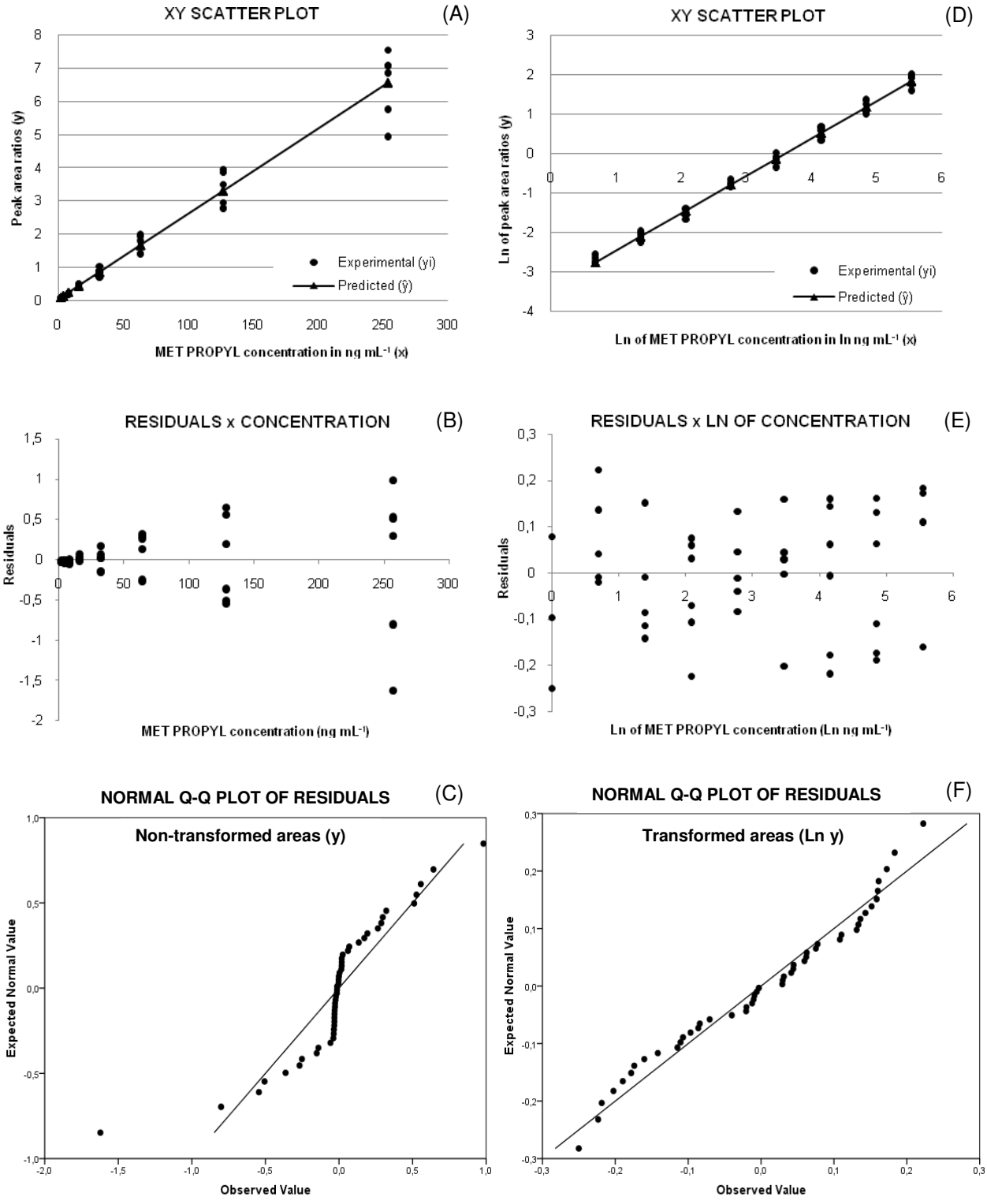


Fig. 3. XY Scatter Plot, Residuals *versus* Concentration Plot and Normal Probability of Residuals Plot (Normal Q-Q Plot) of non-transformed (A,B,C) and transformed (C,D,F) validation data of MET PROPYL (metamphetamine propylcarbamate), subjected to ordinary least squares method. All the tested ATS showed similar charts.

Table 1
Limits of detection (LOD), Pearson correlation coefficients (r), lower (LLOQ) and upper (ULOQ) limits of quantification for calibration results (n=6) [a].

ATS	LOD (ng mL^{-1})	Linear regression with ordinary least squares model				ULOQ (ng mL^{-1})
		Non-transformed data (x,y)		Transformed data (Ln x, Ln y)		
		r	LLOQ (ng mL^{-1})	r	LLOQ (ng mL^{-1})	
AMP	2	0.997	4	0.998	2	256
MET	0.5	0.984	8	0.997	2	256
FEN	2	0.995	8	0.999	4	256
MPH	1	0.990	16	0.996	2	256
DIE	2	0.993	8	0.997	2	256

ATS, amphetamine-type stimulant; AMP, amphetamine; MET, methamphetamine; FEN, fenproporex; DIE, diethylpropion; MPH, methylphenidate; RSD, relative standard deviation; Ln, natural logarithm.
[a] Concentrations in undiluted oral fluid.

Table 2
Accuracy and precision (RSD) of the proposed method

	Low QC [a]				Middle QC [a]				High QC [a]			
	Intra-day (n=5)		Inter-day (5 days; n=14)		Intra-day (n=5)		Inter-day (5 days; n=14)		Intra-day (n=5)		Inter-day (5 days; n=14)	
ATS	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
AMP	103.1	10.9	100.6	8.7	106.7	2.6	104.4	5.4	111.9	1.4	104.6	6.8
MET	100.8	10.8	104.2	9.1	101.8	12.4	99.6	9.1	108.6	12.8	105.6	10.9
FEN	107.9	4.0	101.1	7.8	104.7	6.1	104.4	11.2	99.6	2.2	102.2	7.2
MPH	110.9	6.0	105.1	7.6	108.6	9.0	101.8	10.9	108.6	13.0	104.8	14.1
DIE	100.3	9.7	106.2	10.1	98.2	13.9	100.2	9.9	108.6	8.3	102.1	10.5

ATS, amphetamine-type stimulant; AMP, amphetamine; MET, methamphetamine; FEN, fenproporex; DIE, diethylpropion; MPH, methylphenidate; RSD, relative standard deviation.
[a] Quality control (QC) oral fluid samples: Low QC (6 ng mL^{-1}), Middle QC (100 ng mL^{-1}) and High QC (200 ng mL^{-1}).

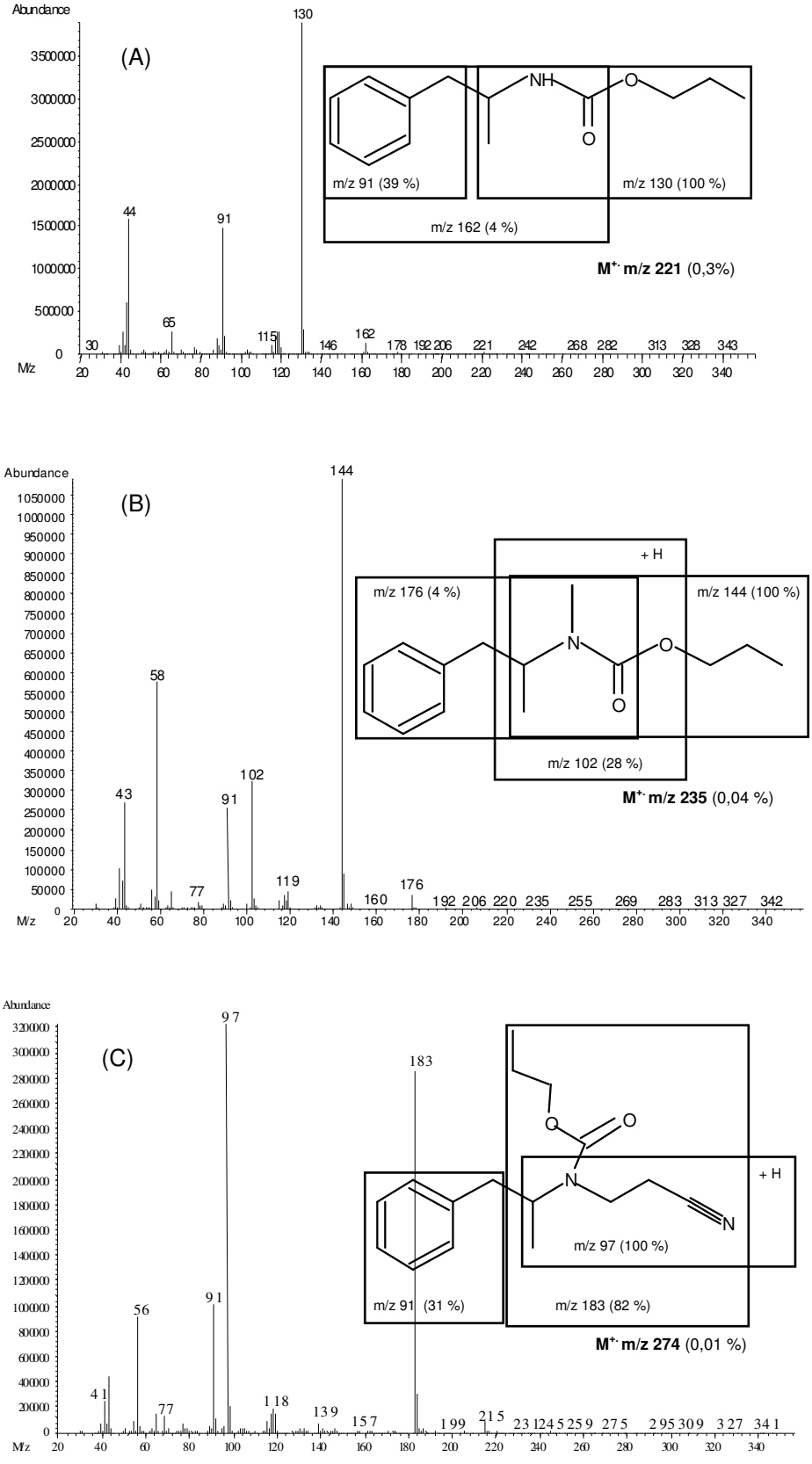
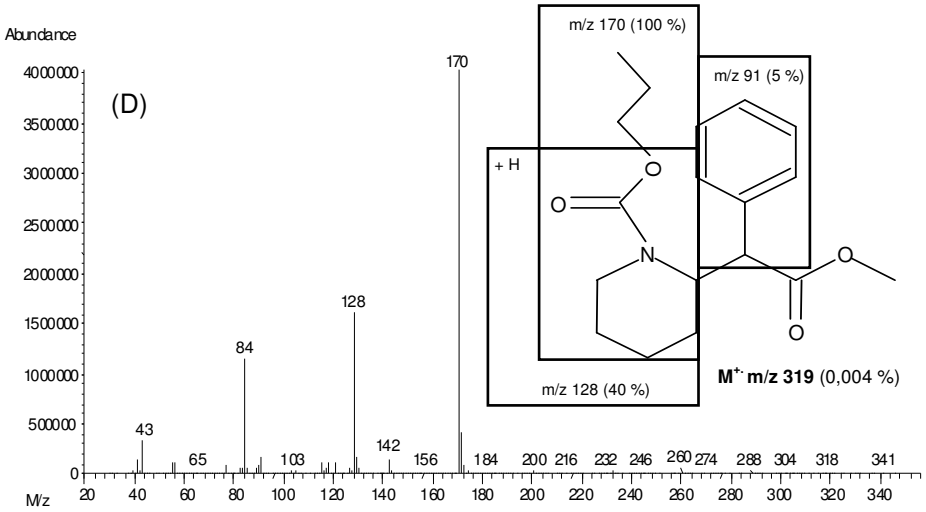


Fig. 4. Mass spectra, proposed molecule fragmentation and relative abundances (%) of m/z fragments selected for monitoring amphetamine propylcarbamate (A), metamphetamine propylcarbamate (B), fenproporex propylcarbamate (C), methylphenidate propylcarbamate (D) and diethylpropion.



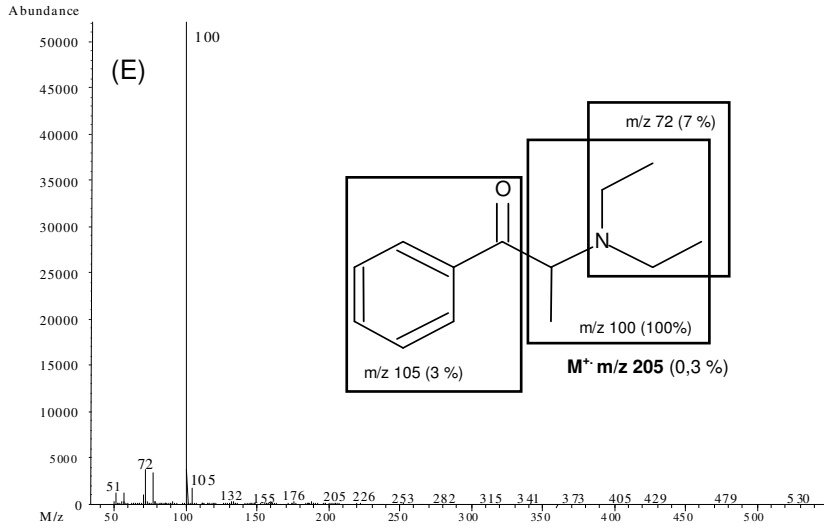


Fig. 4. (continuation) Mass spectra, proposed molecule fragmentation and relative abundances (%) of m/z fragments selected for monitoring amphetamine propylcarbamate (A), metamphetamine propylcarbamate (B), fenproporex propylcarbamate (C), methylphenidate propylcarbamate (D) and diethylpropion.

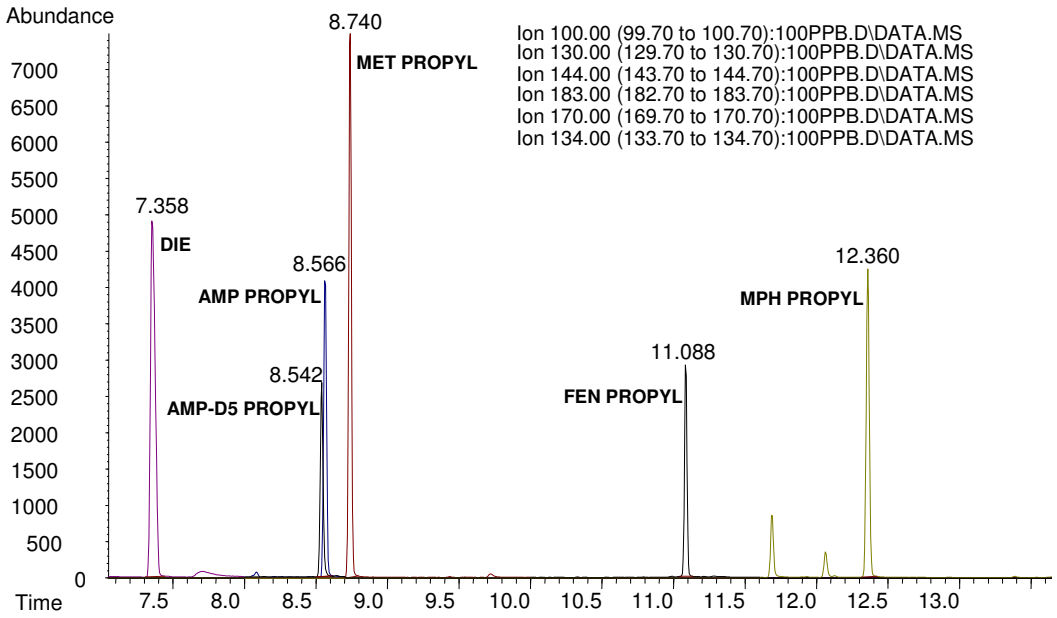


Fig. 5. Extracted ion chromatogram (EIC) of blank oral fluid spiked with 100 ng mL^{-1} of diethylpropion (DIE), amphetamine (AMP), metamphetamine (MET), fenproporex (FEN) and methylphenidate (MPH) analyzed by the proposed method. PROPYL, propylcarbamate derivative.

Table 3
Recovery (n=3) of ATS from Quantisal™ collection Device

	Low QC [a]		Middle QC [a]		High QC [a]	
ATS	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
AMP	91.9	6.9	93.0	1.2	91.0	2.1
MET	90.1	14.4	105.8	3.3	82.9	0.7
FEN	92.7	9.7	84.3	5.2	91.4	7.0
MPH	89.3	15.5	88.5	6.2	77.2	5.8
DIE	100.7	5.9	112.1	2.8	98.9	0.2

ATS, amphetamine-type stimulant; AMP, amphetamine; MET, methamphetamine; FEN, fenproporex; DIE, diethylpropion; MPH, methylphenidate; RSD, relative standard deviation.
[a] Quality control (QC) oral fluid samples: Low QC (6 ng mL^{-1}), Middle QC (100 ng mL^{-1}) and High QC (200 ng mL^{-1}).

6 ARTIGO 3:

"Fenproporex and amphetamine pharmacokinetics in oral fluid after oral administration of fenproporex"

A ser submetido à revista *Clinical Pharmacokinetics*.

Fenproporex and amphetamine pharmacokinetics in oral fluid after oral administration of fenproporex.

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Abstract

Fenproporex hydrochloride (FEN) is an anorectic drug used in the treatment of moderate to severe obesity and its major metabolite is amphetamine (AMP), another central nervous system stimulant. The concentration *versus* time profile of FEN and its metabolite AMP has been described in classical biological matrices, such as plasma and urine, however, there are no reports of such data in oral fluid. The aim of this study is to describe the pharmacokinetics of FEN and AMP in oral fluid, in order to assist in the development, application and interpretation of roadside screening drug tests. Twenty five milligrams of FEN (one capsule of Desobesi®) were orally administrated to six male volunteers and oral fluid samples were collected with Quantisal® device during 24 h after drug ingestion. FEN and AMP were submitted to solid-phase microextraction prior to analysis by gas chromatography-mass spectrometry in the selected ion monitoring mode, using deuterium labeled amphetamine as internal standard. After FEN administration, both analytes could be detected in oral fluid of all volunteers with an initial detection time varying from 0.5 to 1 h. FEN peak concentrations occurred between 1-1.5 h after administration, with maximum between 70.7 and 227.5 µg/L. For AMP, peak concentration occurred between 1.5-4 h, reaching 32.9 to 150.9 µg/L. Using a compartmental approach, FEN data was best fitted by one-compartment model with first order input and output, while AMP followed a two-compartment model with first order input and output.

Keywords: oral fluid, fenproporex, amphetamine, pharmacokinetics, solid-phase microextraction.

1. Introduction

Fenproporex (FEN), chemically known as (±) N-2-cyanoethylamphetamine or (±) 3-[(1-methyl-2-phenethyl)amino]-propionitrile, is an anorectic used for short-term treatment of moderate to severe obesity[1,2] and, as other amphetamine-type drugs, has stimulating effects on the central nervous system.

FEN is the second most frequently consumed amphetamine-based anorectic in the world,[3] and it is currently marketed in countries such as Brazil, Paraguay and

Chile.[4-6] Because of its stimulating properties, FEN is widely misused in Brazil, specially by truck drivers,[7-10] to avoid fatigue and to keep them driving for longer periods.

Kraemer and coworkers[11] proposed a biotransformation pathway to FEN, whereby it is biotransformed to 14 metabolites, with amphetamine (AMP) being the principal. FEN biotransformation is pre-systemic and extensive and occurs by first-pass effect in the liver, enzymatic breakdown by the intestinal flora, or a combination of both processes.[1] Thus, the administration of FEN results in considerable amounts of AMP through the cleavage of nitrogen-cyanoethyl bond (Fig. 1), generally between 27-56% of a FEN oral dose.[12,13]

Fig. 1

FEN and AMP can be analyzed in different biological matrices, which have their own characteristics, with advantages and disadvantages from analytical and collection point of view.[14-15] The interest in the use of oral fluid as biological matrix to analyze amphetamine-type stimulants has increased in recent years, particularly for drugged drivers' control.[16-17] Among the advantages of this matrix are the easy and noninvasive sampling, the difficulty of adulteration because the collection can be supervised by the traffic agent, the lesser interference from endogenous compounds compared to blood or urine, the presence of high concentrations of the parent drug, and the good correlation with serum analytical data, thus informing about recent drug use.[14,15,18-23] Moreover, several studies have shown that oral fluid and serum concentrations well correlate with drivers' impairment symptoms,[16,17,20,24-26] especially for amphetamine-type stimulants.

Transfer of drugs and metabolites from blood to oral fluid occurs primarily by passive diffusion and is dependent upon numerous factors, including chemical properties of drug (lipophilicity, pKa, molecule size), salivary and blood pH, concentration of un-ionized drug (ionized drug does not passively diffuse across cellular membranes) and drug-protein binding (only the free fraction can diffuse).[18,19,27-30] Since salivary glands are highly perfused, rapid transference of drugs from blood to oral fluid occur.[29]

Due to the relative acidity of oral fluid (pH 6.2 to 7.4) compared to plasma (pH 7.35 to 7.45), weak basis with low plasmatic protein binding like AMP (pKa 10.1) and FEN (pKa 7.23) are found in higher concentrations in oral fluid than in plasma, increasing the detectability of this substances.[1,2,31-35] In general, the ratio between oral fluid and plasma concentrations for AMP is greater than unity, about 2 to 3 times,[2,14,30,33] showing an advantage in the use of saliva as a biological matrix for the detection of amphetamine-type stimulants.

Oral fluid is the only specimen that has been successfully used as an alternative to blood in several pharmacokinetic and pharmacotoxicologic studies including drugs of abuse.[33] Pharmacokinetic data are important for correlating with drugged drivers behavioral and performance studies[36] and for establish the detection time of the available oral fluid screening immunoassays directed to roadside testing.[34] Knowledge of drug levels in oral fluid provides the basis for screening tests development and optimization, including the correct setting of cutoff concentrations. The concentration of amphetamine-type stimulants in oral fluid usually correlates well with plasma concentration, showing similar pharmacokinetic profiles.[33,35] This relationship with plasma is important when oral fluid is used as a surrogate to plasma, particularly in situations where invasive collection procedures are too difficult, i.e. at the roadside for drug-using drivers.[33]

By the knowledge of the authors, the pharmacokinetic profile of FEN and its main metabolite, AMP, in oral fluid has not been described so far. The detection of FEN and AMP in biological fluids as blood, urine and gastric contents has already been reported[1] and the behavior of these analytes in urine has been extensively described by Cody and coworkers.[12,37] However, there are no reports of such data in oral fluid. Thereby, this study aims to describe the pharmacokinetics of FEN and AMP in human oral fluid after a single 25 mg oral dose of FEN hydrochloride, in order to determine the average concentrations of FEN and AMP in oral fluid and to estimate their detection window, helping in the improvement of roadside screening drug tests.

2. Materials and Methods

2.1 Materials

AMP and AMP-D_5 (internal standard, IS) were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg/mL methanol solutions and FEN hydrochloride was gently donated by Aché (Guarulhos, SP, Brazil). Desobesi-m® (Aché, SP, Brazil) was purchased in a local pharmacy and consisted of 25 mg FEN hydrochloride gelatin capsules filled with microgranules. Solid-phase microextraction (SPME) manual holder, polydimethylsiloxane (PDMS) coating fibers (30 µm) and propylchloroformate (PRCL) were obtained from Sigma-Aldrich Corporation (São Paulo, SP, Brazil). Methanol and pH indicator strips were purchased from Merck (Darmstadt, Germany), and Na_2CO_3 and Na_2SO_4 from Synth (São Paulo, SP, Brazil).

Quantisal™ oral fluid collection devices, filters and preservative buffer solution were purchased from Immunalysis Corporation (Pomona, CA, USA). Each device contained a collection pad with an indicator that turns blue when 1 mL of oral fluid has been collected, and a plastic transport tube with 3 mL of preservative buffer. After collection, the pad is immersed into the transport buffer and a cylindrical filter is used to separate oral fluid solution from de pad.

2.2 Methods

Drug administration

This study was approved by the Research Ethics Committee of Porto Alegre Clinical Hospital and all the volunteers provided written informed consent.

Twenty five milligrams of FEN hydrochloride (one Desobesi-m® capsule) were orally administered to six healthy male volunteers, non-smokers and without regular use of medications. The mean age of participants was 35 ± 10 years (range, 27-55 years), mean height was 179 ± 8 cm (range, 170-194 cm) and mean weight was 78 ± 8 kg (range, 69-90 kg).

Sample collection

Oral fluid samples were collected with Quantisal™ device according to the manufacturer's instructions. This device provides a collection of about 1 mL of oral fluid, which is diluted in a buffer solution to a final volume of 4 mL.

Samples were collected during 24 hours, one before drug administration and the others in periods at 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 h after ingestion. Three participants had an additional sample collected at 0.75 h. During the collection day, the volunteers kept their usual diet and fluid intake.

Before and after each collection, Quantisal™ sticks with pads were individually weighed, with the collector pads positioned upwards inside a beaker. It was done to determine the exact weight of each oral fluid sample and to verify the accuracy and precision of the sample sizes collected by Quantisal™. After few hours into the preservative buffer, samples were filtered and transferred to glass tubes, remaining frozen at -20 °C until analysis.

The pH of the volunteers' oral fluid was measured before sample collections using pH indicator sticks with increments of 0.2-0.3 pH units. The participants were asked to spit into petri dishes where the indicator sticks were wet with oral fluid.

Sample extraction and analysis

The methodology used for sample preparation and analysis has been previously validated by our research group and is described below.

An aliquot of 1.5 mL of diluted oral fluid was pipetted into a 2 mL vial containing a magnetic stirring bar, 75 mg of Na_2CO_3, 150 mg of Na_2SO_4, 5 µL of PRCL and 10 µL of a 2.4 µg/mL IS solution (final pH 10.1). After vortex for 1 min, a SPME fiber (PDMS 30 µm) was immersed into the solution and left under magnetic stirring (1200 rpm) for 20 min, at room temperature (20 ± 2 °C). Elapsed the extraction time, the fiber was desorbed in a GC-MS injector port (260 °C) for 15 min, and then rinsed with deionized water to remove excess of salts.

The analysis were carried out on a 6890N gas chromatograph coupled to 5973 inert mass selective detector (Agilent Technologies, Palo Alto, CA, USA), equipped with a 900 µL deactivated glass liner and a 30 m x 0.25 mm x 0.25 µm 5% phenyl-methylpolysiloxane column (HP-5MS, Agilent J&W Scientific, Folsom, CA, USA). The oven temperature was programmed at 100 ºC (1 min), increased at 15 °C/min to 250 °C (3 min), and then at 15 °C/min to 280 °C (4 min). Helium (99.9999% purity) was used as carrier gas at flow rate of 1 mL/min. The injector was maintained

at 260 ºC and operated for 1 min in splitless mode. The temperatures of the interface, ion source and quadrupole were 280 ℃, 230 ℃ and 150 ℃, respectively. The MS system was operated in electron impact ionization mode at 70 eV and in selected-ion monitoring (SIM). The following ions were monitored (underlined ions used for quantification): 130, 162, 91 (AMP propylcarbamate); 134, 167, 92 (AMP-D_5 propylcarbamate) and 183, 97, 91 (FEN propylcarbamate). The method was linear ($r>0.998$), the limit of detection (LOD) was 2 µg/L for both analytes and the limits of quantification (LOQ) were 2 µg/L and 4 µg/L for AMP and FEN, respectively.

A new calibration curve was run each day of analysis and the oral fluid samples collected before drug administration were assayed prior to the rest of samples to check for the absence of interfering compounds.

Pharmacokinetic study

Pharmacokinetic parameters were calculated from oral fluid concentration x time curves using non-compartmental (Microsoft Excel 2007®, Microsoft, USA) and compartmental (Scientist®, v.3.0, Micromath, USA) analysis.

3. Results and Discussion

The mean oral fluid pH of the six volunteers was 6.6 ± 0.2 (range, 6.5-7.1), and the average Quantisal™ collected volume, considering an oral fluid relative density of 1.01,[38,39] was 1.105 ± 0.123. The interindividual accuracy (related to the nominal 1 mL volume) and precision (relative standard deviation, RSD) of oral fluid volumes were 110.5% and 11.5%, respectively. The RSD was twice the described by Dickson and coworkers[39] for Quantisal™ (RSD 5.09%), however those authors conducted the experiment immersing the collection pads into a beaker containing oral fluid of a single supplier, without covering the variations due to *in vivo* collection of different individuals. When we look at the collected volumes, ranging from 0,812 mL to 1,429 mL (Table I) and determine the error in using the nominal Quantisal™ dilution of 1:4 for calculating concentrations, we obtain maximum errors of -14.7% and + 29.1% in drug concentrations. Since we have individual weighted all samples and have estimated the individual volumes, we used these data for calculating each sample dilution and concentration, instead of using the nominal Quantisal™ dilution.

Table I

In spite of some studies with controlled administration of AMP, methamphetamine, methylphenidate, 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyphenyl-2-butanamine (MBDB) to humans[30-33,35], and subsequent measure of oral fluid drug concentrations, none has described the pharmacokinetic profile of FEN in oral fluid. According to our results, FEN and AMP could be detected in oral fluid of all six volunteers after the administration of a single 25 mg dose of FEN hydrochloride (Fig. 2).

Fig. 2

The pharmacokinetic parameters, calculated through non-compartmental and compartmental analysis, are shown in tables II and III. Figures 3 and 4 show the average plasma concentration *versus* time profiles.

Table II

Fig. 3

Table III

Fig. 4

FEN was initially detected in four out of six volunteers at 0.5 h after dose administration while AMP was detected after 0.5-1.0 h. In fact, the lag time (t_{lag}) calculated during compartmental analysis was 0.49 h for FEN and 0.72 h for AMP. FEN concentrations reached their maximum at 1.0-1.5 h (t_{max}) and ranged from 70.7 to 227.5 µg/L (C_{max}); peak AMP concentrations attained 32.9 to 150.9 µg/L and occurred between 1.5-4.0 h (Table II). These data are in agreement with the data presented by La Torre and coworkers in a review of amphetamine-type stimulants' pharmacokinetics.[32] According to this review, after oral administration of both the racemate and the individual isomers of AMP, drug oral fluid concentrations peaked between 2 and 4 h, as for plasma, and reached maximum concentrations between 45-120 µg/L after 10 mg oral doses. The calculated elimination half-life ($t_{½}$) for AMP, between 9.9-31.6 h, was also in accordance with the values reported by Cone and Huestis,[29] which ranged from 6.8 h (acidic urine) to 24.6 h (alkaline urine). Despite

we were expecting differences in t_{max} and C_{max} between AMP orally administrated and that generated by FEN biotransformation, it was not observed, probably due to its fast metabolic formation.

With respect to FEN, the only parameter we found reported in the literature to compare with the present data was a $t_{½}$ of 2 h [40] which agrees with the calculated $t_{½}$ by both non-compartmental (2.50±1.77 h, mean±SD) and compartmental (1.91 h) analysis (Tables II and III). The lower FEN $t_{½}$ show that it is eliminated/metabolized about seven times faster than AMP (Table II).

FEN could be detected in oral fluid of all participants up to 12 h after administration of a 25 mg dose. According to the literature, after the administration of a single oral dose (10 or 20 mg) of FEN to healthy volunteers the parent drug could be detected for up to 32 h in urine.[11-12]

In the present study, FEN was detected in oral fluid after 24 h in only one volunteer (at the LOD) while AMP was detected in all six volunteers after this time, with concentrations ranging from 6.7 to 20.7 µg/L. In fact, according to previous data,[29,41,42] AMP can be detected in oral fluid for up to 50 h (LOD 10 µg/L) after an oral administration of 10 mg of AMP hydrochloride. The short monitoring time of AMP concentrations in oral fluid employed in the present study (24 h) may be hampered the accurate determination of AMP pharmacokinetic parameters, generating extrapolated areas under the concentration *versus* time profile curves (AUC_{extrap}) of about 34.3 % (table II).

The oral fluid concentration profile of FEN and AMP presented high interindividual variability (table II) and the variations observed for AMP can be in part related to differences in hepatic cytochrome P450 enzimes involved in FEN N-dealkylation.[43]

After compartmental analysis, the best models that fitted the data were one-compartment for FEN and two-compartment for AMP (table III and fig. 4). The calculated parameters for FEN and AMP are in accordance with those determinated by non-compartmental analysis (tables II and III).

Schepers and co-workers[31] reported the adequacy of the one-compartment model to describe the data obtained after oral administration of 10 and 20 mg of

methamphetamine (MET) sustained release formulation. In the same study, AMP generated in MET biotransformation was not submitted to compartmental analysis.

When FEN/AMP oral fluid concentration ratios for the six participants were plotted against administration time (Fig. 5) it was verified a decrease in ratio values as well as in ratio variability along time. In the FEN absorption fase (between 1 and 2 h) it was seen the largest values and variation in FEN/AMP ratios.

Fig. 5

The detection time is the typical duration of time a drug can be detected in a biological specimen by an analytical test and serves as a useful guideline to establish the probable time that drug use occurred prior to specimen collection.[29] This parameter is often obtained from controlled drug administration to healthy volunteers and depend mainly on the dose, the pharmaceutical preparation, the route of administration, the duration of use (acute or chronic), the matrix that is analyzed, the nature of the molecule or the metabolite, the pH, the concentration of the matrix, the interindividual variation, as well as the detection limit of the analytical method used.[42]

With the chromatographic method described in this paper, the detection window of FEN and AMP in oral fluid, after a single oral administration of 25 mg of FEN, was estimated in 0.5 to 12 h and 0.75 to greater than 24 h, respectively. If we consider the 50 µg/L initial screening cutoff concentration for amphetamine-type stimulants in oral fluid, already proposed by US Substance Abuse and Mental Health Services Administration[44] and by Standards Australia,[23] the detection time in the evaluated volunteers will be between 0.5-4.0 h for FEN and 1.0-8.0 h for AMP. At exactly 0.5 h (FEN) and 1 h (AMP) after oral administration of 25 mg of FEN hydrochloride, the oral fluid concentrations of FEN and AMP were lower than 14 µg/L and 37 µg/L, respectively, for all participants. However, most of the oral fluid immunoassays currently marketed[45-53] has (+)-AMP as the target drug and presents cross-reactivity of about 50% for (±)-AMP, the metabolite of FEN.[12,29,43] Employing these kits, with an average 100 µg/L (±)-AMP cutoff, the detection time of AMP in the evaluated volunteers would be 1.5-4.0 h.

5. Conclusions and perspectives

Oral administration of FEN results in significant amounts of FEN and AMP in oral fluid, showing that this matrix might be useful for pharmacokinetic studies of these substances.

The detection window of FEN and AMP in oral fluid, after a single oral administration of 25 mg of FEN, was estimated in 0.5 to 12 h and 0.75 to greater than 24 h, respectively, using a sensitive analytical method (detection limit of 2 µg/L).

According to the obtained data, FEN and its metabolite AMP follow different pharmacokinetic models. FEN and AMP oral fluid profiles were better described by one and two-compartment models, respectively. Due to the high interindividual variability found in the present study, especially for AMP, more consistent pharmacokinetic results may be obtained with the assessment of a higher number of subjects for a minimum 48 h period. It is important to notice that these variabilities are expected for highly metabolized drugs, such as FEN.

In order to establish the average ratio between oral fluid and plasma concentrations of FEN and AMP and to verify whether plasma concentrations can be predicted in some way by oral fluid concentrations, a simultaneous evaluation of oral fluid and plasma data is necessary.

Acknowledgements

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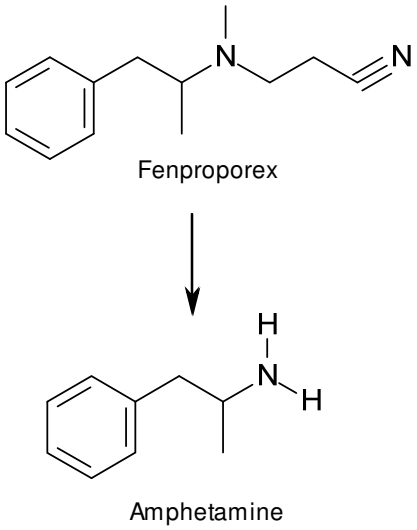


Fig. 1. Biotransformation of fenproporex to amphetamine through nitrogen-cyanoethylated bond cleavage.

Table I. Error in estimating drug concentration using the nominal Quantisal™ collection device dilution of 1:4.

	Oral Fluid volume (mL)	Total volume (mL)	Dilution factor	Error in drug concentration (%)
Minor volume	0,812	3,812	4,7	-14,7
Major volume	1,429	4,439	3,1	29,1
Nominal	1,000	4,000	4,0	

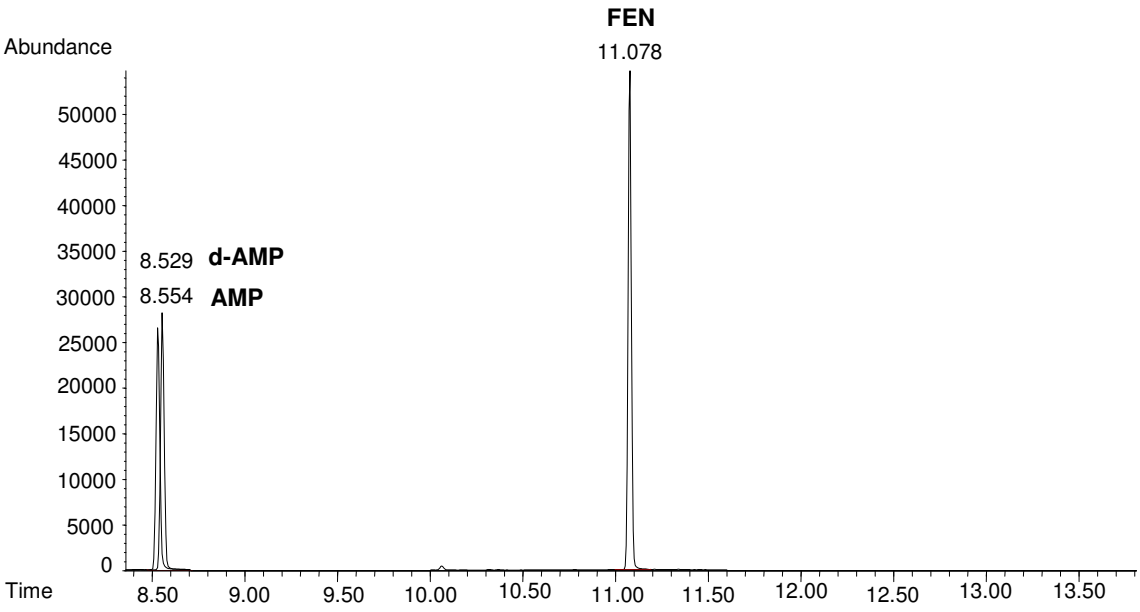


Fig. 2. Extracted ion chromatogram of a volunteer's oral fluid sample after 1.5 h of the administration of a 25 mg fenproporex hydrochloride capsule. **FEN=** fenproporex; **AMP=** amphetamine; **d-AMP=** amphetamine-D_3.

Table II. Non-compartmental analysis of oral fluid fenproporex (FEN) and amphetamine (AMP) concentrations after administration of a single 25 mg dose of FEN hydrochloride (n=6).

	FEN		**AMP**	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**
t_{max} (h)	1.42 ± 0.20	1.00-1.50	2.42 ± 1.24	1.50-4.00
C_{max} (µg/L)	175.79 ± 56.21	70.67-227.50	78.68 ± 43.06	32.97-150.94
K_e (h^{-1})	0.36 ± 0.15	0.12-0.54	0.05 ± 0.02	0.02-0.07
$t_{½}$ (h)	2.50 ± 1.77	1.29-6.00	16.90 ± 8.02	9.97-31.60
AUC_{24} (h·µg/L)	387.74 ± 119.31	239.39-539.50	611.20 ± 217.32	384.34-901.97
% AUC_{extrap} (%)	7.29 ± 6.11	2.74-19.26	34.35 ± 11.84	19.91-51.15
AUC_{∞} (h·µg/L)	421.00 ± 135.74	252.81-558.68	969.48 ± 419.66	680.15-1593.16
CL/F (L/h)	65.80 ± 24.25	44.75-98.89	30.37 ± 13.38	15.69-51.43
V_d/F (L)	223.11 ± 125.89	96.87-387.36	662.98 ± 225.52	347.66-1019.15

SD = standard desviation; **t_{max}** = time to reach maximum (peak) oral fluid concentration following drug administration; **C_{max}** = maximum (peak) oral fluid drug concentration; **k_e** = elimination rate constant from the central compartment; **$t_{½}$** = elimination half-life; **AUC_{24}** = area under the oral fluid concentration-time curve from time zero to time 24 hours; **% AUC_{extrap}** = area under the oral fluid concentration-time curve extrapolated from time 24 hours to infinity as a percentage of total AUC; **AUC_{∞}** = area under the oral fluid concentration-time curve from time zero to infinity; **CL/F** = apparent total clearance of the drug from oral fluid after oral administration; **V_d/F** = apparent volume of distribution after non-intravenous administration.

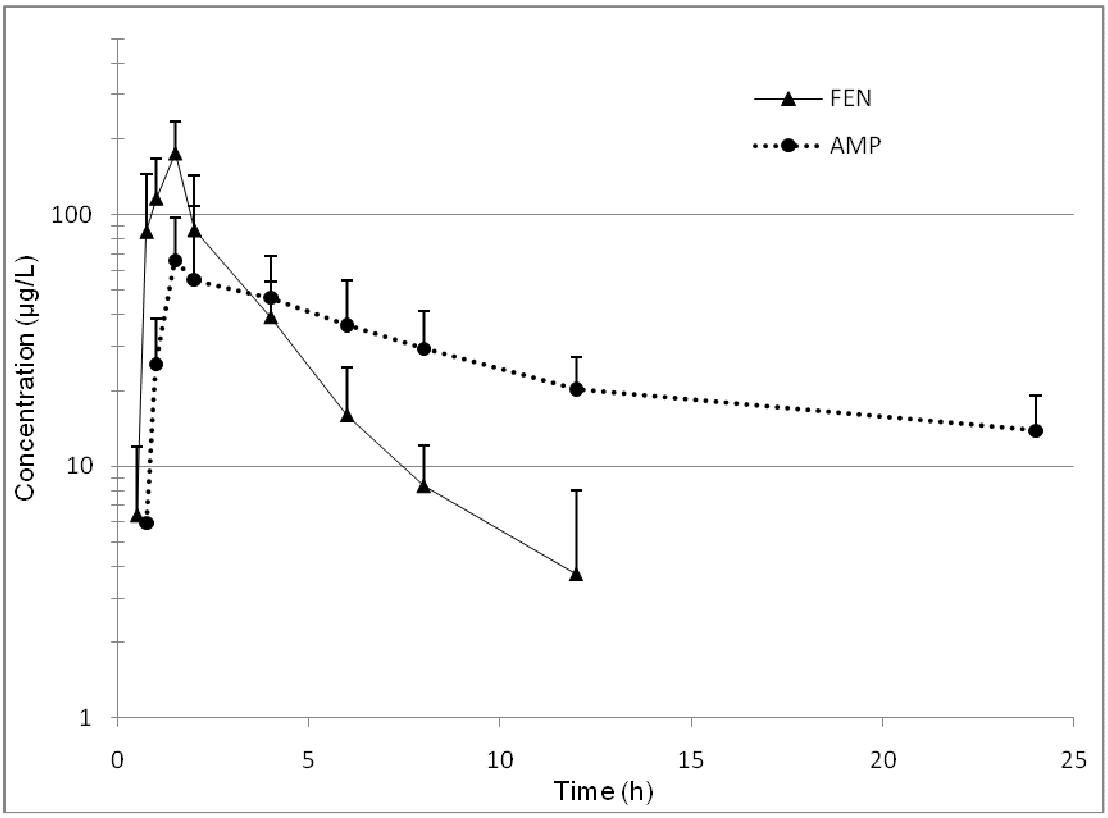


Fig. 3. Average profile of fenproporex (FEN) and amphetamine (AMP) concentrations in oral fluid after single-dose administration of 25 mg of FEN hydrochloride (n=6). Error bars represent standard deviation.

Table III. Compartmental analysis of oral fluid fenproporex (FEN) and amphetamine (AMP) mean concentrations (n=6) fitted to one-compartment and two-compartment model, respectively.

	FEN	**AMP**
t_{max} (h)	1.08	
C_{max} (µg/L)	126.18	
K_a (h^{-1})	4.67	2.01
K_e (h^{-1})	0.36	
$t_{½}$ (h)	1.91	
t_{lag} (h)	0.49	0.72
MSC	2.52	1.51
AUC_{∞} (h·µg/L)	479.43	
CL/F (L/h)	52.14	
V_d/F (L)	143.65	
A		60.95
B		26.91
C		87.86
α		0.30
β		0.03
$t_{½\alpha}$ (h)		2.30
$t_{½\beta}$ (h)		23.55
$t_{½abs}$ (h)		0.34

t_{max} = time to reach maximum (peak) oral fluid concentration following drug administration; **C_{max}** = maximum (peak) oral fluid drug concentration; **k_a** = absorption rate constant; **k_e** = elimination rate constant from the central compartment; **$t_{½}$** = elimination half-life; **t_{lag}** = lag time; **MSC** = model selection criteria; **AUC_{∞}** = area under the oral fluid concentration-time curve from time zero to infinity; **CL/F** = apparent total clearance of the drug from oral fluid after oral administration; **V_d/F** = apparent volume of distribution after non-intravenous administration; **A**, **B and C** = macroconstants; **α** =distribution phase slope; **β** = elimination phase slope; $t_{½\alpha}$ = disposition half-life; **$t_{½\beta}$** = terminal elimination half-life; **$t_{½abs}$** = absorption half-life.

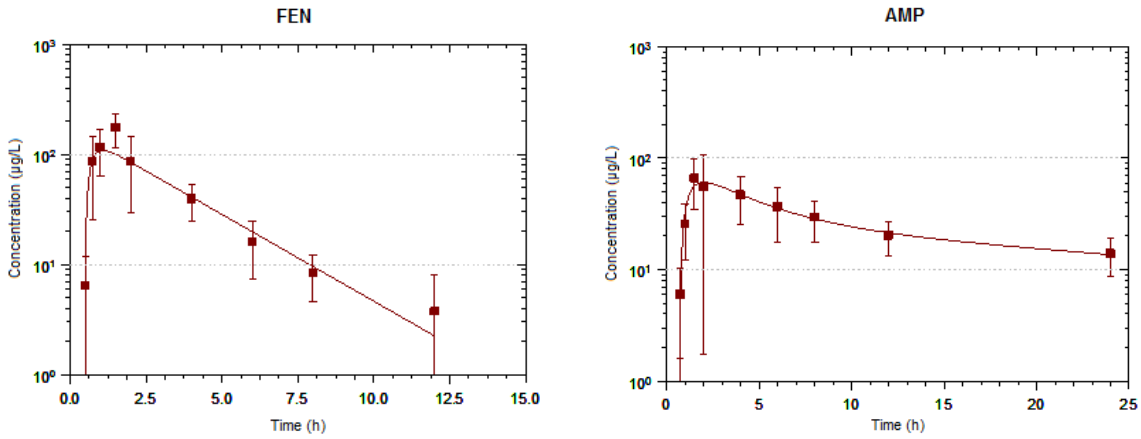


Fig. 4. Mean oral fluid fenproporex (FEN) and amphetamine (AMP) concentrations (n=6) fitted to one-compartment and two-compartment model, respectively.

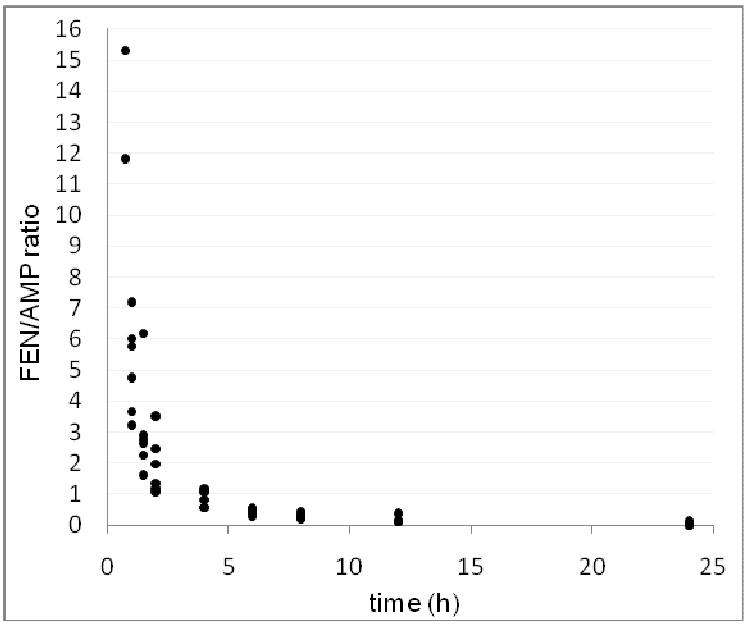
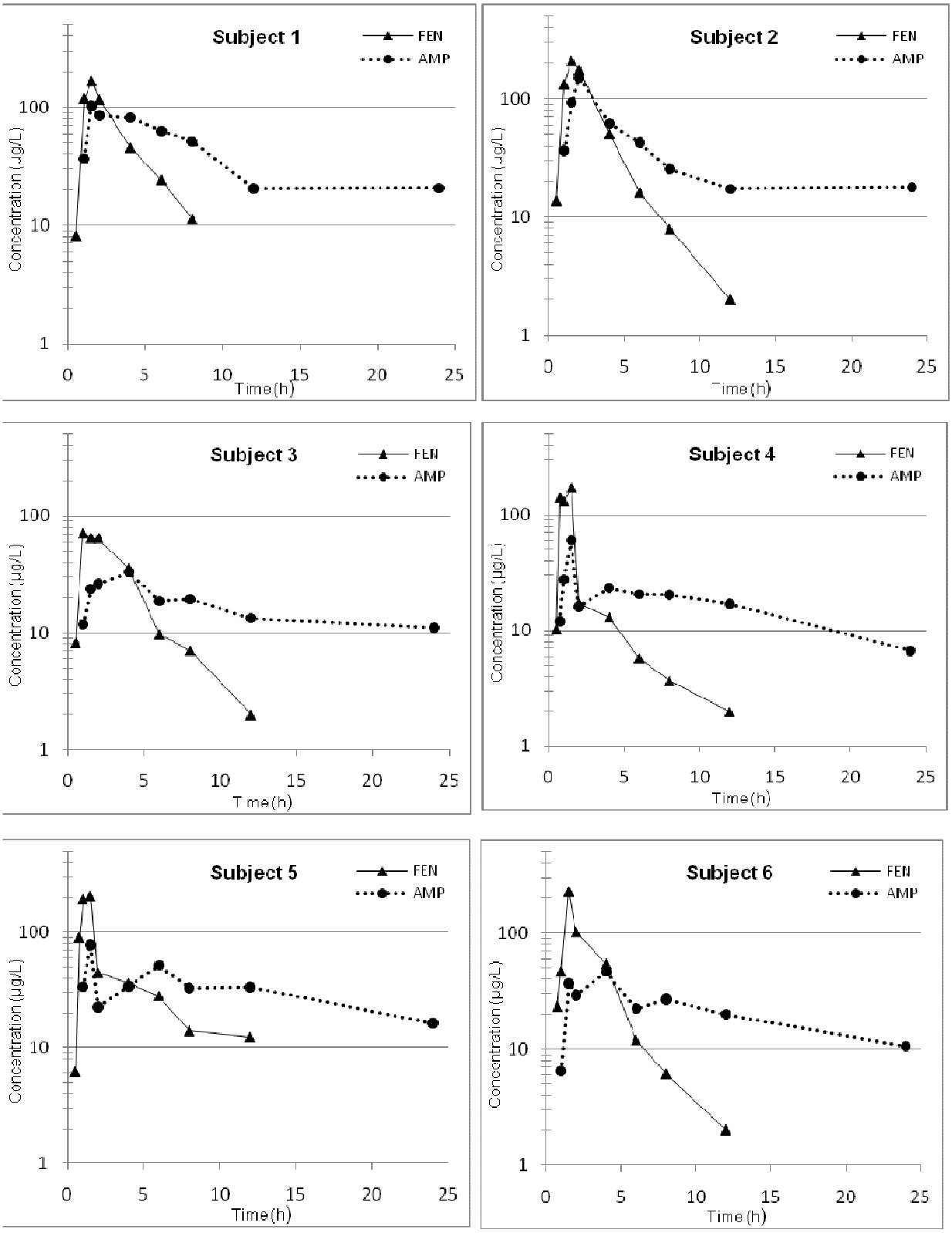


Fig. 5. Ratios between fenproporex and amphetamine concentrations in oral fluid (FEN/AMP ratios) follow administration of single 25 mg doses of fenproporex hydrochloride to six subjects.

Data supplement: Individual profile of fenproporex (FEN) and amphetamine (AMP) concentrations in oral fluid after single-dose administration of 25 mg of FEN hydrochloride.



7 DISCUSSÃO GERAL

A avaliação da especificidade analítica de imunoensaios destinados à triagem de amostras de OF quanto à presença de ATS constituiu etapa essencial do projeto “Estudo do impacto do uso de bebidas alcoólicas e outras substâncias psicoativas no trânsito brasileiro” (PECHANSKY; DE BONI, 2007). Sem ela, os ensaios imunológicos de triagem seriam aplicados a um vasto número de amostras de fluído oral (mais 3.398 amostras) sem detectar os ATS passíveis de consumo pelos motoristas brasileiros, representando um grande desperdício de dinheiro público (PECHANSKY; DUARTE; DE BONI, 2010). A partir da verificação da inadequabilidade dos immunoensaios comerciais à realidade brasileira, foi possível redirecionar as análises laboratoriais do projeto para a triagem e confirmação diretamente por métodos cromatográficos.

A utilização da derivatização em métodos analíticos baseados em SPME-CG permite a obtenção de baixos limites de detecção para substâncias polares e reativas, como os ATS (STASHENKO; MARTÍNEZ, 2004). Além disso, a derivatização melhora a precisão e a especificidade da técnica, tornado os espectros de massas dos ATS mais complexos e discriminativos (STALIKAS; FIAMEGOS, 2008; STASHENKO; MARTÍNEZ, 2004). Nos experimentos realizados, os ALCL se mostraram reagentes eficazes, práticos e de baixo custo para a derivatização de ATS em OF, reagindo rápido e diretamente na matriz em estudo, sob condições reacionais brandas e sem o emprego de solventes orgânicos (HUSEK, 1998; MEATHERALL, 1995; UGLAND; KROGH; RASMUSSEN, 1997). Ainda, todos os ALCL estudados (à exceção do PHCL) reagiram formando derivados únicos para cada analito, com sinais cromatográficos bastante superiores aos ATS não derivatizados.

Durante o desenvolvimento do método analítico por SPME-CG/EM, foi testada a utilização de *liner* de 0,75 mm de diâmetro interno, ao invés do *liner* convencional *splitless* de 900 µL, com a finalidade de aumentar a resolução e a sensibilidade do método (PAWLISZYN, 1997; VAS; VÉKEY, 2004). De fato, foi observado um acréscimo nas áreas dos picos cromatográficos dos ATS estudados, no entanto acompanhado da elevação da imprecisão da técnica. Em decorrência disso, optou-se pelo emprego de *liner* convencional. Ainda, apesar de não ser recomendada a utilização de lã de vidro no interior dos *liners* para SPME, o uso de

pequena quantidade deste material, em posição bastante distanciada da fibra, mostrou-se importante para reter os sais precipitados no interior do *liner*.

A maioria dos métodos de SPME (DI-SPME ou HS-SPME) para a extração de derivados ALCA de ATS descritos na literatura utiliza tempos de dessorção da fibra de até 3 minutos em temperaturas que variam de 225ºC a 300ºC (BROWN; RHODES; PRITCHARD, 2007; NISHIDA et al. 2006; UGLAND; KROGH; RASMUSSEN, 1999; YAHATA et al., 2006), sendo restritos os relatos de tempos de dessorção superiores (YONAMINE et al., 2003). No entanto, nos experimentos realizados, tempos de dessorção inferiores a 15 minutos ocasionavam *carryover* em amostras em branco analisadas subseqüentemente, o que é impeditivo para a aplicação da técnica com finalidade forense e confirmatória. Tal fato vai ao encontro de recente revisão crítica realizada por Nováková e Vlčková (2009), que ressaltam que a técnica de SPME é bastante propensa a *carryover*.

Com objetivo de reduzir ao máximo o tempo de dessorção e aumentar a durabilidade das fibras de SPME expostas ao pH alcalino (SUPELCO, 1999), optou-se pela utilização de fibras de PDMS 30 µm em substituição às de 100 µm. De fato, a menor espessura de filme polimérico facilitou a dessorção das fibras, no entanto não aumentou a sua durabilidade em pH 10 conforme esperado (SUPELCO, 1999). Se por um lado a utilização de pH elevados provê o ambiente necessário à eficiente derivatização dos ATS, por outro danifica o recobrimento das fibras de SPME quando utilizado a técnica de DI-SPME (PAWLISZYN, 1997). Nos presentes experimentos as fibras de SPME puderam ser reutilizadas por no máximo 100 extrações, quando então se verificou um decaimento na eficência de sorção e dessorção das fibras.

Vários autores já descreveram a utilização de vidraria silanizada (KASPRZYK-HORDERN; KONDAKAL; BAKER, 2010; LEE et al., 2000; RÖHRICH; KAUERT, 1997) para a análise de traços de ATS, no entanto a aquisição de vidraria já tratada representa um elevado custo. Neste trabalho, foi testada a utilização de dimetildiclorosilano (DMDCS) para a silanização *in house* da vidraria, seguido de lavagens com tolueno e metanol. Além do elevado volume de solventes orgânicos necessários ao processo, verificou-se grande instabilidade das soluções padrões de ATS quando armazenadas em *vials* silanizados por este método. De fato, as áreas

dos picos cromatográficos caíram 20% após 24 horas à temperatura ambiente e 50% após 7 dias a -18ºC. A preparação subseqüente de novas soluções padrões e o armazenamento em *vials* não silanizados solucionou o problema, confirmando que a instabilidade dos ATS decorreu do processo de silanização, provavelmente devido a resíduos remanescentes de DMDCS.

O método desenvolvido também se mostrou adequado para a determinação de cocaína em OF, no entanto, devido ao equipamento de CG/EM utilizado nos experimentos ser empregado para análise de rotina de amostras de cocaína apreendidas pela Polícia Federal, tal determinação acabou prejudicada em virtude da contaminação do equipamento. De fato, a análise de traços requer a utilização de vidraria e instrumental analítico dedicados a este fim, com cuidados de limpeza e manutenção bastante diferenciados.

Por fim, os ensaios farmacocinéticos comprovaram que o método por SPME-CG/EM validado é suficientemente sensível para a monitorização das concentrações de FEN e AMP no OF após a administração de 25 mg de cloridrato de FEN. Apesar da eliminação do FEN e de seu principal metabólito AMP na urina já ter sido bastante estudada (CODY; VALTIER, 1996, 1999), não foram encontrados trabalhos relatando o perfil de concentração *versus* tempo do FEN e da AMP no OF após a administração controlada de FEN.

8 CONCLUSÕES E PERSPECTIVAS

Os testes imunológicos atualmente disponíveis para a detecção preliminar de ATS em OF são importados e inadequados para a identificação de FEN, DIE e MPH, principais ATS consumidos no Brasil. Considerando o elevado custo que representa a realização de exames cromatográficos na totalidade das amostras coletadas, há necessidade do emprego de testes de triagem para separar as amostras negativas das potencialmente positivas. Neste sentido, os imunoensaios são considerados os testes de escolha devido à elevada sensibilidade, praticidade, simplicidade e possibilidade de automação. Portanto, para que no futuro seja possível a utilização do fluído oral para monitorar o consumo de ATS no trânsito brasileiro, é necessário o desenvolvimento de imunoensaios adequados à realidade nacional, produzidos com anticorpos voltados à detecção de FEM, DIE, MPH.

Apesar das diversas vantagens apresentadas pela SPME como técnica de extração e concentração, tais como a não utilização de solventes orgânicos e o reduzido número de etapas de preparação de amostra, as fibras são bastante frágeis e requerem certa habilidade do analista. Ainda, os elevados tempos necessários para a sorção e dessorção de substâncias pouco voláteis como os ATS derivatizados inviabilizam a aplicação manual da técnica para um grande número de amostras. Considerando que os derivados ALCA de ATS são bastante estáveis em soluções aquosas alcalinas, a utilização de amostradores automáticos do tipo CombiPal (Varian, Palo Alto, USA) pode sobrepujar os problemas relacionados a SPME manual e permitir a aplicação do método desenvolvido para a triagem e confirmação simultâneas de ATS em fluído oral.

Ressalta-se a importância da avaliação gráfica dos resíduos para verificação da adequabilidade do modelo de regressão linear simples na inferência estatística dos dados. A regressão linear simples e a análise da variância (ANOVA) estão baseados em pressuposições básicas associadas aos resíduos (normalidade, homocedasticidade e independência), as quais devem ser ao menos em parte atendidas para que sejam obtidos bons estimadores (intercepto e coeficiente de regressão linear) e para que as conclusões estatísticas da ANOVA tenham validade. No presente trabalho, comprovou-se a necessidade da observação destes pressupostos antes da aplicação do modelo de regressão linear simples aos resultados experimentais da validação. Com os dados adequados ao modelo, foi

possível obter melhores coeficientes de correlação (r) e reduzir os limites de quantificação, devido ao aumento da exatidão na região inferior da curva padrão.

Os ensaios farmacocinéticos permitiram estimar a janela de detecção do FEN e da AMP no OF após a ingestão de uma dose da especialidade farmacêutica comercializada no país contendo FEN, o Desobesi-m® (BRASIL, 2010b). Considerando a ausência na literatura de estudos avaliando o perfil de concentração *versus* tempo do FEN no OF, ou mesmo da AMP gerada como seu metabólito, os dados gerados no presente trabalho contribuem para o entendimento do comportamento destes fármacos no organismo, servindo de base para o desenvolvimento e otimização de testes de triagem e de confirmação toxicológica. No entanto, faz-se necessário a realização de estudos farmacocinéticos mais amplos, com a coleta concomitante de OF e sangue, a fim de calcular as razões entre as concentrações de FEN e AMP nas duas matrizes. Ainda, devido à grande variabilidade interindividual observada no presente estudo, um número maior de sujeitos devem ser avaliados, por um período mínimo de 48 horas, para determinar com maior exatidão o perfil farmacocinético do FEN e de seu metabólito AMP no organismo humano.

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10 ANEXOS

Comitê de Ética

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:

Projeto: 09-005 **Versão do Projeto:** 20/03/2009 **Versão do TCLE:** 20/03/2009

Pesquisadores:
FLAVIO PECHANSKY
RENATA PEREIRA LIMBERGER
DANIELE ZAGO SOUZA

Título: DETERMINAÇÃO DE AMINAS PSICOATIVAS NA SALIVA POR SPME-CG

Este projeto foi Aprovado em seus aspectos éticos e metodológicos, inclusive quanto ao seu Termo de Consentimento Livre e Esclarecido, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde. Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA. Somente poderão ser utilizados os Termos de Consentimento onde conste a aprovação do GPPG/HCPA.

Porto Alegre, 07 de maio de 2009.

Profª Nadine Clausell
Coordenadora do GPPG e CEP-HCPA

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

RESOLUÇÃO

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB00000921) analisaram o projeto:

Projeto: 09-005

Pesquisador Responsável:
FLAVIO PECHANSKY

Título: DETERMINAÇÃO DE AMINAS PSICOATIVAS NA SALIVA POR SPME-CG

	Data da Versão:
EMENDA 1 - EFETIVIDADE DA TÉCNICA	23/10/2009
TCLE EMENDA 1 - FENPROPOREX	23/10/2009
TCLE EMENDA 1 - ANFEPRAMONA	23/10/2009

Este documento referente ao projeto acima foi Aprovado em seus aspectos éticos e metodológicos, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, 23 de outubro de 2009.

Profª Nadine Clausell
Coordenadora do GPPG e CEP-HCPA

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

RESOLUÇÃO

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB00000921) analisaram o projeto:

Projeto: 09-005

Pesquisador Responsável:
FLAVIO PECHANSKY

Título: DETERMINAÇÃO DE AMINAS PSICOATIVAS NA SALIVA POR SPME-CG

Data da Versão:

CORREÇÃO DA EMENDA 1 - TAMANHO DA AMOSTRA 17/12/2009

Este documento referente ao projeto acima foi Aprovado em seus aspectos éticos e metodológicos, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, 17 de dezembro de 2009.

Profª Nadine Clausell
Coordenadora GPPG e CEP/HCPA

Ementa Metodológica ao Projeto "Determinação de Aminas Psicoativas na Saliva por Microextração em Fase Sólida e Cromatografia à Gás" (Projeto GPPG/HCPA 09-005)

1. Introdução e Justificativa

Ementa metodológica em relação ao projeto de pesquisa "Determinação de Aminas Psicoativas na Saliva por Microextração em Fase Sólida e Cromatografia a Gás", aprovado pela Comissão Cientifica/Comissão de Pesquisa e Ética em Saúde do Grupo de Pesquisa e Pós-graduação do Hospital de Clínicas de Porto Alegre (GPPG/HCPA) sob o número 09-005, em 07 de maio de 2009.

A presente ementa visa comprovar a efetividade da técnica de detecção desenvolvida no projeto supracitado, bem como estimar a janela de detecção do femproporex, da anfepramona e de um dos seus principais metabólitos (anfetamina) na saliva.

Inicialmente o referido projeto foi desenvolvido com a utilização de amostras de saliva negativas adicionadas de padrões de fármacos, entre eles o femproporex e a anfepramona. A metodologia assim otimizada não foi ainda aplicada em amostras reais de indivíduos que sabidamente consumiram os medicamentos, e este é um passo importante na finalização da validação da metodologia analítica proposta. Entretanto, poucos são os dados da literatura científica acerca destes fármacos e sua análise em matriz biológica saliva.

Durante as etapas de revisão bibliográfica realizadas observou-se que alguns autores já relataram a metabolização de femproporex à anfetamina no organismo humano e seu perfil farmacocinético em urina (CODY et al, 1996; CODY et al, 1999) . Porém, não foram encontrados estudos que relatem o perfil farmacocinético do femproporex e da anfepramona em saliva e faltam dados sobre o intervalo de tempo após administração oral em que podem ser detectados os fármacos inalterados e seus metabólitos na saliva. Isto é fundamental visto que há possibilidade de drogas que se metabolizam a anfetamina terem uma interpretação de resultados de testes de drogas positivos para anfetamina e as drogas inalteradas não, fornecendo resultados falso-negativos em etapas de triagem, o que pode vir a comprometer todo o projeto original ao qual este específico está inserido, coordenado pelo Prof. Dr. Flavio Pechansky.

Neste contexto, esta ementa propõe o estudo da toxicocinética da administração oral de dose única (25mg) de femproporex e de anfepramona em 10 voluntários masculinos, bem como acompanhamento através de coletas em tempos pré-estabelecidos durante 24h,

correlacionando-os com dados urinários. Tendo em vista que o projeto já desenvolveu metodologia analítica que permite a quantificação de femproporex, anfepramona e anfetamina em saliva, pretende-se utilizá-la para gerar este dado inexistente na literatura e fundamental para interpretação dos resultados finais do projeto original. Cabe ressaltar que a metodologia para a quantificação de femproporex, anfepramona e anfetamina em urina já se encontra validada por nosso grupo de pesquisa (SEBBEN, 2007).

2. Objetivo

Comprovar a efetividade da técnica analítica desenvolvida para detecção de femproporex, anfepramona e o metabólito anfetamina na saliva, a partir da realização de estudos toxicocinéticos neste fluido. Paralelamente pretende-se comparar os dados gerados com dados urinários produzidos no mesmo estudo.

4. Materiais e Métodos

O estudo será realizado na Faculdade de Farmácia da UFRGS, situada na Av. Ipiranga, 2752, Bairro Santa Cecília, Porto Alegre/RS, a qual disponibilizará todos os materiais de coleta (devidamente identificados), recursos humanos e informações necessárias aos voluntários.

Serão selecionados 10 voluntários homens, saudáveis, não-fumantes, sem nenhum tipo de doença crônica (hipertensão, diabetes, problemas cardiovasculares ou endócrinos, etc.), que não fazem uso regular de medicamentos e que aceitarem participar do estudo, conforme Termo de Consentimento Livre e Esclarecido anexo.

Os voluntários receberão esclarecimentos a qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa e terão a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo. Os pesquisadores assegurarão a privacidade dos voluntários quanto aos dados de identificação fornecidos na pesquisa. Os fluidos biológicos (saliva e urina) coletados não serão utilizados para outros fins senão os previstos nesta ementa.

O estudo consistirá na administração de dose única via oral de 25 mg de femproporex ou anfepramona, 1 comprimido para cada voluntário, sendo coletadas amostras de saliva e urina, em intervalos de tempo pré-estabelecidos (total de doze coletas) num período de 24 horas. As coletas de saliva serão realizadas através de um dispositivo de coleta Quantisal® (Immunalysis®, EUA), o qual consiste em um chumaço de algodão preso a uma haste plástica, que é inserido sob a língua por cerca de 5 minutos, período em que são coletados cerca de 1 mL de saliva. Após a coleta o algodão é inserido em solução

conservante e congelado. As amostras de urina (cerca de 100 ml) serão coletadas em frasco plástico estéril com tampa rosca e congeladas. Durante as coletas, os voluntários serão orientados por integrantes do grupo de pesquisa.

Os possíveis efeitos adversos relacionados ao femproporex já relatados na literatura incluem vertigem, tremor, irritabilidade, reflexos hiperativos, fraqueza, tensão, insônia, confusão, ansiedade, dor de cabeça, calafrios, palidez ou rubor das faces, palpitação, arritmia cardíaca, hipertensão ou hipotensão, boca seca, gosto metálico na boca, náusea, vômito, diarréia, câimbras abdominais, alteração da libido (ACHÉ, 2007). Os possíveis efeitos adversos relacionados à anfepramona já relatados na literatura incluem insônia, fraqueza, cansaço, dor de cabeça, vertigem, nervosismo, irritabilidade, manifestações depressivas, alteração do paladar, boca seca, náuseas, vômitos, diarréia ou constipação, taquicardia, perturbações das funções sexuais e urticária, agranulocitose, disritmia cardíaca, cardiomiopatia, isquemia cerebral, acidente cerebrovascular, leucopenia, hipertensão pulmonar primária e desordens psicóticas (MICROMEDEX, 2009).

Cabe ressaltar que em relação ao uso de uma única e pequena dose (25 mg) de femproporex e ou de anfepramona em indivíduos saudáveis, como proposto nesta ementa, não são esperados efeitos adversos importantes e que causem risco a saúde.

5. Referências

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Micromedex® Healthcare Series: MICROMEDEX, Greenwood Village, Colorado, 2009.

Termo de Consentimento Livre e Esclarecido

Você está sendo convidado a participar de um estudo que objetiva avaliar por quanto tempo o femproporex e seu metabólito pode ser detectados na saliva e urina após administração oral de uma única dose (25 mg). Este estudo está inserido no projeto "Determinação de aminas psicoativas na saliva por microextração em fase sólida e cromatografia a gás" aprovado pela Comissão Científica/Comissão de Pesquisa e Ética em Saúde do Grupo de Pesquisa e Pós-graduação do Hospital de Clínicas de Porto Alegre (GPPG/HCPA) sob o número 09-005, em 07 de maio de 2009.

Objetivos: determinar as variações das concentrações salivares e urinárias de femproporex e seu metabólito com o passar do tempo, após administração única, via oral, deste medicamento; bem como comprovar a efetividade de técnicas analíticas previamente desenvolvidas para a detecção destes medicamentos.

Justificativa: faltam dados sobre o intervalo de tempo após administração oral de femproporex em que podem ser detectados o fármaco inalterado e seu metabólito na saliva.

Como será realizado o estudo: você deverá comparecer ao local do estudo na hora e dias agendados, onde deverá ingerir um comprimido de 25 mg de femproporex de especialidade farmacêutica aprovada para comercialização no Brasil e permanecer durante um período de 12 horas para o fornecimento das amostras com retorno para a ultima coleta ao final de 24 horas. Sob supervisão dos pesquisadores responsáveis, serão coletadas amostras de saliva e urina, em intervalos de tempo pré-estabelecidos (total de doze) num período de 24 horas. As coletas de saliva serão realizadas através de dispositivo de coleta Quantisal®, o qual consiste em um chumaço de algodão preso a uma haste plástica, que é inserido sob a língua por cerca de 5 minutos, período em que são coletados cerca de 1 mL de saliva. Após a coleta o algodão é inserido em solução conservante e congelado. As amostras de urina (cerca de 100 ml) serão coletadas em frasco plástico estéril com tampa rosca e congeladas. Serão fornecidos aos voluntários todos os materiais (devidamente identificados) e informações necessários para coleta.

Formas de Ressarcimento das Despesas decorrentes da Participação na Pesquisa: podem ser ressarcidas as despesas com alimentação e transporte relacionadas ao período da coleta.

Desconforto ou Riscos Esperados: os possíveis efeitos adversos relacionados ao femproporex já relatados na literatura incluem vertigem, tremor, irritabilidade, reflexos hiperativos, fraqueza, tensão, insônia, confusão, ansiedade, dor de cabeça, calafrios, palidez ou rubor das faces, palpitação, arritmia cardíaca, hipertensão ou hipotensão, boca seca, gosto metálico na boca, náusea, vômito, diarréia, câimbras abdominais, alteração da libido. Em relação ao uso de uma única e pequena dose (25 mg) de femproporex em indivíduos saudáveis não são esperados efeitos adversos importantes e que causem risco a saúde.

Informações: o voluntário tem garantia que receberá respostas a qualquer pergunta ou esclarecimento a qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa. Também os pesquisadores supracitados assumem o compromisso de proporcionar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando.

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Métodos Alternativos Existentes: não há outra metodologia de estudo para a obtenção dos dados requeridos senão a proposta.

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

Aspectos Regulatórios: elaborados de acordo com as diretrizes e normas regulamentadas de pesquisa envolvendo seres humanos atendendo à Resolução n.º 196, de 10 de outubro de 1996, do Conselho Nacional de Saúde do Ministério de Saúde – Brasília – DF.

Garantia de Proteção dos Dados dos Voluntários: os pesquisadores asseguram a privacidade dos voluntários quanto aos dados de identificação fornecidos na pesquisa.

Local e Horário da Realização do Estudo: Faculdade de Farmácia da UFRGS, situada na Av. Ipiranga, 2752, Bairro Santa Cecília, Porto Alegre/RS, às 7 horas e 30 minutos do dia estipulado, a ser combinado.

Nome Completo e telefones dos Pesquisadores para contato: Prof. Dr. Renata Pereira Limberger (051) 3308-5297 ou 8199-1189; Mestranda Daniele Zago Souza (051) 3235-9069 ou 92167801; Aluna de Graduação Eloisa Comiran (051) 3308-5297 ou 98323726. Contato com o CEP/HCPA: 3359-8304.

Consentimento Pós-Informação:

Eu, _____, após leitura e compreensão deste termo de informação e consentimento, entendo que minha participação é voluntária, e que posso sair a qualquer momento do estudo, sem prejuízo algum. Confirmo que recebi cópia deste termo de consentimento, e autorizo a execução do trabalho de pesquisa e a divulgação dos dados obtidos neste estudo no meio científico.

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

Porto Alegre,_____de_____de 2009.

Nome (por extenso):_____

Assinatura:_____

1ª via: Instituição

2ª via: Voluntário

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