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Programa de Pós-graduação em Química

MARIANE GONÇALVES SANTOS

Síntese, caracterização e emprego de Polímeros Molecularmente Impressos na extração de fármacos em amostras humanas de plasma e urina

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À Deus, pai eterno e misericordioso;
a meus pais amados, que fizeram de mim a pessoa que sou hoje;
à minha filha Júlia, minha motivação para sempre ir além;
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ao meu querido Jesper, por todo apoio.

Dedico.

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Não sei quantas almas tenho.
Cada momento mudei.
Continuamente me estranho.
Nunca me vi nem achei.
De tanto ser, só tenho alma.
Quem tem alma não tem calma.
Quem vê é só o que vê,
Quem sente não é quem é,

Atento ao que sou e vejo,
Torno-me eles e não eu.
Cada meu sonho ou desejo
É do que nasce e não meu.
Sou minha própria paisagem;
Assisto à minha passagem,
Diverso, móbil e só,
Não sei sentir-me onde estou.

Por isso, alheio, vou lendo
Como páginas, meu ser.
O que segue não prevendo,
O que passou a esquecer.
Noto à margem do que li
O que julguei que senti.
Releio e digo: "Fui eu ?"
Deus sabe, porque o escreveu.

Fernando Pessoa

RESUMO

A análise de compostos orgânicos em matrizes complexas requer a aplicação de metodologias com boa seletividade e detectabilidade, que levem a resultados confiáveis. Baseando-se nesta demanda, a química analítica tem proposto soluções viáveis e inovadoras para tornar esses ensaios mais dinâmicos. Dentre elas podemos destacar as técnicas cromatográficas, a espectrometria de massas e os sistemas multidimensionais. Acompanhando este desenvolvimento, sorventes seletivos como os polímeros de impressão molecular (MIP), os materiais de acesso restrito (RAM), os polímeros de impressão molecular de acesso restrito revestidos com monômeros hidrofílicos (RAMIP) e os polímeros de impressão molecular de acesso restrito revestidos com monômeros hidrofílicos e albumina sérica bovina (RAMIP-BSA) vêm sendo adotados com sucesso no preparo de amostras. Neste contexto, essa tese de doutorado teve como objetivo sintetizar, caracterizar e avaliar as potencialidades do emprego de diferentes tipos de polímeros molecularmente impressos na extração fármacos em amostras biológicas, bem como associar estes materiais a técnicas de preparo de amostras *online* à cromatografia e/ou espectrometria de massas. O primeiro artigo trata de um estudo comparativo de polímeros de impressão molecular seletivos a beta- bloqueadores. Os polímeros foram sintetizados pelo método de precipitação utilizando ácido metacrílico como monômero funcional, hidróxi-etilmetacrilato e glicidil dimetacrilato, como comonômeros hidrofílicos, etilenoglicol dimetacrilato como agente de ligação cruzada, 2,2-azoisobutironitrila como iniciador radicalar e acetonitrila como solvente. Parte dos materiais obtidos foi revestida com BSA (albumina sérica bovina). Os polímeros foram caracterizados e pode-se concluir que a maneira pela qual a síntese dos materiais é conduzida influencia na forma e tamanho de partículas e que a adição de co-monômeros hidrofílicos, bem como o revestimento com BSA não altera a estrutura química dos mesmos. Os estudos de adsorsão mostraram que os polímeros alcançam o equilíbrio de adsorção em 60 min, que o revestimento com BSA não altera o perfil de adsorção dos mesmos, que é evidente a diferença de adsorção entre polímeros impressos e não impressos e que o modelo que melhor se adequa, a fim de descrever o perfil de adsorção, é o modelo de Langmuir. O RAMIP foi o polímero que melhor adsorveu os beta-bloqueadores, comparado à MIP, MIP-BSA (polímero molecularmente impresso revestido com BSA) e RAMIP-BSA, sendo esta a razão pela qual ele foi escolhido para o desenvolvimento da metodologia que é trazida no segundo artigo, no qual beta-bloqueadores são analizados em urina para monitoramento de doping. No método desenvolvido, uma pré-coluna de HPLC vazia

foi preenchida com RAMIP e utilizada para a montagem de um sistema *Column Switching* acoplado à espectrometria de massas. As fases móveis utilizadas foram tampão formiato de amônio 10 mmol L^{-1}, como fase de carregamento e recondicionamento e uma solução ácido fórmico 0,01% - metanol (v/v) na proporção 30:70 (v/v) como fase de eluição e lavagem; ambas a uma vazão de 0,4 mL min^{-1}. O sistema foi utilizado para a extração *online* e simultânea de oxprenolol (1,0-75,0 µg L^{-1}), atenolol, propranolol, nadolol, pindolol, labetalol e metoprolol (todos na faixa de 3,0-50,0 µg L^{-1}), em amostras de urina. O coeficiente de correlação foi superior a 0,99 para todos os analitos. O método foi otimizado, validado e apresentou todos os parâmetros em conformidade com o que exigido pela legislação. No terceiro artigo um RAMIP seletivo a antidepressivos tricíclicos foi sintetizado pelo método *in bulk*, usando amitriptilina como molécula modelo, ácido metacrílico como monómero funcional e glicidilmetacrilato como co-monômero hidrofílico. Posteriormente, foi realizada a abertura do anel epóxido e o polímero foi recoberto com BSA. Uma pré-coluna de HPLC foi preenchida com RAMIP-BSA e foi acoplada a um detector de ultravioleta/visível e a um espectrômetro de massas. Foram usadas água como fase de carregamento e recondicionamento e uma solução aquosa de ácido acético a 0,01% (v/v) - acetonitrila 30:70 (v/v) como fase de eluição. O sistema foi utilizado para a extracção *online* e identificação/quantificação de nortriptilina, desipramina, amitriptilina, imipramina, clomipramina (faixa de 15,0 a 500,0 µg L^{-1}), simultaneamente, a partir de amostras de plasma. O coeficiente de correlação foi superior a 0,99 para todos os analitos. Os valores de desvio padrão relativo variaram de 1,34% a 19,13% para precisão intra-dias e de 1,32% a 19,77% para precisão inter-dias. Os valores de erro relativo variaram de -19,15% a 19,51% para exatidão intra-dias e -9,04% a 16,22% para exatidão inter-dias.

Palavras chave: MIP. Espectrometria de massas. Doping. Beta-bloqueadores. Antidepressivos Tricíclicos. *Column Switching*. RAMIP. RAMIP-BSA.

ABSTRACT

Analysis of organic compounds in complex matrices requires the application of methods with good selectivity and detectability, to lead reliable results. Based on this demand, analytical chemistry has proposed viable and innovative solutions to make these tests more dynamic. Among them, we can highlight the chromatographic techniques, mass spectrometry and multidimensional systems. Following this development, selective sorbents such as molecularly imprinting polymers (MIPs), restricted access materials (RAM), restricted access molecularly imprinting polymers obtained by covering the surface with hidrofilic comonomers (RAMIP) and restricted access molecularly imprinting polymers obtained by covering the surface with hidrofilic comonomers and bovine serum albumin (RAMIP-BSA), have been successfully used in sample preparation. In this context, this PhD thesis aimed to synthesize, characterize and evaluate the different types of molecularly imprinted polymers potentialities for drugs extraction from biological samples and associate these materials with online sample preparation techniques, combined to chromatography and/or mass spectrometry. In paper one we proposed a comparative study of different molecularly imprinted polymers selective to beta-blockers. The polymers were synthesized by precipitation method using methacrylic acid as functional monomer, hydroxy-ethyl methacrylate and glycidyl dimethacrylate, as hydrophilic co-monomer, ethylene glycol dimethacrylate as crosslinking agent, 2,2-azoisobutironitrile as radical initiator and acetonitrile as solvent. One part of the obtained materials was coated with BSA (bovine serum albumin). The polymers were characterized and it was possible to conclude that the synthesis procedure influences the size and shape of particles and that hydrophilic co-monomer addition as well as coating with BSA do not alter the chemical recognition ability of the material. The difference between imprinted and non-imprinted polymers' adsorption was evident (suggesting that imprinted polymers have a better capacity to bind the template than the non-imprinted ones). The Langmuir model presents the best fit to describe the materials' adsorption profile. The polymer covered with hydrophilic monomers presented the best adsorption for the template in an aqueous medium, probably due to a hydrophilic layer on its surface. We also concluded that an association of the hydrophilic monomers with the BSA coating is important to obtain materials with higher capacity of macromolecule exclusion. In paper two, the RAMIP selective to oxprenolol was chosen as sorbent in a online method for beta blockers analyses from human urine for doping monitoring. A column filled with RAMIP was coupled to an LC-MS/MS instrument under the multidimensional configuration, with 10.0

mmol L^{-1} ammonium formate buffer (pH 5.0) as the loading and reconditioning mobile phase and a 0.01% formic acid aqueous solution – methanol (30:70 v/v) as the elution mobile phase. The system was used for on-line extraction and quantization of oxprenolol (from 1.0 to 75.0 μg L^{-1}), atenolol, propranolol, nadolol, pindolol, labetalol and metoprolol (all from 3.0 to 50 μg L^{-1}) simultaneously, from urine samples. The correlation coefficient was higher than 0.99 for all the analytes. Suitable precision and accuracy were obtained. In paper three a RAMIP, selective to tricyclic antidepressant, was synthesized by the in bulk method. Amitriptyline was used as template molecule, methacrylic acid as the functional monomer and glycidyl methacrylate as hydrophilic co-monomer. Afterwards the epoxide ring opening was made and the polymer was covered with BSA. A column filled with RAMIP-BSA was coupled to an MS/MS instrument under the multidimensional configuration, with water as the loading and reconditioning mobile phase and a 0.01% acetic acid aqueous solution - acetonitrile at 30:70 as the elution mobile phase. The system was used for online extraction and quantization of nortriptyline, desipramine, amitriptyline, imipramine and clomipramine, simultaneously, from plasma samples. The correlation coefficient was higher than 0.99 for all the analytes. The RSD (relative standard deviation) values ranged from 1.34% to 19.13% for intra assay precision and 1.32% to 19.77% for inter assay precision. The E% (relative error) values ranged from -19.15% to 19.51% for intra assay accuracy and from -9.04% to 16.22% for inter assay accuracy.

Keywords: MIP. Mass spectrometry. Doping. Beta-blockers. Tricyclic antidepressants. Column Switching. RAMIP. RAMIP-BSA.

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LISTA DE ABREVIATURAS E SIGLAS

ABDV	Azo-bis-dimetilvaleronitrila
AIBN	2,2-azoisobutironitrila
ALC	Agente de Ligação Cruzada
BSA	Albumina bovina sérica
Ce	Concentração do adsorbato no equilíbrio
EGDMA	Etileno glicol dimetacrilato
FT-IR	Espectroscopia de infra-vermelho com transformada de Fourier
GC-MS/MS	Cromatografia gasosa acoplada a espectrometria de massas sequencial
GDMA	Glicidil dimetacrilato
GMA	Glicidil metacrilato
HEMA	Hidróxi-etilmetacrilato
HPLC	Cromatografia líquida de alta performance
K_F	Constante de Freundlich
K_L	Constante de Langmuir
LC-MS/MS	Cromatografia líquida acoplada a espectrometria de massas sequencial
MAEL	Octa-acetato de lactose
MF	Monômero funcional
MIP	Polímeros molecularmente impressos
MM	Molécula modelo
MS	Espectrometria de massas
Qe	Quantidade adsorvida por grama do adsorvente
Qm	Constante relacionada com a energia de adsorção
RAM	Materiais de acesso restrito
RAMIP	Polímeros de acesso restrito molecularmente impressos revestido com comonômeros hidrofílicos

RAMIP-BSA	Polímeros de acesso restrito molecularmente impressos revestido com comonômeros hidrofílicos e albumina sérica bovina
R_L	Parâmetro de equilíbrio
WADA	Agência mundial anti doping

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1 INFORMAÇÕES ADICIONAIS SOBRE O FORMATO DA TESE

Conforme descrito nas normas do Programa de Pós-graduação em Química da UNIFAL-MG, artigo 79, a critério do orientador e do discente, e após análise do CPPGQ, a tese poderá ser apresentada sob a forma de 01 (um) volume contendo:

I- Uma revisão e discussão ampla da literatura;

II- No mínimo um artigo aceito ou publicado em revista indexada, "CAPES-Qualis B1 ou superior" e um artigo aceito ou publicado em "CAPES-Qualis B2 ou superior". O discente deverá ser o primeiro autor do artigo e o orientador, obrigatoriamente deverá figurar entre os autores. Além disso, o artigo aceito ou publicado deverá ser representativo dos resultados obtidos no desenvolvimento do projeto de pesquisa.

Por atender aos critérios do artigo acima citado, contido nas normas do PPGQ, a parte que compreende aos OBJETIVOS, MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO E CONCLUSÃO desta tese, serão apresentados na forma de 3 artigos científicos, sendo que os dois primeiros estão publicados no periódico "ANALYST", DOI: 10.1039/C5AN01482D e 10.1039/C4AN02066A, CAPES-Qualis A2 e o terceiro ainda não submetido a um periódico da área.

2 INTRODUÇÃO

A análise de compostos de baixo peso molecular em amostras biológicas tais como fármacos, metabólitos e toxicantes, presentes em fluidos biológicos e tecidos corporais, requer métodos analíticos simples e rápidos que possam ser empregados na monitorização terapêutica, em toxicologia forense, em toxicologia clínica e em toxicologia social. Neste contexto, a análise quantitativa de fármacos e seus metabólitos tem sido utilizada extensivamente em testes de bioequivalência e farmacocinética, se tornado cada vez mais importantes devido à necessidade de se compreender os efeitos terapêuticos e tóxicos destes compostos. A pesquisa de fármacos e seus metabólitos em amostras biológicas mostra-se presente também em outros segmentos, como para a finalidade esportiva, em que o controle de dopagem precisa monitorar e fiscalizar o uso de substâncias proibidas pelos atletas (KATAOKA; SAITO, 2011).

Contudo, a complexidade das matrizes biológicas e a presença de muitos concomitantes (muitas vezes em concentrações maiores do que a das substâncias de interesse) fazem com que seja necessário um pré-tratamento da amostra, a fim de se eliminar os interferentes da matriz e pré-concentrar o analito. Sem esta etapa de preparo, as análises tornam-se pouco seletivas e consequentemente não confiáveis.

A busca por técnicas seletivas de análise é uma tendência atual. No entanto, quando materiais seletivos para extração eram ainda pouco conhecidos, a seletividade de uma análise era, muitas vezes, garantida através do emprego de reações químicas que se processam acompanhadas de variação das propriedades físicas ou químicas do analito. A partir destas, os detectores até então dotados de pouca seletividade, eram capazes de identificar essas modificações e, por conseguinte, quantificar e identificar os analitos de interesse. Contudo, devido à especificidade de algumas reações, a análise se limitava, em grande maioria, a apenas uma substância, pois os demais compostos que pudessem também reagir de forma similar ao analito de interesse poderiam interferir na análise. Assim, o detector, isoladamente, era incapaz de atribuir um sinal de forma a identificar que modificações seriam oriundas de cada um desses componentes, a não ser que uma técnica de separação, como a cromatografia, fosse acoplada a ele.

Dessa forma, acompanhando o grande desenvolvimento tecnológico dos últimos anos, a química analítica vem apresentando soluções inovadoras que facilitam este processo, tornando-o mais dinâmico e mais seletivo, e permitindo o estudo de diversos compostos

orgânicos em amostras cada vez mais complexas. A espectrometria de massas (MS), devido a sua alta sensibilidade, seletividade e rapidez, conquista cada vez mais espaço nas análises de compostos em matrizes complexas.

Atualmente, estudos sobre o emprego de materiais seletivos na purificação de amostras biológicas vêm crescendo, dentre os quais se destaca a elaboração de adsorventes de extração (para SPE) eficientemente capazes de purificar amostras complexas (HE et al., 2007). Nesse sentido, dois adsorventes vem ganhando espaço: i) os polímeros molecularmente impressos (do inglês *Molecularly Imprinted Polymers* - MIP), que são polímeros sintéticos capazes de se moldar tridimensionalmente à molécula de interesse, deixando cavidades impressas de acordo com sua forma estrutural, extraindo-a seletivamente (ANDERSON, 2000) e ii) os materiais de acesso restrito (do inglês *Restrict Access Materials* - RAM), que são adsorventes capazes de reter diversos analitos de baixo peso molecular e eliminar macromoléculas como proteínas e polipeptídios (YIM; JEONG; PARK, 2001).

Como principal vantagem dos MIP pode-se destacar sua alta seletividade a uma molécula ou classe de moléculas, sendo muito eficientes para o estudo de amostras que não apresentam macromoléculas em sua constituição, como a urina por exemplo. Contudo, estes materiais são susceptíveis à ligação de macromoléculas, o que inviabiliza sua utilização na extração direta de fármacos em fluidos biológicos como plasma. Já os RAM, embora eficientes na eliminação de macromoléculas, não apresentam reconhecimento molecular e por isso são pobres em seletividade. Dessa forma, Haginaka et al., (1999), propuseram um material que associa as características vantajosas dos RAM e dos MIP em um único polímero, capaz de reter seletivamente uma dada molécula e eliminar significativamente as macromoléculas, bem como ter um elevado reconhecimento molecular em meio aquoso. Esse polímero, denominado de RAMIP (do inglês *restricted access molecularly imprinted polymer*), apresenta sítios específicos de reconhecimento molecular, bem como grupos hidrofílicos externos que bloqueiam a ligação com proteínas e melhoram a seletividade em meio aquoso.

É sabido que os fármacos bloqueadores de adrenorreceptores têm grande importância terapêutica no tratamento de várias desordens cardiovasculares, tais como hipertensão, angina *pectoris* e arritmia cardíaca. No entanto, esses fármacos também podem ser usados por atletas a fim de reduzir a atividade simpática em esportes que exijam alta precisão e controle pisíquico, como por exemplo provas de tiro, arco e flecha, ginástica artística, dentre outros. O uso destas subtâncias diminui consideravelmente a ocorrência de tremores que podem prejudicar a performance do atleta. Como consequência, tais fármacos foram adicionadas à lista de

substâncias proibidas pela World Anti Doping Agency (WADA) (WADA, 2014; MURRAY; DANACEAU, 2009).

Os antidepressivos tricíclos são fármacos comumente usados no tratamento e alívio dos sintomas da depressão. Contudo, alguns efeitos colaterais podem ser observados, principalmente aqueles relacionados à sua ação nos receptores muscarínicos. Os efeitos adversos mais relatados estão associados à boca seca, visão borrada, constipação, tremores, efeitos cardiovasculares e retenção urinária (HÄRTTER; HIEMKE, 1992; FURLANUT; BENETELLO, 1990; WOLF et al., 2012). O monitoramento dos níveis plasmáticos desses fármacos tem sido requerido para um melhor ajuste de dose, eviatando ou detectando a ocorrência de níveis tóxicos ou subterapêuticos (monitorização terapêutica) e para a realização de estudos farmacocinéticos.

Diante do exposto e baseando-se na necessidade de métodos simples, precisos, exatos e seletivos para análises de compostos orgânico em amostras biológicas, foram desenvolvidos novos métodos para análise de beta-bloqueadores em urina e de antidepressivos tricíclicos em plasma. Em ambos, o preparo das amostras (de urina e plasma) foi *online*. Para as análises em urina foi utilizado uma polímero de impressão molecular revestido por co-monômeros hidrofílicos e com seletividade melhorada para análise em matrizes aquosas. As análises foram feitas através de um sistema de column switching com detecção/quantificação por MS. Também foi realizado um estudo comparativo que abordou modificações realizadas na superfície de polímeros de impressão molecular e suas consequências, por meio da caracterização de diferentes materiais sintetizados com a finalidade de extrair beta-bloqueadores em amostras biológicas. Para análise dos antidepressivos, um polímero de impressão molecular de acesso restrito, que permite a eliminação das proteínas plasmáticas e retenção de analitos de baixo peso molecular foi utilizado. As análises foram feitas sem prévia separação cromatográfica e com quantificação/identificação realizada por MS.

3 REVISÃO DE LITERATURA

A síntese bibliográfica dessa Tese refere-se aos seus pontos mais relevantes, tais como conceitos e possibilidades de uso de cetros materiais no preparo de amostras complexas (MIP, RAM e RAMIP), definição e utilização dos fármacos analisados, bem como a abordagem de algumas técnicas para análise de compostos orgânicos de baixo peso molecular.

3.1 *Polímeros molecularmente impressos (MIP)*

A biomimetização de interações bioquímicas é um dos maiores desafios em várias áreas da ciência. A partir deste ponto de vista, os polímeros molecularmente impressos (MIP), tem atraído considerável atenção nas últimas décadas, pois aparecem como uma ferramenta promissora para o desenvolvimento de sistemas com reconhecimento molecular semelhantes aos sistemas específicos enzima-substrato ou antígeno-anticorpo (VERLI; BARREIRO, 2005).

O conceito de impressão molecular surgiu a partir da teoria de Linus Pauling para formação de anticorpos, em que um antígeno era usado como uma molécula molde para moldar a cadeia polipeptídica de anticorpos, resultando numa configuração complementar ao antígeno na respectiva cadeia de anticorpos. A partir desta concepção, surgiu a ideia de produzir uma estrutura rígida tridimensional (um polímero) ao redor de uma molécula molde que pudesse atuar de forma similar ao anticorpo, ou seja, que pudesse efetuar seletivamente o reconhecimento molecular. Os MIP são materiais rígidos e tridimensionais sintetizados ao redor de uma molécula molde, por meio de ligação covalente ou não covalente, resultando em sítios seletivos (TARLEY; SOTOMAYOR; KUBOTA, 2005; MAGALHÃES et al., 2007; DIAS et al., 2008).

Os sítios de reconhecimento são obtidos pelo arranjo de monômeros funcionais polimerizáveis ao redor da molécula molde resultando na formação de complexos. Estes são fixados através de reações de entrecruzamento entre o reagente de ligação cruzada e o monômero funcional. A remoção da molécula molde da matriz polimérica forma lacunas (sítios de reconhecimento) que irão exibir afinidade pelo analito/analitos de interesse (ANDERSON; PAPRICA; ARDVIDSSON, 1997; WALSHE et al., 1997). A reação de polimerização é

iniciada após a adição de um iniciador radicalar. Por fim a molécula molde é removida por meio de soluções de lavagens (Figura 1) (MAYES; MOSBACH, 1997; YE; MOSBACH, 2001).

Na literatura, são relatadas três diferentes abordagens para a preparação dos MIP, em termos da natureza das interações entre a molécula molde e os monômeros funcionais: **i) covalente, ii) não-covalente e iii) semi-covalente**. A primeira abordagem envolve a formação de ligações covalentes reversíveis entre a molécula molde e o monômero funcional. Após a polimerização, a molécula molde é retirada do polímero pela clivagem das ligações correspondentes. A forte ligação entre a molécula molde e o monômero funcional é a principal vantagem desta forma de síntese, o que garante uma maior seletividade do polímero impresso. Em contrapartida, essa forte ligação dificulta o processo de eluição dos analitos, sendo necessário, em muitos casos, procedimentos drásticos de hidrólise (TURIEL; MARTÍN-ESTEBAN, 2010).

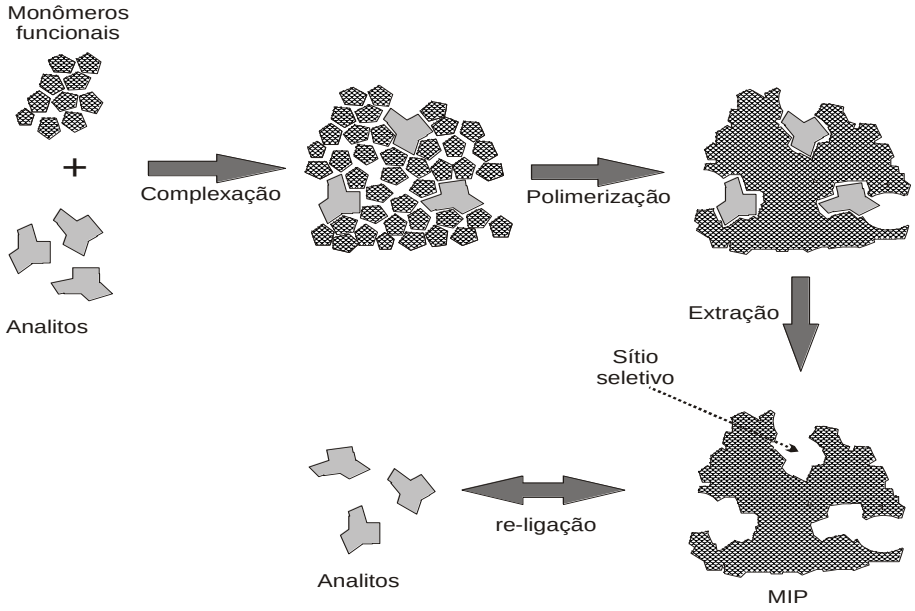


Figura 1. Esquema genérico da síntese de MIP.
Fonte: Adaptado de ANDERSON (2000a).

A síntese não covalente se baseia na formação de interações relativamente fracas (ligações de hidrogênio, interações eletrostáticas, etc.) entre a molécula molde e monômeros funcionais. Esta abordagem é a mais usada para a preparação de MIP, devido à possibilidade de aplicação para analitos que pertençam a uma mesma classe e à facilidade de eluição. Entretanto, uma menor seletividade é observada nesses polímeros, uma vez que as interações entre molécula molde e monômero funcional são mais fracas quando comparadas com a ligação covalente (TURIEL; MARTÍN-ESTEBAN, 2010).

Buscando aliar as vantagens que as duas abordagens anteriores oferecem, foi criada a síntese semi-covalente, que estabelece que o processo de polimerização seja mediado por ligações covalentes, que garante a maior seletividade ao MIP; enquanto as demais interações, durante a utilização do material, sejam estabelecidas por ligações não-covalentes, facilitando assim o processo de eluição (NAVARROA et al., 2011).

O primeiro passo para a síntese dos MIP consiste em estabelecer criteriosamente a escolha do monômero e do analito. O analito necessita conter em sua estrutura molecular grupos funcionais capazes de interagir com os monômeros, a fim de formar um complexo estável (AL-KINDY et al., 2000).

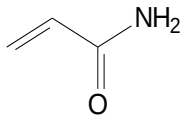
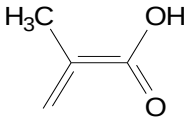
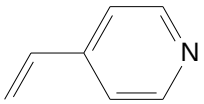
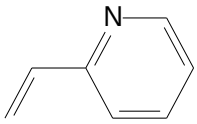
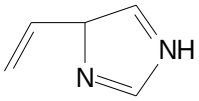
A molécula molde, na maioria das vezes, é o próprio analito, usado para moldar estereoquimicamente os sítios de ligação. Três características são de suma importância na escolha desta molécula: ela não deve conter grupos envolvidos na polimerização, deve manter a estabilidade química do polímero e apresentar grupos funcionais bem adaptados para a ligação com o monômero funcional (GUO et al., 2006).

Os monômeros funcionais são responsáveis pela ligação entre a molécula molde e a rede polimérica. Sua seleção está intimamente relacionada às características do analito em questão. A força das interações entre eles determina a seletividade do material, enquanto que a proporção molar determina o número de sítios seletivos disponíveis para as ligações (RACHKOV; MINOURA, 2001). Na Tabela 1 é possível observar alguns dos principais monômeros funcionais empregados na síntese dos MIP.

Outro parâmetro que influencia a estabilidade da formação do complexo “analito-monômero” é o solvente empregado durante a síntese do polímero. Este possui influência direta nas características morfológicas do material. O solvente ideal deve fornecer um meio onde analitos e monômeros sejam solúveis e não deve interferir na interação analito-monômero. Como determina a porosidade do material, este recebe o nome de solvente porogênico. Para que os MIP apresentem características desejáveis com relação à seletividade, é fundamental que haja um estudo acerca do solvente utilizado na síntese (TARLEY; SOTOMAYOR; KUBOTA, 2005).

O agente de ligação cruzada é responsável pela rigidez do polímero, uma vez que promove a interligação das cadeias poliméricas formadas pelos monômeros funcionais. Sua relação com o monômero funcional deve ser bem estudada para favorecer o número de cavidades efetivas e diminuir a quantidade de cavidades não seletivas.

Tabela 1. Alguns dos principais monômeros funcionais empregados na síntese dos MIP.

Fórmula estrutural	Nome do monômero
	Acrilamida
	Ácido metacrílico
	4-Vinilpiridina
	2-Vinilpiridina
	4-Vinilimidazol

Fonte: Do autor.

O etilenoglicol dimetacrilato (EGDMA) é um reagente de ligação amplamente utilizado. Ele possibilita a formação de materiais estáveis e com ligeira transferência de massa. Na Figura 2 é apresentada a estrutura química do EGDMA (HOGENDOORN; ZOONEN, 2000).

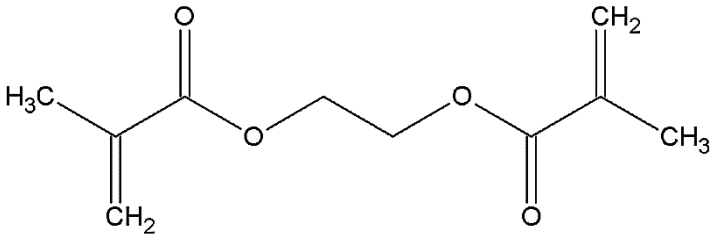


Figura 2. Estrutura química do etilenoglicol dimetacrilato (EGDMA).
Fonte: Do autor.

Em uma reação de polimerização, após adição do reagente de ligação cruzada, é acrescentado um iniciador radicalar ao meio reacional. Esse, por sua vez, sofre quebra em sua estrutura, gerando radicais responsáveis pelo início da reação de polimerização (HOGENDOORN; ZOONEN, 2000). O iniciador radicalar 2,2'-azo-bis-isso-butironitrila é mais empregado na síntese dos MIP (CORMACK; ELORZA, 2004).

A Figura 3 mostra a estrutura de alguns iniciadores mais utilizados.

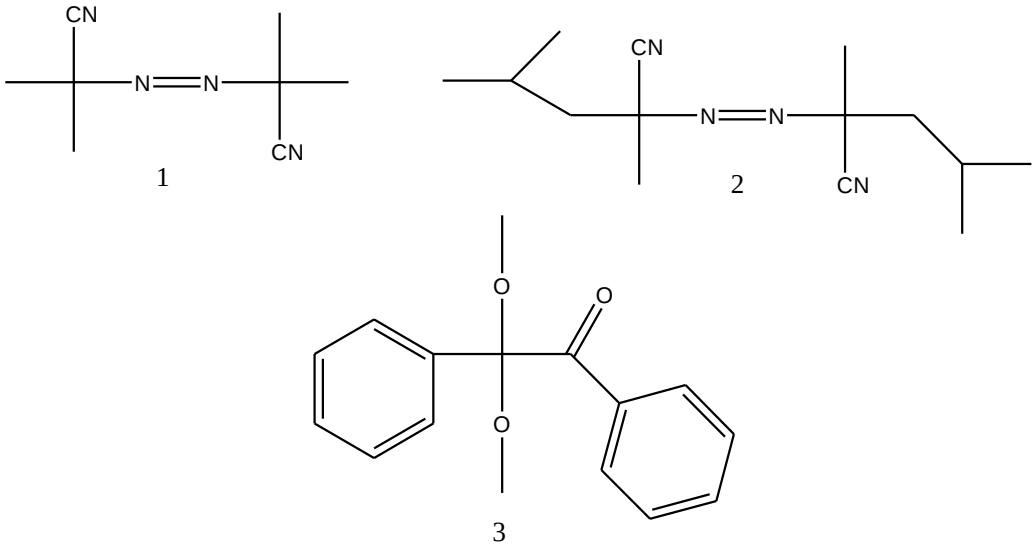


Figura 3. Exemplos de iniciadores radicalares empregados na impressão molecular: 1) 2,2'-azo-bis-iso-butironitrila (AIBN); 2) azo-bis-dimetilvaleronitrila (ABDV); 3) dimetilacetal de benzíla.
Fonte: Do autor.

A escolha do método de síntese dos polímeros de impressão molecular é, normalmente, influenciada pelas características de cada um deles em função da aplicação pretendida para o MIP nas diferentes áreas. Na literatura, a síntese do MIP pode ser feita de quatro maneiras, mas convencionalmente ela é realizada pelo método conhecido como polimerização em "*in bulk*", que ocorre num sistema homogêneo. Neste método, a síntese é conduzida em uma ampola onde são adicionados o monômero funcional, a molécula molde, o solvente, o reagente de ligação cruzada e o iniciador radicalar. Em seguida o oxigênio presente no sistema é retirado borbulhando-se argônio ou nitrogênio. A ampola é lacrada e submetida a aquecimento ou radiação UV. Para o melhor rendimento na produção do MIP, a seleção dos reagentes bem como suas concentrações e volumes devem ser selecionados de forma criteriosa. Ao final da síntese uma massa rígida é formada (monólito), sendo em seguida triturada, peneirada e lavada com solvente para a retirada da molécula molde (CORMACK; ELORZA, 2004).

O método de polimerização por suspensão é o mais simples e comum para produção de MIP com partículas de tamanho uniforme. Nesta técnica, a reação polimérica ocorre dentro das gotas do monômero suspensos normalmente em água. Esta reação é conduzida quando monômero, analito, reagente de ligação cruzada e iniciador radicalar são solubilizados em solvente orgânico apolar e posteriormente adicionados em água. O sistema é mantido homogêneo pela adição de um agente dispersante (tensoativo) que previne a separação de fases. O meio reacional é aquecido e mantido sob agitação. O polímero obtido é separado da fase aquosa apresentando formato esférico de 5 a 50μm de tamanho. Devido à uniformidade de tamanho e formato das partículas, alguns trabalhos têm sido desenvolvidos empregando-se a

polimerização por suspensão em métodos de separação *online*, obtendo-se um empacotamento uniforme das colunas (HE et al., 2007).

Na síntese pelo método de precipitação, o volume de solvente é aumentado, em relação à síntese *in bulk* e a reação deve ser processada em um recipiente dotado de sistema de alívio de pressão, evitando assim possíveis explosões. Outra característica desse processo é a agitação constante que favorece a formação de partículas mais homogêneas. Ao final do processo, o resultado é um material com partículas de tamanho e forma mais regulares em relação à síntese *in bulk* (HE et al., 2007).

O método de polimerização por expansão em multi-etapas é uma alternativa aos acima descritos, devido à viabilidade de obtenção de polímeros com tamanhos e poros regulares e maiores. Monômero e solvente passam por uma etapa de expansão sobre um polímero com diâmetro bem definido, normalmente poliestireno (diâmetro de 1µm), antes da polimerização do MIP. Desta forma, as partículas poliméricas serão responsáveis pela formação de partículas regulares e de tamanho reduzido, diferente dos métodos anteriores nos quais isto é atribuído ao meio reacional (TARLEY; SOTOMAYOR; KUBOTA, 2005).

3.2 *Materiais de acesso restrito (RAM)*

Para análise de compostos orgânicos em matrizes complexas, como plasma e soro humano, a presença de macromoléculas (mais frequentemente proteínas), exige uma etapa prévia de preparo, antes da extração propriamente dita, para que seja feita a eliminação destes componentes. Com o intuito de dinamizar a análise nestes tipos de matrizes, os materiais de acesso restrito (RAM) foram desenvolvidos, sendo usados principalmente para a extração de substâncias com baixa massa molecular (SADÍLEK; SATÍNSKÝ; SOLICH, 2007).

A expressão meio de acesso restrito (do inglês *restricted acces media* – RAM) foi utilizada primeiramente em 1991 como um termo geral para designar suportes cromatográficos que permitiam a injeção direta de amostras biológicas e limitando a interação dos analitos de baixo peso molecular dentro dos poros. A criação deste material é atribuída a Hagestam e Pinkerton em 1985. (HAGESTAM; PINKERTON, 1985; DESILETS; ROUNDS; REGNIER, 1991; SANTOS-NETO, 2007).

O emprego desses adsorventes em sistemas *online* tem permitido a injeção direta de amostras biológicas (sem tratamento prévio) no sistema de análise (RIEUX et al., 2007). A

grande vantagem deste tipo de material sobre outros sorventes é a capacidade de suportar repetidas injeções de grandes volumes de amostras sem alterar suas propriedades de retenção.

De um modo simplificado, o mecanismo de adsorção de moléculas de baixo peso molecular ocorre devido à presença de poros dotados de grupos ligantes (no interior do RAM) capazes de capturar essas moléculas (MACHTEJEVAS, 2006). A superfície externa dos RAM pode ser revestida com grupos hidrofílicos (barreira química), evitando assim a adsorção da matriz proteica (alto peso molecular). Desse modo, no momento da extração, os analitos de interesse (de baixo peso molecular) penetram nos poros e são retidos pelos grupos ligantes, enquanto que as macromoléculas são transportadas para o descarte sem que haja retenção das mesmas na coluna RAM (WANG et al., 2011). Como exemplo, podemos citar o RAM revestido com BSA (RAM-BSA). A BSA é imobilizada sobre um suporte por meio de reações de entrecruzamento, por meio do uso do glutaraldeído. É graças a esse revestimento que o acesso das macromoléculas hidrofílicas com o suporte fica restrito. A C18 é muito utilizada como suporte, apresentando resultados significativos com relação a eliminação de macromoléculas e à retenção dos compostos de baixo peso molecular (CASSIANO, 2006).

A Figura 4 mostra um RAM denominado de suporte de alquil diol sílica - ADS (A) e um RAM denominado de superfície coberta por proteína - PCS (B).

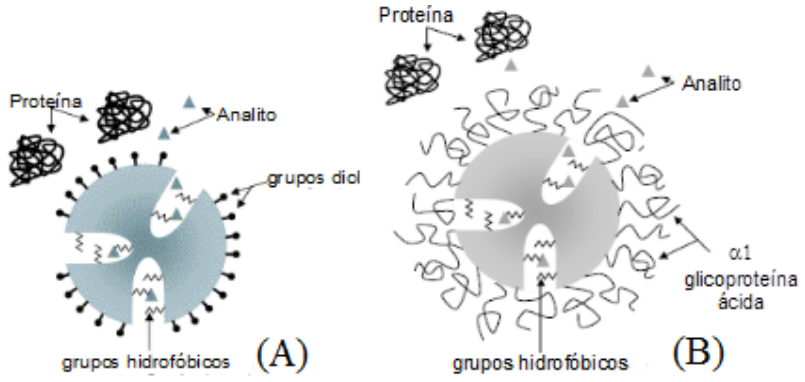


Figura 4. Exemplos de RAM com suporte de alquil diol sílica - ADS (A) e denominado de superfície coberta por proteína - PCS (B).
Fonte: Adaptado de SOUVERAIN; RUDAZ; VEUTHEY, 2004.

3.3 *Polímeros de acesso restrito molecularmente impressos (RAMIP)*

De acordo com o que foi descrito anteriormente, os MIP são materiais altamente seletivos a um composto ou uma classe de compostos, contudo, devido a sua capacidade de

reter macromoléculas, a extração direta de matrizes como plasma ou soro se torna inviável, uma vez que a ligação desses materiais às proteínas irá diminuir a sua seletividade drasticamente. Os RAM por sua vez, são capazes de eliminar macromoléculas, porém são pobres em seletividade, podendo reter qualquer molécula de baixo peso molecular (BOOS; FLEISCHER, 2001; KOEBER et al., 2001). Assim, a fim de congregar as vantagens de ambos os materiais, foi proposta a associação dessas tecnologias, com o intuito de se obter um polímero seletivo para purificação de amostras complexas, que tenham o seu reconhecimento molecular em meio aquoso potencializado, e que ao mesmo tempo sejam incapazes de se ligar a macromoléculas. Esses materiais foram chamados de polímeros de impressão molecular de acesso restrito (RAMIP), que apresentam sítios específicos de reconhecimento molecular, bem como grupos hidrofílicos/protetores externos que bloqueiam a ligação com macromoléculas (HAGINAKA et al., 1999).

Os materiais encontrados podem ser classificados de acordo com os reagentes de síntese que são empregados, sendo divididos entre aqueles que utilizam monômeros hidrofílicos e aqueles que utilizam monômeros que após um tratamento se tornam hidrofílicos.

Para a obtenção do primeiro, os MIP são sintetizados pelos métodos tradicionais de síntese de MIP e posteriormente são revestidos para a formação da camada hidrofílica que irá impedir o acesso de macromoléculas aos grupos ligantes do MIP. A Figura 5 ilustra o esquema genérico da síntese deste tipo de polímero. Haginaka et al. (1999) foram os precursores dessa tecnologia quando propuseram, em 1999, um RAMIP seletivo a *(S)*-naproxeno.

Já para se obter um RAMIP do segundo tipo são empregados monômeros que se tornam hidrofílicos após um tratamento, como glicidil metacrilato (GMA). A abertura no anel epóxido do GMA, realizada com o uso do ácido perclórico, é a responsável pela criação da camada hidrofílica. Puoci et al., em 2009, propuseram a síntese de um RAMIP baseada nesta técnica. O principal objetivo dos autores foi obter um polímero para ser empregado em ambientes fisiológicos (PUOCI et al., 2009).

Também se encaixa na segunda categoria os materiais que utilizam um glico-monômero para dar caráter hidrofílico ao polímero. O primeiro passo é a síntese do glico-monômero empregando-se octa-acetato de lactose (MAEL), um monômero funcional com caráter hidrofílico o hidróxi metilmetacrilato (HEMA), solvente e trifluoreto de boro (HUA et al., 2011). O restante da síntese ocorre de acordo com o modelo do trabalho de Haginaka et al., (1999). Ao final, o material passa por um processo chamado alcoólise, quando o metóxido de sódio reage com o MAEL retirando os grupos acetila e deixando OH livre no polímero, que dá o caráter hidrofílico ao mesmo. Ainda que as vantagens dos RAMIP no preparo de amostras

sejam claras, este tipo de tecnologia, por enquanto, não é muito utilizado. Ademais, poucos estudos foram conduzidos no sentido de compreender melhor o comportamento destes materiais em comparação com os MIP tradicionais.

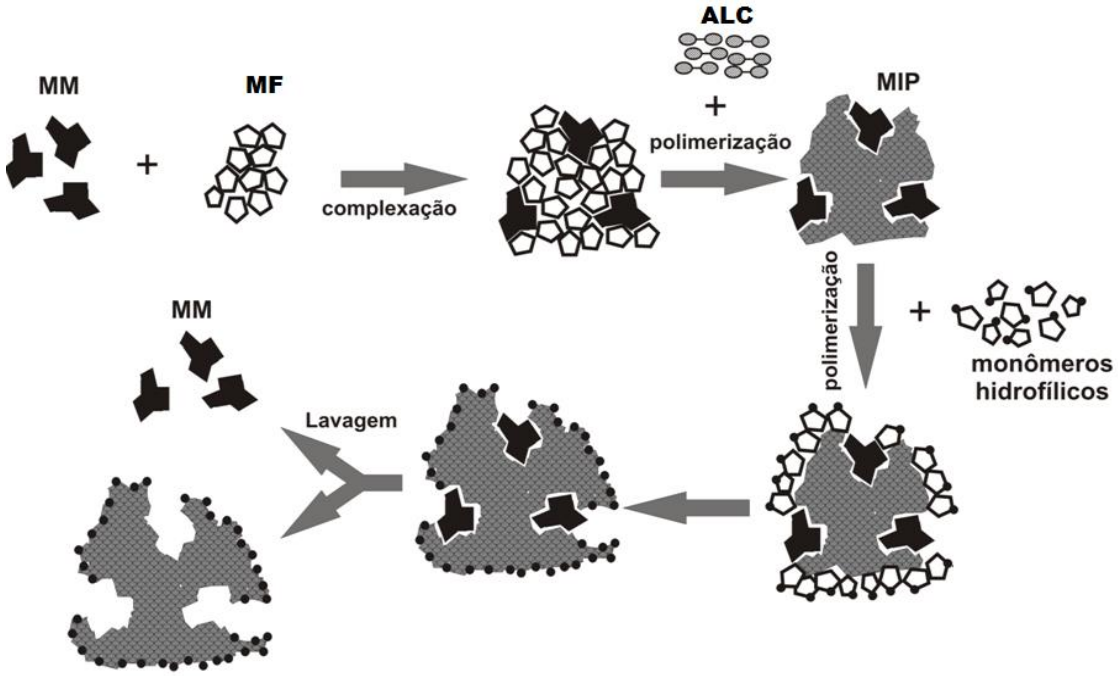


Figura 5. Esquema genérico da síntese de um RAMIP do primeiro tipo.
Fonte: Do autor.

Por fim, uma estratégia de obtenção RAMIP foi desenvolvida em nosso grupo de pesquisa e consiste no revestimento externo de um MIP com uma cápsula de albumina de soro bovino (BSA), resultando em um polímero de impressão molecular restrito à ligação de macromoléculas por meio deste revestimento (RAMIP-BSA) (MORAES et al., 2013).

O revestimento externo foi obtido por meio do entrecruzamento das cadeias de BSA empregando glutaraldeído. Assim, formou-se em torno do polímero uma cápsula proteica, que impediu a ligação de proteínas na superfície do RAMIP-BSA. O mecanismo de exclusão deve-se à repulsão eletrostática entre a capa de BSA e as proteínas da amostra, que se encontram negativamente ou positivamente ionizadas quando o pH do meio é maior ou menor que o ponto isoelétrico das proteínas, respectivamente. Os analitos de baixo peso molecular conseguem se difundir por entre as cadeias de BSA e ligar-se aos sítios seletivos. O polímero obtido neste trabalho apresentou excelente capacidade de eliminação das macromoléculas e recuperação adequada do analito, tendo sido empregado com sucesso na extração *on-line* de clorpromazina em amostras de plasmas sanguíneo humano para análise por HPLC-UV (MORAES et al., 2013)

3.4 Caracterização dos MIP

Para o sucesso da utilização dos MIP, é necessário conhecer bem o processo de síntese destes materiais e o seu comportamento. Assim, são necessários estudos que avaliem as características físicas e físico-químicas destes polímeros.

A microscopia é uma das ferramentas que pode ser usada no estudo de polímeros com capacidade de reconhecimento molecular. A microscopia eletrônica de varredura e a microscopia eletrônica de transmissão permitem verificar a integridade estrutural, o formato e o tamanho das partículas poliméricas e alguns casos a visualização dos macroporos (CORMACK; ELORZA, 2004).

A espectroscopia de infravermelho com transformada de Fourier (FT-IR) dos MIP pode ser realizada de uma forma simples (por exemplo, em disco de KBr ou diretamente nas próprias partículas do MIP), sendo utilizada para a obtenção de informações relativas aos grupamentos funcionais do polímero. O método apresenta uma importância particular quando os diferentes ambientes químicos (por exemplo, devido ao monômero funcional, ao agente reticulante e aos agentes de recobrimento num MIP) originam sinais com boa resolução. A técnica de FT-IR apresenta utilidade na determinação da incorporação de grupos funcionais, especialmente na quantificação do grau de polimerização e de reatividade de cada tipo de grupo polimerizável dos monômeros (CORMACK; ELORZA, 2004).

A adsorção pode ser definida como: **i)** processo pela qual moléculas de uma substância se acumulam em uma interface (DABROWSKI, 2001), **ii)** fenômeno que ocorre na interface de duas fases em que uma espécie química se concentra na superfície de uma outra espécie química com consequente troca de calor e **iii)** transferência de um ou mais constituintes de uma fase fluida para a superfície de um adsorvente (CORNÉLIO; FERRUA; GUERREIRO, 2004). O primeiro passo é para investigar as possibilidades de uso desses sorventes em um determinado processo, é conhecer o equilíbrio de adsorção. Estudos da cinética de adsorção irão indicar o tempo necessário para que o equilíbrio de adsorção entre analito e sorvente sejam alcançados, enquanto as isotermas de adsorção vão indicar a forma como o adsorvente efetivamente adsorverá o soluto e fornecerá uma estimativa da quantidade máxima de soluto adsorvida (MORENO-CASTILHA, 2004).

Existem vários modelos que descrevem as isotermas de adsorção e dentre estes, os mais conhecidos são o modelo de Langmuir e o modelo de Freundlich.

Para explicar a adsorção sobre uma superfície uniforme simples, infinita e não porosa, em 1918, Langmuir propôs uma teoria que se baseia na hipótese de movimento das moléculas adsorvidas pela superfície do adsorvente, de modo que, à medida que mais moléculas são adsorvidas, há uma distribuição uniforme formando uma monocamada que recobre toda a superfície (AMUDA et al., 2007). Esta proposta utiliza o conceito dinâmico do equilíbrio de adsorção que estabelece a igualdade nas velocidades de adsorção e dessorção. As seguintes aproximações são usadas: a adsorção é monomolecular, a superfície é energeticamente homogênea e não existe interação entre as partículas adsorvidas (RADHIKA; PALANIVELU, 2006; PORPINO, 2009). A expressão da isoterma de Langmuir é representada pela equação (1):

(1)

$$Q_e = \frac{Q_m \,.\, K_L \,.C_e}{1 + Q_m \,.\, C_e}$$

Onde:

K_L é a constante de Langmuir que dá a capacidade de adsorção teórica na monocamada (L g^{-1});

Q_m é o parâmetro que representa a capacidade de adsorção na monocamada (mg.L^{-1});

C_e é a concentração do adsorbato no equilíbrio (mg L^{-1});

Q_e é a quantidade adsorvida por grama do adsorvente (mg g^{-1});

Linearizando a equação de Langmuir é possível determinar os parâmetros de adsorção, Q_m e K_L. A equação linearizada de Langmuir é expressa na Equação (2).

(2)

$$\frac{C_e}{Q_e} = \frac{1}{Q_m \,.\, K_L} + \frac{1}{K_L}\, C_e$$

O gráfico de C_e/Q_e em função de C_e é uma reta com interseção $1/(K_L\, Q_m)$ e inclinação $1/ K_L$.

Através do parâmetro de equilíbrio RL é possível prever a forma da isoterma de adsorção, indicando se a adsorção é favorável ou desfavorável. O parâmetro R_L pode ser calculado pela Equação (3). Se $R_L > 1$, o processo de adsorção é desfavorável, $R_L = 1$ é linear, $0 < R_L < 1$ é favorável e $R_L = 0$ caracteriza adsorção irreversível (PORPINO, 2009).

$$R_L = \frac{1}{1 + Q_m K_L} \quad (3)$$

Freundlich por sua vez propôs um modelo que uma distribuição em multicamadas de sítios ativos, que constitui um tratamento válido quando não existe interação apreciável entre as moléculas de adsorbato (KALAVATHY et al., 2005; PORPINO, 2009). A capacidade de adsorção Qe pode ser calculada pela equação (4).

$$Q_e = K_F \,.\, C_e^{1/n} \quad (4)$$

Em que K_F é a constante de Freundlich (mg g^{-1}); n é um parâmetro empírico.

A constante de Freundlich (K_F) relaciona-se com a capacidade de adsorção, enquanto que a constante n relaciona-se com a intensidade desta adsorção. Valores de n na faixa 1< n <10 indicam adsorção favorável (PORPINO, 2009). A equação de Freundlich linearizada assume a forma da equação (5).

$$\ln Q_e = \ln K_F + \frac{1}{n} \ln C_e \quad (5)$$

O gráfico de ln Q_e em função de ln C_e é uma reta com interseção igual a ln K_F e inclinação igual a 1/n.

3.5 Beta-bloqueadores

Os beta-bloqueadores são drogas clinicamente importantes, utilizadas no tratamento de distúrbios como angina, hipertensão e arritmia. Os beta-bloqueadores são classificados como antagonistas β1 ou β2 adrenérgicos e agem relaxando músculos e abrandando o ritmo cardíaco.

O efeito de relaxamento muscular dos beta-bloqueadores é um atrativo para atletas que competem em categorias de esportes que exigem equilíbrio e destreza como ginástica, tiro ao

alvo e arco e flecha. Os competidores fazem uso de forma indevida destes fármacos a fim de melhorar o seu desempenho (MURRAY; DANACEAU, 2009; WADA, 2014).

Para extrair estes componentes da urina, muitos métodos de preparo de amostras têm sido empregados, como a extração em fase sólida convencional (C18) e a extração líquido-líquido. No entanto, estas técnicas, além dos compostos de interesse, são capazes de extrair um amplo espectro de substâncias dentro de uma gama grande de diferentes valores de pKa. Por isso, eles são capazes de extrair outros componentes orgânicos presentes na amostra, levando a fortes efeitos da matriz, resultando na supressão ou aumento do sinal dos analitos. A seletividade de fases estacionárias é um parâmetro importante a se considerar quando se analisa compostos orgânicos a níveis de traço em matrizes complexas, porque a redução na extração de contaminantes resulta em melhor sensibilidade e, consequentemente, limites de detecção mais baixos. Por estas razões, o uso de adsorventes seletivos, como os MIP, é indicado para este tipo de estudo (GROS et al., 2008).

A análise de rastreio de beta-bloqueadores no controle de doping tem sido preferencialmente feita por cromatografia gasosa acoplada a espectrometria de massas (GC/MS) após derivatização dos analitos. Mais recentemente, a utilização de cromatografia gasosa acoplada a espectrometria de massas sequencial (GC-MS/MS) e cromatografia líquida acoplada a espectrometria de massas sequencial (LC-MS/MS) melhorou a seletividade e sensibilidade do método de rastreamento de beta-bloqueadores na urina (DELAMOYEA et al., 2004). A Figura 6 expõe alguns beta-bloqueadores comumente utilizados com a finalidade de doping.

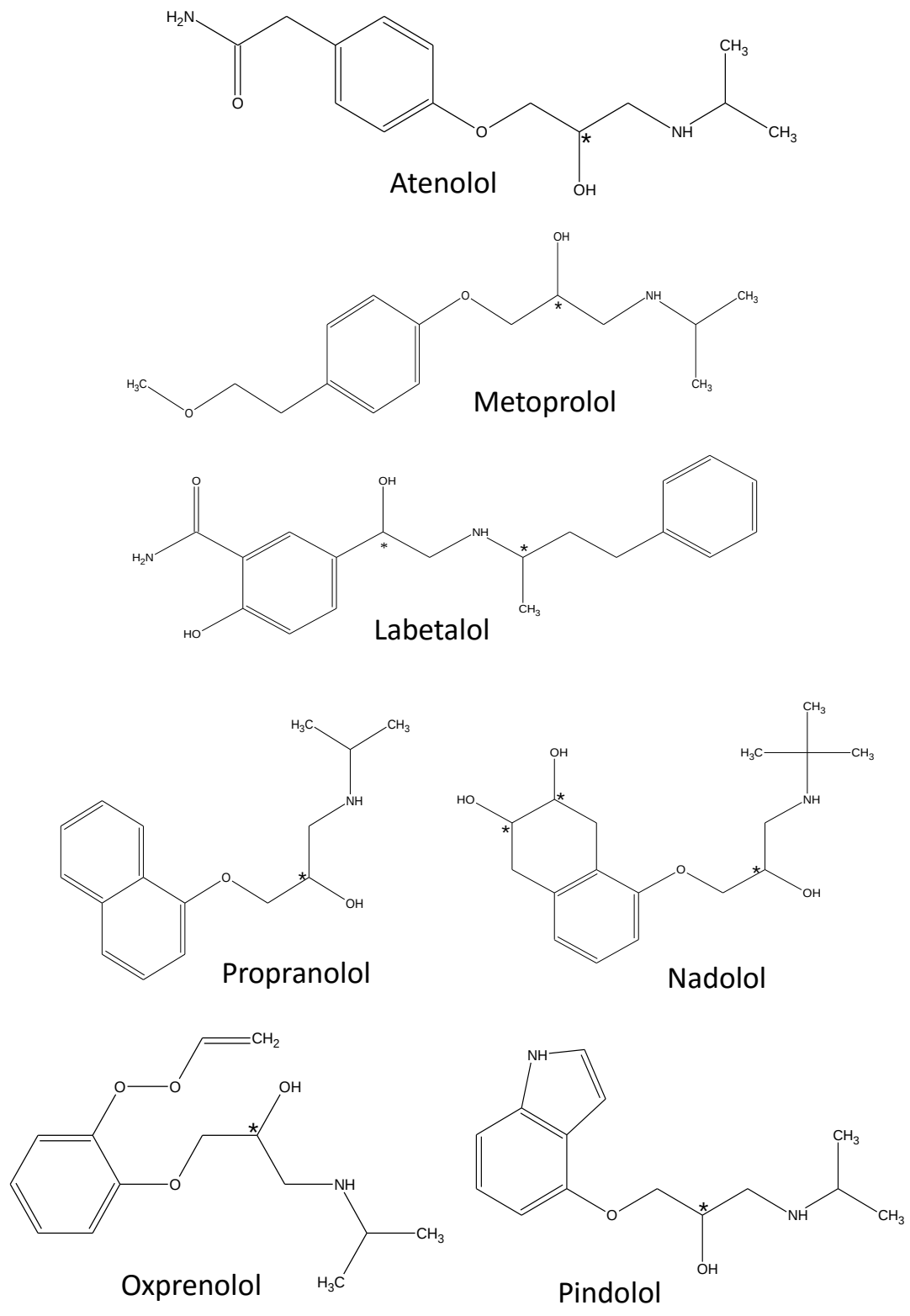


Figura 6. Estrutura molecular de alguns beta-bloqueadores proibidos para alguns esportes pela Agência Mundial Anti-Doping (WADA, 2014).
Fonte: Do autor.

3.6 Atidepressivos tricíclicos

Os antidepressivos tricíclicos são fármacos utilizados para tratar e aliviar os sintomas oriundos dos distúrbios da depressão. Estes são classificados em duas classes: as aminas secundárias e as aminas terciárias. A primeira impede preferencialmente a receptação de noradrenalina, enquanto a segunda classe, a recaptação de serotonina. Todos eles apresentam em sua estrutura química comum três anéis, sendo o anel central com sete ou oito átomos de carbono, uma cadeia lateral e um grupo amina terminal. A Figura 7 mostra a estrutura química de alguns antidepressivos tricíclicos comumente usados na terapêutica. Além disso, estes fármacos apresentam alta taxa de ligação a proteínas plasmáticas. Seus níveis terapêuticos estão usualmente entre 50 a 300 ng mL^{-1} e níveis de detecção tão baixos quanto 1 ng mL^{-1} podem ser requeridos em estudos farmacocinéticos após dose única de alguns desses compostos (IVANDINI et al., 2002; YOSHIDA et al., 2000).

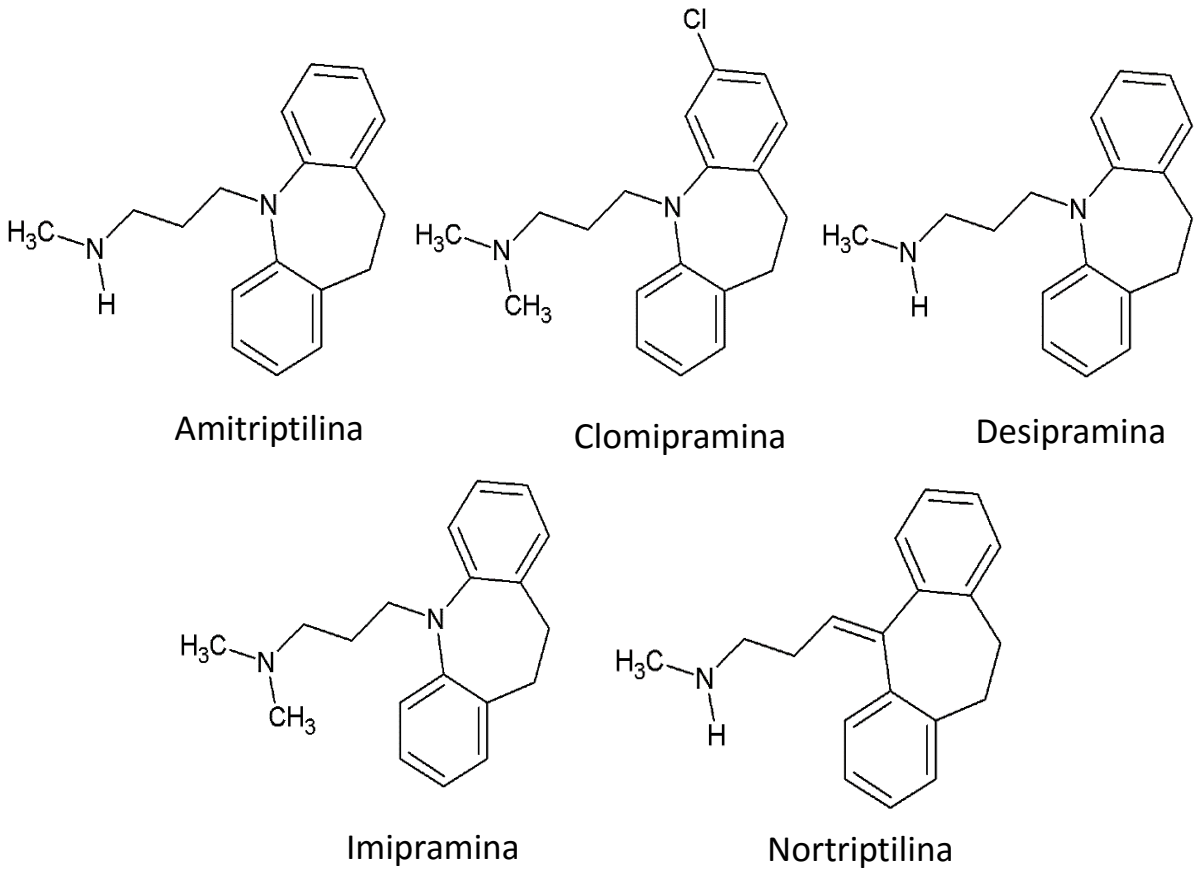


Figura 7. Estrutura molecular de alguns antidepressivos tricíclicos usados na terapêutica.
Fonte: Do autor.

Para estudos farmacocinéticos ou para estudos de monitorização terapêutica, normalmente estes fármacos são analisados em plasma, sendo necessária uma etapa de precipitação das proteínas plasmáticas que antecede a extração propriamente dita. O preparo da amostra, usualmente é feito por ELL ou SPE convencional, mas já há relatos do emprego de MIP na extração desses compostos. As análises são feitas por técnicas cromatográficas (gasosa ou líquida) acopladas ou não a detectores de massas (simples ou sequenciais) ou por técnicas eletroanalícas (ULRICH; ISENSEE; PESTER, 1996; WEN; MA; ZHU, 2008; XU et al., 2009). Dessa forma, o emprego de adsorventes seletivos com restrição a ligação de macromoléculas seria muito útil para tornar a análise desses compostos mais simples e rápida.

3.7 Cromatografia Líquida Multidimensional

A necessidade de análises rápidas tem estimulado o desenvolvimento de técnicas em que as etapas de preparo de amostra, separação analítica, identificação e quantificação sejam associadas. Dentre as estratégias empregadas pode-se citar a utilização de cromatografia líquida multidimensional no modo denominado *column switching*, que permite a injeção direta de matrizes complexas no sistema analítico (KILLGORE, 1996).

Nesse sistema geralmente são usadas válvulas de seis vias para integrar colunas extratoras e colunas analíticas. Inicialmente a amostra é percolada através da coluna extratora para a retenção dos analitos de interesse e exclusão dos interferentes, que são conduzidos para um descarte. A válvula é acionada e a fase móvel elui os analitos da coluna extratora, envia-os para a coluna analítica (de separação) e subsequentemente para um detector (KILLGORE, 1996).

Como vantagem dos sistemas *column switching* pode-se destacar a economia de solventes, uso de menor quatidade de amostra, além da alta frequência analítica.

3.8 Espectrometria de massas

Para a identificação e quantificação de fármacos, uma das técnicas mais comumente empregadas atualmente é a espectrometria de massas. Esta consiste na ionização das moléculas

de interesse, e separação dos íons baseado em suas diferentes razões massa/carga (m/z), energia cinética ou momento de íons. Um instrumento típico é constituído de diversas partes: interface de ionização, analisador de massas, detector e sistema de processamento de dados (SILVERSTEIN; WEBSTER; KIEMLE, 2005; SANTOS-NETO, 2007).

No que tange à instrumentação, muitos avanços foram alcançados, tanto no desenvolvimento de técnicas de ionização mais eficientes, quanto de analisadores com alta resolução. Para estudos de fármacos a ionização por *eletrospray* à pressão atmosférica (ESI) tem sido empregada com sucesso.

A ESI é basicamente uma técnica de transferência dos analitos de uma fase líquida para a fase gasosa. Ela é, para a maioria das moléculas, a técnica mais amena de ionização e, geralmente, é bem sucedida com moléculas iônicas de polaridade mediana. Ademais, a ESI produz com frequência íons com múltiplas cargas para moléculas grandes tais como peptídeos e proteínas (SANTOS-NETO, 2007).

Os mecanismos da ESI ainda estão sob estudo e não estão totalmente compreendidos. Basicamente, um campo elétrico é aplicado entre o líquido que flui por um capilar e um contra eletrodo, causando a separação dos íons com carga positiva e negativa e formando uma dupla camada de íons no menisco do líquido. A atração eletrostática dos íons em excesso na camada exterior do líquido, em direção ao contra eletrodo, causa uma distorção da sua superfície no formato de um cone (chamado de Cone de Taylor). Ao mesmo tempo, a tensão superficial do líquido atua para manter a superfície não deformada. A partir de certa voltagem, a tensão superficial não é capaz de reter a superfície coesa e um fino filamento líquido alonga-se do Cone de Taylor, emitindo um jato de gotículas carregadas em direção ao contra eletrodo. A medida que as gotículas viajam em direção ao contra eletrodo, o solvente tende a evaporar, aumentando a sua razão carga-volume. Quando a repulsão das cargas ultrapassa a força da tensão superficial das gotículas, a fissão coulômbica passa a ocorrer e gotículas menores são ejetadas das gotículas iniciais (SANTOS-NETO, 2007).

Atualmente duas propostas tentam explicar a obtenção de íons isolados em fase gasosa. No modelo de carga residual, considera-se que as gotículas vão se dividindo e ficando menores, até que somente reste o íon dessolvatado (DOLE et al., 1968). Por sua vez, o modelo de evaporação do íon, assume que os íons dessolvatados são emitidos das gotículas carregadas de pequeno diâmetro (IRIBARNE; THOMSON, 1976; THOMSON; IRIBARNE, 1979) .

Quantos aos analisadores de massas, muitos estão disponíveis e a escolha do analista dependendo do tipo de aplicação desejada e das características dessa demanda. Para análise de fármacos o quadrupolo tem sido empregado com êxito. Esse analisador consiste de quatro

hastes simetricamente arranjadas às quais é aplicado um potencial alternado por rádio frequência sobreposto a um potencial de corrente contínua. Pela interação desses potenciais aplicados, um campo elétrico/magnético é gerado, estabilizando os íons axialmente introduzidos no centro do quadrupolo, de acordo com suas m/z. Assim dependendo da intensidade e frequência das voltagens aplicadas, diferentes m/z podem ser filtradas através do quadrupolo. Íons estabilizados irão oscilar harmonicamente em um movimento complexo perpendicularmente às hastes do quadrupolo e sendo mantidos em posição central, irão atravessar o filtro de massas, enquanto que os íons não estabilizados pela escolha dos potenciais irão oscilar erraticamente e atingir as hastes do quadrupolo, não sendo transmitidos através dele (HERBERT; JOHNSTONE, 2003; SANTOS-NETO, 2007).

3.9 Determinação direta de analitos sem separação cromatográfica

A determinação direta de analitos, sem prévia separação cromatográfica, tem sido efetuada, principalmente, por técnicas espectrofotométricas, quimioluminescentes ou eletroquímicas. Essas técnicas, por serem baseadas normalmente em reações químicas seletivas ou características físicas/químicas individuais dos componentes a serem analisados, são empregadas normalmente para análise de uma substância por vez, já que qualquer outro composto que possua grupos funcionais susceptíveis a reação, será também identificado e quantificado, uma vez que os detectores não são capazes de distinguir se o sinal é proveniente dos compostos de interesse ou dos demais constituintes da amostra. Além disso, algumas destas técnicas apresentam baixa sensibilidade e não conseguem detectar e quantificar com eficiência os compostos de interesse, principalmente quando estão presentes em baixíssimas concentrações na matriz analisada. Neste sentido, visando melhorar a seletividade e a sensibilidade destas técnicas, os MIP vêm sendo empregados como alternativa para extração seletiva e pré-concentração dos analitos.

Alguns trabalhos que empregam os MIP juntamente com técnicas espectrofotométricas são encontrados na literatura, dentre os quais podemos destacar o estudo realizado por FIGUEIREDO et al. (2009), que utilizou a extração em fase sólida molecularmente impressa (MISPE) aplicada em amostras biológicas para determinação espectrofotométrica seletiva de nicotina em amostras de urina de fumantes. O método se baseia na redução de manganês VII a manganês VI, promovida pela nicotina, em meio alcalino. Como se trata de uma reação

inespecífica, a seletividade obtida foi atribuída ao MIP. A exatidão do método foi avaliada comparando-se a técnica de injeção em fluxo – MISPE com um método de cromatografia líquida de alta eficiência. Não foram observadas diferenças significativas, considerando-se um intervalo de confiança de 95%. É interessante ressaltar os bons resultados de frequência analítica (11 h^{-1}) e limite de quantificação (1,1 μmol L^{-1}) do método proposto quando comparados com os mesmos parâmetros para o método por HPLC (2 h^{-1} e 3,0 μmol L^{-1}).

Outra técnica que vem sendo associada aos MIP para análise direta é a quimiluminescência. Assim um sistema de quimiluminescência acoplado à injeção em fluxo para determinação de 2,4-diclorofenol, foi desenvolvido por FENG et al., (2008). O polímero para este composto foi preparado através de técnicas não covalentes de impressão molecular utilizando-se 4-VP e etilenoglicoldimetacrilato como monômero e agente de ligação cruzada respectivamente. O composto 2,4-diclorofenol foi seletivamente adsorvido pelo MIP e, então, determinado pelo efeito aumentado sobre a reação de quimioluminescência entre permanganato de potássio e luminol.

As técnicas eletro analíticas também têm sido empregadas com sucesso em conjunto com os MIP. Assim um sensor químico piezo-elétrico foi recoberto com um filme de MIP, por eletropolimerização, para determinação seletiva de dopamina. A análise foi realizada por injeção em fluxo. Quando comparado ao sensor não impresso, o sensor impresso demonstrou um aumento na sensibilidade em torno de cinco vezes. O sensor também mostrou eficiência ao discriminar análogos estruturais da dopamina, tais como a histamina, o ácido ascórbico e a 2-feniletilamina (PIETRZYK et al., 2010).

4 ARTIGO 1

POLÍMEROS MOLECULARMENTE IMPRESSOS DE ACESSO RESTRITO OBTIDOS POR MODIFICAÇÃO NA SUPÉRFICIE COM ALBUMINA SÉRICA BOVINA E/OU CO-MONÔMEROS HIDROFÍLICOS: UM ESTUDO COMPARATIVO RELACIONADO A PROPRIEDADES FÍSICAS E QUÍMICAS

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Restricted access molecularly imprinted polymers obtained by bovine serum albumin and/or hydrophilic monomers' external layers: a comparison related to physical and chemical properties

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Molecularly imprinting polymers (MIPs) can be modified with external layers in order to obtain restricted access molecularly imprinted polymers (RAMIPs) able to exclude macromolecules and retain low weight compounds. These modifications have been frequently achieved using hydrophilic monomers, chemically bound on the MIP surface. Recently, our group proposed a new biocompatible RAMIP based on the formation of a bovine serum albumin coating on the surface of MIP particles. This material has been used to extract drugs directly from untreated human plasma samples, but its physicochemical evaluation has not been carried out yet, mainly in comparison with RAMIPs obtained by hydrophilic monomers. Thus, we proposed in this paper a comparative study involving the surface composition, microscopic aspect, selectivity, binding kinetics, adsorption and macromolecule elimination ability of these different materials. We concluded that the synthesis procedure influences the size and shape of particles and that hydrophilic co-monomer addition as well as coating with BSA do not alter the chemical recognition ability of the material. The difference between imprinted and non-imprinted polymers' adsorption was evident (suggesting that imprinted polymers have a better capacity to bind the template than the non-imprinted ones). The Langmuir model presents the best fit to describe the materials' adsorption profile. The polymer covered with hydrophilic monomers presented the best adsorption for the template in an aqueous medium, probably due to a hydrophilic layer on its surface. We also concluded that an association of the hydrophilic monomers with the bovine serum albumin coating is important to obtain materials with higher capacity of macromolecule exclusion.

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Introduction

The low molecular weight compounds' analysis, in biological samples, requires simple, reliable and quick analytical methods. However, the complexity of a biological matrix makes it necessary to have a sample pretreatment in order to remove the concomitants (often at high concentrations) as well as to concentrate the analytes.[1–3]

For this reason, studies about selective sorbents that are able to purify complex samples have been growing continuously,[4] where two sorbents have been gaining space: (i) molecularly imprinted polymers – MIPs, which are synthetic polymers capable of selectively binding to the target analytes[5] and (ii) restrict access materials – RAMs, which are able to retain low molecular weight analytes and remove macromolecules such as proteins and polypeptides.[6] Despite their high selectivity to a molecule or a class, MIPs can retain macromolecules on their surfaces. Therefore, biological fluids, such as plasma or serum, can only be extracted by MIPs after eliminating the macromolecules by a previous sample preparation step. With respect to RAMs, they are very efficient in removing macromolecules, but they have no molecular recognition and, therefore, they are poor in selectivity.[7,8] Thus, in order to obtain the advantages of both MIPs and RAM, some researchers have proposed the combination of these technologies. Their aim was to obtain a polymer for the selective extraction of analytes from complex samples, with molecular recognition enhanced in aqueous medium and, at the same time, capable of eliminating macromolecules.[9]

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Haginaka and co-workers were the pioneers. They proposed the synthesis of a new kind of material called restricted access molecularly imprinted polymer (RAMIP) selective to (S)-naproxen,[10,11] (S)-ibuprofen,[10] and propranolol.[12] Their proposal was to cover a conventional MIP with a layer of hydrophilic co-monomers. The obtained polymer presented good sensitivity and selectivity to extract the target analytes from biological samples and good capability to eliminate macromolecules due to the presence of polar groups on the particle surfaces. Similarly, Puoci and co-workes[13] as well as Hua and co-workers[14] obtained RAMIPs by using different hydrophilic co-monomers and achieved good results in terms of selectivity and the exclusion of macromolecules was obtained in both cases.

The most recently addressed strategy was developed by Moraes and co-workers.[15] They synthesized a MIP that was coated with bovine serum albumin (BSA) using glutaraldehyde as a cross-linker, resulting in a protein chemical shield around it (restricted access molecularly imprinted polymer covered by bovine serum albumin – RAMIP-BSA). At high pH, this biocompatible material was able to selectively retain a template molecule. At the same time, *ca.* 99% of the protein from human plasma samples were eliminated, due to the electrostatic repulsion between these proteins and the BSA layer fixed on the polymer surface (both negatively charged).

Thereby, to better understand the behavior of the RAMIPs obtained by hydrophilic monomers and/or the BSA external layers, as well as the consequences of each superficial modification, we proposed a comparative study involving the characterization of different polymers selective to oxprenolol, with and without each one of the above-mentioned surface modifications.

Experimental

Chemicals and solutions

HPLC-grade acetonitrile and methanol were obtained from Vetec (Rio de Janeiro, Brazil). The work solutions and buffers were prepared by using deionized water (18.2 MΩ cm) obtained from a Milli-Q water purification system (Millipore, Bedford, USA). For the syntheses of the MIPs, oxprenolol (OXP), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and 2,2′-azobisisobutyronitrile (AIBN) were used as the template, functional monomer, cross-linker and initiator, respectively (all from Sigma-Aldrich, Steinheim, Germany). HPLC-grade acetonitrile was used as the porogenic solvent. Glycerol dimethacrylate (GDMA) and hydroxyethyl methacrylate (HEMA) (both from Sigma-Aldrich) were used as hydrophilic co-monomers in the syntheses of the RAMIPs. Methanol and acetic acid (Merck, Darmstadt, Germany) were employed during the polymer washing steps. To perform the external coating of the materials with proteins, bovine serum albumin (BSA) (Sigma-Aldrich), glutaraldehyde (Rioquímica, São José do Rio Preto, Brazil) and sodium borohydride (Sigma-Aldrich) were used. Monobasic and dibasic potassium phosphates (Synth Diadema, Brazil) were used to prepare a phosphate buffer pH 7.0, 0.01 mol L^{-1}.

Stock solutions of OXP, metoprolol (MET), labetalol (LAB), propranolol (PROP) and nadolol (NAD) (all from Sigma-Aldrich, and at concentrations of 1.0 and 2.0 mg L^{-1}) were prepared in HPLC-grade methanol, placed in amber flasks and stored at −5.0 °C for up to 30 days. HPLC-grade methanol and ammonium formate (Fluka, Seelze, Germany) were used to prepare the mobile phases for the LC-MS/MS analyses.

Syntheses of the polymers

Four imprinted polymers and their corresponding non-imprinted polymers (NIPs) were synthesized in this study. Table 1 summarizes each one of them, according to the presence or absence of each synthesis step. The detailed procedures are described below.

For the MIP synthesis, 1.0 mmol of OXP and 4.0 mmol of MAA were dissolved in 48.0 mL of acetonitrile in a 250 mL three-mouth glass flask. Then, 7.0 mmol of EGDMA and 25.0 mg of AIBN were added, and the mixture was purged with nitrogen for 20 min. The flask was connected to a condenser in order to avoid the solvent loss due to evaporation. The apparatus was immersed in a glycerin bath, agitating at 65 °C, for 24 h. A NIP was obtained as MIP, but in the absence of OXP (template).

The RAMIP was obtained employing the same quantities of OXP, MAA, EGDMA and AIBN previously used in the MIP synthesis. Initially, the reagents were dissolved in 24.0 mL of acetonitrile and the synthesis was carried out as it was for MIP, but for only 1 h. At this moment, a mixture of 7.5 mmol of HEMA, 0.5 mmol of GDMA (both hydrophilic monomers) and 24.0 mL of acetonitrile was purged with nitrogen for 20 min and added into the synthesis flask. The polymerization was carried out for 23 h more. A restricted access non-imprinted polymer (RANIP) was synthesized the same way as the RAMIP was, but in the absence of the template molecule.

For each polymer, particles from 75.0 to 106.0 μm in size were selected using steel sieves. Approximately 2.0 g of each were washed in an ultrasonic bath with 10.0 mL of a 9 : 1 (v : v) methanol : acetic acid solution for 1 h. The washing procedure was repeated 10 times, and the washing solution was renewed for each repetition. After this, MIP, NIP, RAMIP and RANIP were dried at 70.0 °C for 24 h.

Table 1 Compositions of each polymer in terms of molecularly imprinting, presence of hydrophilic monomers and presence of BSA layers

Polymer	Presence of template during the synthesis	Presence of hydrophilic monomers	Presence of BSA layers
MIP	Yes	No	No
NIP	No	No	No
RAMIP	Yes	Yes	No
RANIP	No	Yes	No
MIP-BSA	Yes	No	Yes
NIP-BSA	No	No	Yes
RAMIP-BSA	Yes	Yes	Yes
RANIP-BSA	No	Yes	Yes

Five hundred milligrams of each previously obtained polymer (MIP, NIP, RAMIP and RANIP) were placed in 5 mL polypropylene cartridges separately (one cartridge for each polymer). Then, 20 mL of 1% (w/v) albumin solution (prepared in 0.05 mol L^{-1} phosphate buffer, pH 6.0) were percolated through each cartridge at a 1.0 mL min^{-1} flow rate. Subsequently, 25 mL of a 5% (w/v) glutaraldehyde aqueous solution were percolated through each one cartridge at a 1.0 mL min^{-1} flow rate, and they were maintained in a standby state for 5 h. Finally, 10 mL of sodium borohydride 1% (w/v) aqueous solution were percolated through the cartridges at a 1.0 mL min^{-1} flow rate. The coated polymers (MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA) were washed with water to remove residues of the reagents.

Polymer characterization

Initially, the materials were characterized by using a scanning electron microscope (Zeiss LEO 440, Cambridge, England) equipped with an OXFORD detector, operating at a 15 kV electron beam. The samples were coated with a 10 nm layer of gold, using a Coating System BAL-TEC MED 020 (BAL-TEC, Liechtenstein) and maintained in a desiccator before analysis.

The infrared (IR) spectra of the polymers were obtained using a Fourier transform infrared (FT-IR) spectrometer (Shimadzu, Kyoto, Japan). Pellets were made, using 200 mg of KBr and 10 mg of MIP, NIP, RAMIP RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA or RANIP-BSA. The analyses were carried out at a spectral range from 500 to 4000 cm^{-1}, with a resolution of 4 cm^{-1} (20 scans).

The adsorption pH was studied by adding 10.0 mg of each polymer in separate glass tubes containing OXP aqueous solutions at a concentration of 1.0 mg L^{-1}, in different electrolytes (acetic acid aqueous solution 0.01 mol L^{-1}, pH 3.5; phosphate buffer solution 0.01 mol L^{-1}, pH 7.0; and sodium hydroxide aqueous solution 0.01 mol L^{-1}, pH 10.5). The tubes were shaken for 15 min and centrifuged at 1000*g* for 10 min. The supernatant was collected and the remaining concentration of OXP was quantified by spectrophotometry. The mass, retained by the polymers (adsorption capacity – Qe), was calculated by subtraction. The same test was carried out individually for each polymer.

A kinetic study was carried out by adding 10.0 mg of each polymer (MIP, NIP, RAMIP, RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA or RANIP-BSA) to test glass tubes containing 1.0 mL of 100.0 mg L^{-1} OXP phosphate buffer solution (0.01 mol L^{-1}, pH 7.0). The tubes were shaken for 0, 15, 30, 45, 60, 75, 90 or 105 min at room temperature (approximately 25.0 °C) and centrifuged at 1000*g*. The OXP, remaining in the supernatant (equilibrium concentration – Ce), was quantified by spectrophotometry. The Qe for each polymer was calculated by subtraction.

Adsorption isotherms were constructed in order to evaluate the extraction capacities of all obtained materials. Each material was studied individually. OXP standard solutions (25.0, 50.0, 75.0, 100.0, 200.0, 300.0, 500.0, 1000.0 and 1500.0 mg L^{-1}) were prepared in a phosphate buffer solution (0.01 mol L^{-1}, pH 7.0). One milliliter of each solution was transferred to glass tubes containing 10.0 mg of each polymer. The tubes were shaken for 60.0 min at room temperature (approximately 25 °C), and then each sample was centrifuged at 1000*g* for 10.0 min. The Ce for each polymer was determined by spectrophotometry and the Qe was calculated by subtraction. The data were modeled according to the Freundlich and Langmuir models, with the accepted adequacy standard being the linear correlation coefficient (*r*). The Langmuir isotherm was given by: $Ce/Qe = Ce/M + 1/(KM)$, where Qe was obtained in mg g^{-1} and Ce in mg L^{-1}. *K* and *M* were the OXP maximum adsorption capacity and binding constant, respectively.[16,17]

To perform the selectivity tests, a LC-MS/MS method, for the detection and quantification of LAB, NAD, MET, OXP and PROP, was initially developed. A LC-MS 8030 instrument from Shimadzu® (Kyoto, Japan), equipped with a Shim-Pack XR-ODS C18 (100 × 3 mm, 2.2 μm) chromatographic column and a triple-quadrupole mass analyzer was used for this. The positive electrospray ionization mode was selected with the SRM (selected reaction monitoring) transitions and optimal collision energies optimized for each analyte (Table 2). The identification criterion was the simultaneous presence of the three fragments of each molecule (Table 2), the ratio between these fragments when compared to beta-blockers' standard analyses and the fragments' relative abundance. The quantitative analyses were carried out using the TIC (total ion chromatogram) of the three SRM transitions of each molecule. The oven, interface and heat block temperatures were set to 40.0, 250.0 and 400.0 °C, respectively. The nebulizing and drying gas flow rates were 1.5 and 15.0 mL min^{-1}, respectively. A gradient elution starting with 5 : 95% methanol : ammonium formate buffer solution 0.01 mol L^{-1}, pH 3.5 was used. The methanol proportion was linearly increased, first to 20% reaching at the mark of 0.5 min, then to 43% until reaching at 1.5 min, to 58% until 3.5 min, to 98% until 5.5 min, then to 10% until 6.5 min and finally turning back to 5% until

Table 2 Analytes and their precursors, fragments and collision energies optimized for detection using LC-MS/MS in the SRM mode

Analyte	Precursor (*m*/*z*)	Fragments (*m*/*z*)	Collision energy (kV)
LAB	329.2	91.1	−35
		162.1	−25
		294.1	−20
NAD	310.1	254.2	−20
		74.1	−25
		201.1	−25
MET	268.2	116.5	−25
		98.1	−25
		133.2	−25
OXP	266.2	72.1	−25
		224.9	−15
		116.1	−20
PROP	260.2	116.1	−20
		98.1	−20
		183.2	−20

7.0 min. The flow rate was 0.5 mL min^{-1}. The volume of the sample loop was 100.0 μL and the data files were acquired using LabSolutions® software. A calibration curve of LAB, NAD, MET, OXP and PROP was constructed at concentrations of 0.5, 0.7, 1.0, 1.5, 2.0 and 3.0 mg L^{-1}.

For the selectivity test, 10 mg of each polymer (MIP, NIP, RAMIP, RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA) were individually placed in test tubes containing 1.0 mL of LAB, NAD, MET, OXP and PROP phosphate buffer solution (0.01 mol L^{-1}, pH 7.0) at a concentration of 500 mg L^{-1} each. The tubes were shaken for 60 min at room temperature (approximately 25 °C) and centrifuged at 1000*g*. Subsequently, the supernatant was removed and the polymers were placed in contact with 1 mL methanol. Once again, the tubes were shaken for 10 min, centrifuged at 1000*g* and the supernatant was collected. An aliquot of this supernatant was diluted about 100 times and 25 μL of this solution was injected into LC-MS/MS to determine the beta-blockers' concentrations. The data were acquired by using LabSolutions® software. The Qe was calculated by subtraction. The selectivity constants were calculated as the Qe for OXP per the Qe of other beta-blockers (LAB, NAD, MET, and PROP). Values greater than one (1) indicate that more OXP (template) was retained by the studied materials, compared to others, while values less than 1 (one) indicate a lower retention of the template.

To evaluate the macromolecules' elimination capacity, first, 25 μL of 44 mg mL^{-1} BSA standard in phosphate buffer 0.01 mol L^{-1}, pH 7.0 (approx. the same concentration found in human plasma) was injected into a HPLC system without an analytical column and with phosphate buffer 0.01 mol L^{-1}, pH 7.0 as the mobile phase at 1 mL min^{-1} and a UV detector operating at 254 nm. Subsequently, 70 mg of each polymer were individually packed into HPLC pre-columns (10 × 4.6 mm i.d.). Each column was assembled in the analytical pathway and 25 μL of 44 mg mL^{-1} BSA standard in phosphate buffer 0.01 mol L^{-1}, pH 7.0 was injected, following the same conditions described earlier. The percentage of protein exclusion was defined as the ratio between the peak areas obtained in the system with and without the columns, multiplied by 100.

Results and discussion

The syntheses of the MIP and RAMIP, selective to OXP, were based on non-covalent interactions between functional monomers and templates. The synthesis procedures were carried out by the precipitation method, when a large volume of the solvent is used.[18]

Two chemical reactions are involved in the BSA covering process (for MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA): (i) reaction 1 occurs between the amine groups from the BSA and the aldehyde groups from glutaraldehyde (cross-linker); and (ii) reaction 2 occurs between the free aldehyde groups of the BSA–glutaraldehyde complex and the amino groups of another molecule of BSA, forming a BSA polymeric network around the materials.[15] The reaction between glutaraldehyde and albumin results in imines, which are very labile functions. This fact justifies the use of sodium borohydride solution 1% (w/v), which reduces the imines to amines (more stable compounds).[15]

Scanning electron micrographs (Fig. 1) revealed that the morphological structures of the MIP, RAMIP, NIP, RAMIP, MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA presented macropores formed by microsphere agglomerates, as frequently obtained by the precipitation method.[19] In MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA, the microspheres were more regular and velvety, probably due to the presence of the BSA layer on the surface of the polymers.

The infrared spectra of the polymers (Fig. 2) did not present significant chemical differences in terms of the presence or absence of specific chemical groups. The use of the hydrophilic monomers (GDMA and HEMA) in the RAMIP and RANIP syntheses resulted in a hydrophilic layer on each polymer, but with a chemical composition similar to the MIP and NIP. For the MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA, we believe that thin BSA layers were formed on the polymer surfaces, and their chemical nature was not adequately detected. Probably most of the radiation penetrated through it, reaching the polymeric cores. Moreover, possible

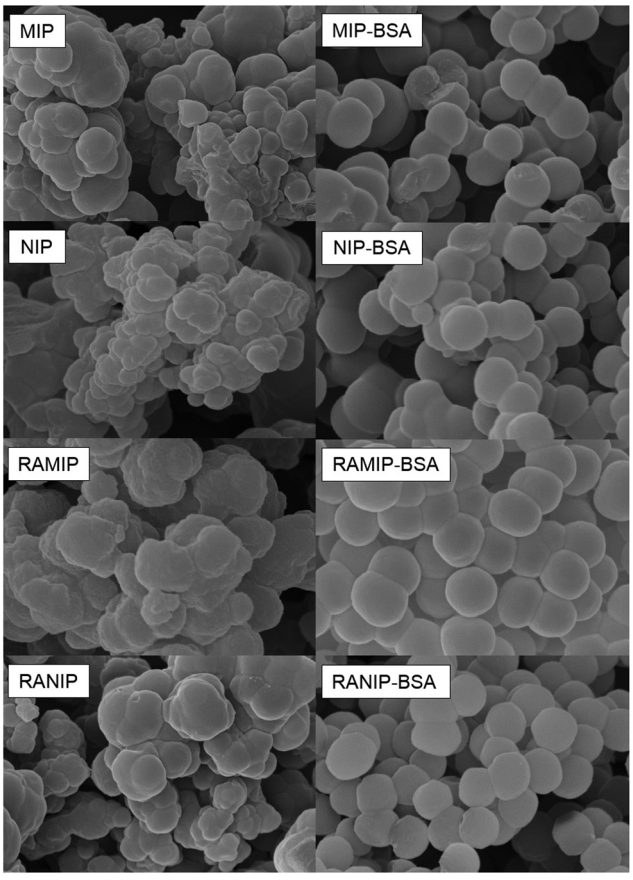

Fig. 1 Scanning electron micrographs of the MIP, MIP-BSA, NIP, NIP-BSA, RAMIP, RAMIP-BSA, RANIP and RANIP-BSA magnified 50 000×.

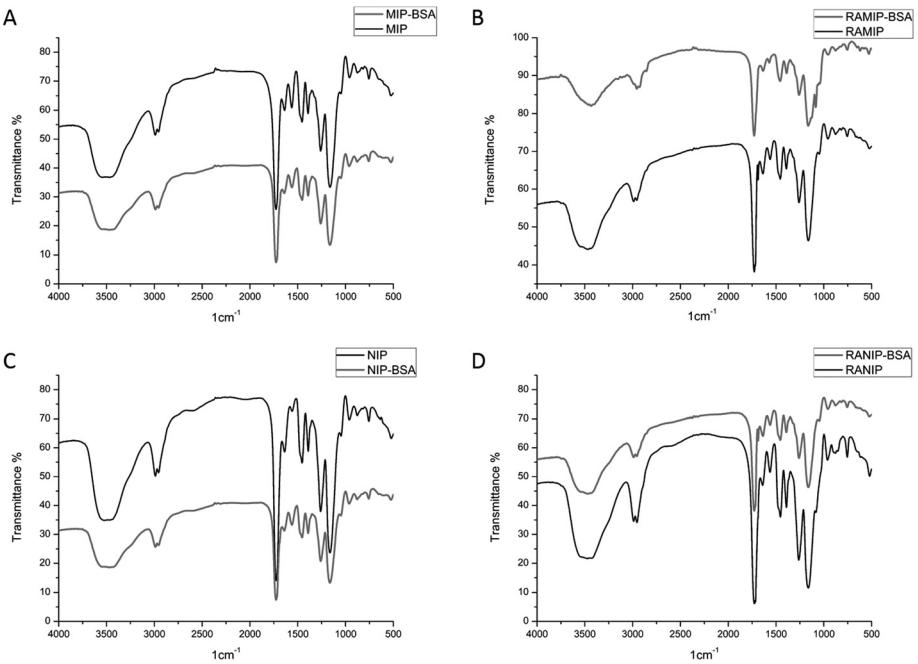

Fig. 2 Infrared spectra of (A) MIP and MIP-BSA, (B) NIP and NIP-BSA, (C) RAMIP and RAMIP-BSA and (D) RANIP and RANIP-BSA.

evidence for the BSA layer existence can be that the transmittances decreased for all the polymers after their covering with the BSA. All the spectra presented strong and broad bands between 3600 cm^{-1} and 3300 cm^{-1} approximately, which indicates the presence of associated OH, possibly due to the polymeric association; bands between 2960 cm^{-1} and 2850 cm^{-1}, indicating the presence of aliphatic CH; bands between 1760 cm^{-1} and 1710 cm^{-1}, indicating the presence of C═O of esters, likely from the polymeric association (a functional monomer and a cross-linker); bands at about 1635 cm^{-1}, indicating the presence of vinyl groups (also association between a functional monomer and a cross-linker) and bands at about 1160 cm^{-1} indicating the presence of C–O from ester groups.[20]

Adsorption can be defined as a process in which a component present in a solution adheres to a solid surface. The intensity of the adsorption effect depends on the adsorption temperature, adsorbent nature, adsorbate concentration, pH solute solubility, contact time, and agitation, among others.[21] The adsorption phenomena can be classified as physical or chemical adsorptions. In the physical adsorption, the process occurs reversibly and quickly, and the adsorbate binds to the adsorbent surface. By the way, chemical adsorption or chemisorption involves the interaction between the adsorbent and adsorbate, with energy almost as high as the chemical bond formation.[21,22]

All the polymers retained the OXP from the solutions at pH 7.0 more efficiently. It was also observed that the adsorption equilibrium was reached in 60.0 min, according to the adsorption kinetic studies (Fig. 3). Thus, pH 7.0 and adsorption time of 60 min were chosen as the optimum for carrying out the adsorption studies.

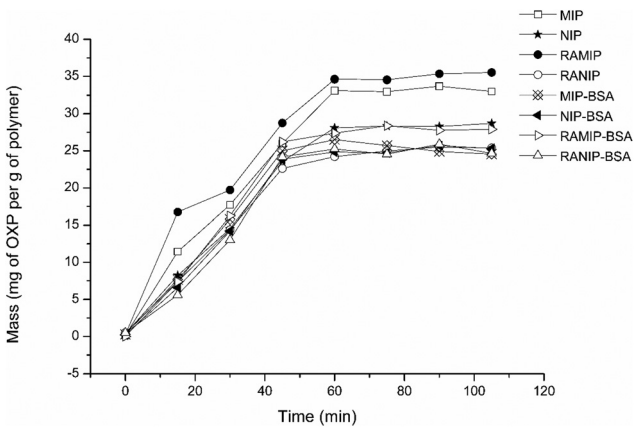

Fig. 3 Adsorption kinetics for the imprinted and non-imprinted polymers.

At a constant temperature, the adsorbed amount increases with the adsorbate concentration increasing and the relationship between the adsorbed amount and the remaining concentration is known as the adsorption isotherm.[23] Adsorption isotherms were constructed for MIP, NIP, RAMIP, RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA at different concentrations of OXP. As shown in Fig. 4, the adsorption presented a linear relationship with the OXP concentration until equilibrium was reached (approximately 100.0 and 200.0 mg L^{-1}, respectively for the imprinted and non-imprinted polymers). Based on the molecular recognition, imprinted polymers showed higher adsorption capacities, probably because there were selective interactions between these materials and

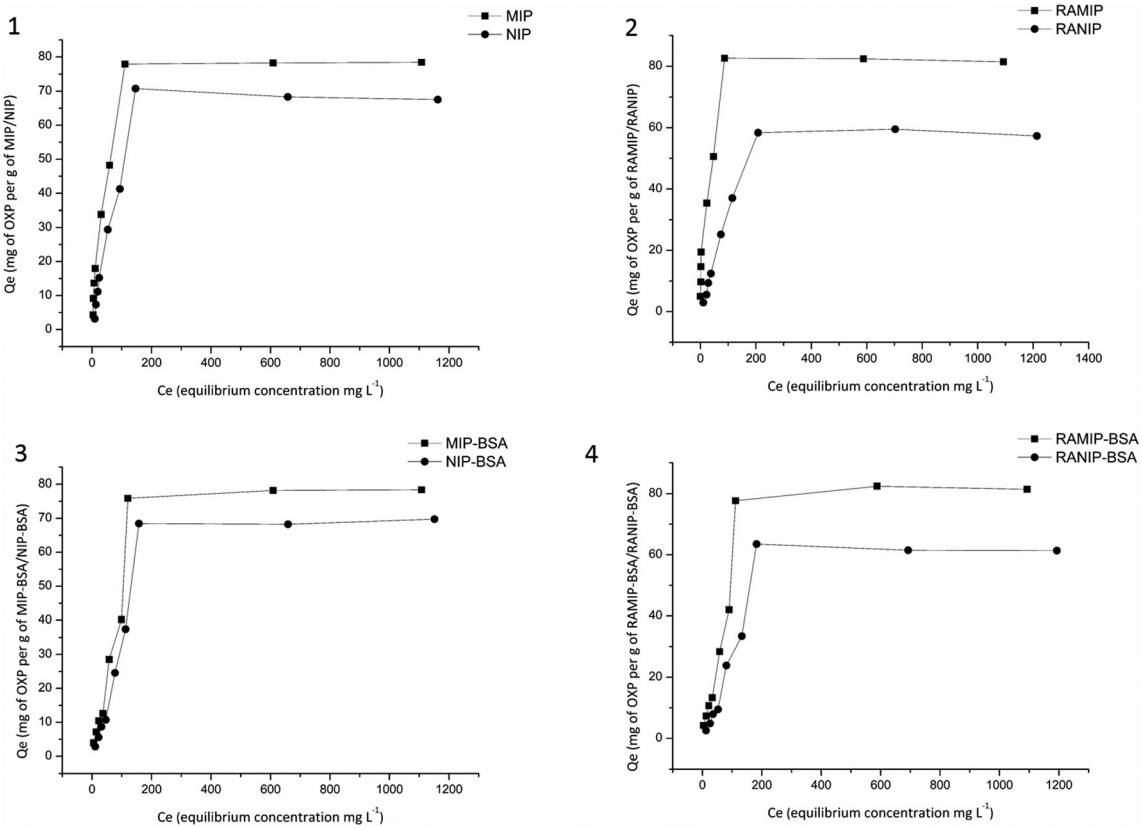

Fig. 4 Adsorption isotherms of OXP for (1) MIP and NIP, (2) RAMIP and RANIP, (3) MIP-BSA and NIP-BSA and (4) RAMIP-BSA and RANIP-BSA.

OXP, whereas only nonspecific interactions prevailed between non-imprinted polymers and OXP.[4,24,25] However, these adsorption differences are more evident in materials that were coated with hydrophilic comonomers (RAMIP, RANIP, RAMIP-BSA and RANIP-BSA). This fact corroborates with the theory that affirms that hydrophilic comonomers (like HEMA and GDMA) contribute to a better molecular recognition in aqueous media. In fact, water molecules can interact with the surface instead of the selective binding sites. In this way, the solvent interferes less in the template/binding site interactions.[26,27]

The Freundlich and Langmuir models were tested for all the obtained isotherm data. Table 3 shows the correlation coefficients (r) for both models, as well as the maximum adsorption capacities and the equilibrium parameters for the Langmuir model. It is possible to see that the best fit was obtained with the Langmuir model for the imprinted and non-imprinted polymers (higher correlation coefficients). The maximum adsorption capacities showed that all polymers had an OXP satisfactory retention. The Langmuir model indicated that the molecules had a uniform distribution in the binding sites around the polymer and that each binding site was able to receive only one molecule. Furthermore, according to the model, the analytes were retained in a monolayer on the MIP surface, and the energy involved in this process was the same for all binding sites surrounding the polymer.[27–30]

Table 3 Langmuir and Freundlich linear coefficients, maximum adsorption capacities and equilibrium parameter values for MIP, NIP, RAMIP, RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA

Polymer	Linear coefficient (r) Langmuir	Linear coefficient (r) Freundlich	Maximum adsorption capacities (mg of OXP per g of polymer)
MIP	0.99	0.91	81.30
NIP	0.99	0.90	75.19
RAMIP	0.99	0.93	82.64
RANIP	0.99	0.92	67.11
MIP-BSA	0.99	0.93	90.09
NIP-BSA	0.97	0.92	87.72
RAMIP-BSA	0.99	0.93	92.59
RANIP-BSA	0.96	0.92	79.37

In the selectivity studies (Fig. 5), the RAMIP presented the best beta-blockers' adsorption, which suggest that molecular recognition in aqueous media is improved with the addition of hydrophilic comonomers. The NAD adsorption was negatively influenced by BSA coating and it was little retained by MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA. Thus, we believe that the NAD binds to the NIP and RANIP surface by non-specific interactions and, when the surface is blocked by the BSA layer, the NAD adsorption is impaired. The NAD interaction with the binding sites of the OXP imprinted polymers is difficult because NAD presents 3 chiral centers, and

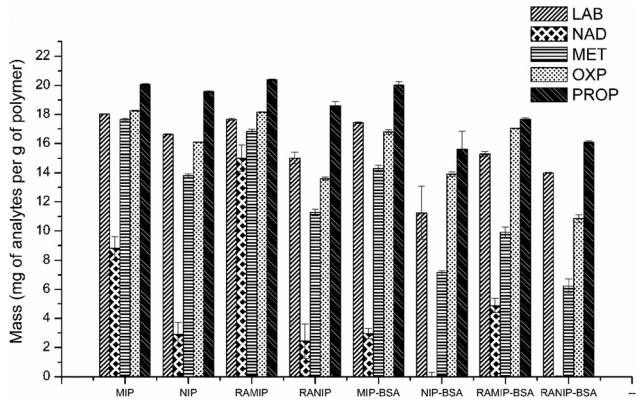

Fig. 5 Retention graph of LAB, NAD, MET, OXP and PROP by MIP, NIP, RAMIP, RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA.

may take 8 different conformations. Fig. 6 shows the studied beta-blockers' chemical structure.

From the selectivity constants (K_s) shown in Table 4, it is possible to observe that more OXP was retained by the studied polymers than the other beta-blockers, except when compared to PROP. A possible explanation is that PROP has a very similar chemical structure compared to OXP, as well as due to its lower molecular weight, which can facilitate the adaptation to the OXP imprinted binding site.

Macromolecule elimination tests were carried out with all the polymers (Table 5), in order to understand the influence of the hydrophilic comonomers or BSA layers in the exclusion mechanisms. By these results, it is possible to see that the

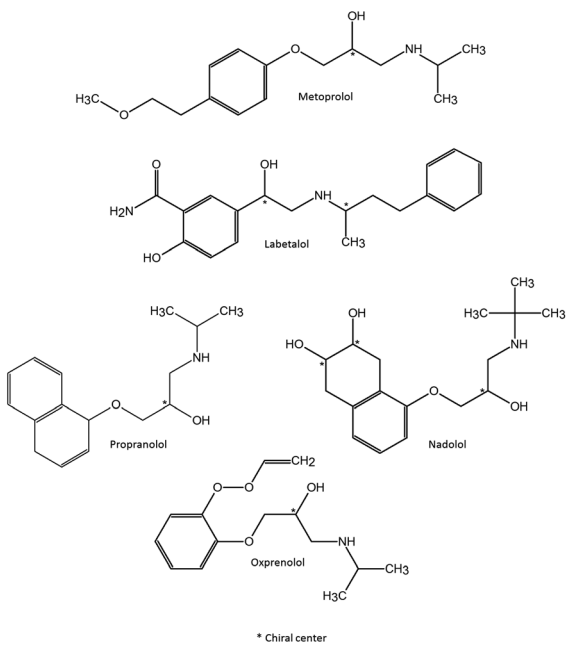

Fig. 6 Molecular structures of beta-blockers used to perform selectivity tests.

Table 4 Polymers' selective constants for LAB, NAD, MET and PROP

Polymer	KK_s LAB	K_s NAD	K_s MET	K_s PROP
MIP	1.01	2.07	1.03	0.91
NIP	0.97	5.54	1.16	0.82
RAMIP	1.03	1.21	1.08	0.89
RANIP	0.90	5.56	1.21	0.73
MIP-BSA	1.01	5.69	1.17	0.84
NIP-BSA	1.24	Not determined	1.95	0.89
RAMIP-BSA	1.16	3.50	1.73	0.97
RANIP-BSA	0.78	Not determined	1.75	0.68

Table 5 BSA elimination percentages for MIP, NIP, RAMIP, RANIP, MIP-BSA, NIP-BSA, RAMIP-BSA and RANIP-BSA

Polymer	BSA elimination (%)
MIP	14.7
NIP	18.1
RAMIP	87.8
RANIP	84.5
MIP-BSA	87.3
NIP-BSA	89.4
RAMIP-BSA	98.7
RANIP-BSA	98.3

hydrophilic comonomers' addition (HEMA and GDMA) or the BSA coating alone is ineffective for satisfactory macromolecule elimination. Probably because, in pH 7.0, the negative charge's density, derived from comonomers' addition, as well as from the BSA coating is not enough to generate the negative charge amount needed to repel all proteins present in the samples. However, the combination of these processes produces a material (RAMIP-BSA) able to eliminate almost 100% of the macromolecules from the sample. Thus, this sorbent is suitable for direct biological sample extractions of specific analytes from plasma and serum, for example. As a possible explanation, in pH 7.0, the BSA (isoelectric point of about 4.7[31]) and hydrophilic monomers (hydroxyl group $pK_a < 7.0$) from the RAMIP-BSA surface are negatively charged and thus repel the proteins from the sample.[15]

Conclusion

The synthesized polymers were efficient for beta-blockers' adsorption. Through physical characterization, it was observed that the way in which the material synthesis is conducted influences the size and shape of the particles, and that hydrophilic comonomers' addition, as well as BSA coating, do not alter their chemical recognition. Adsorption studies showed that: (i) adsorption equilibrium is reached in 60 minutes, (ii) BSA coating does not alter its adsorption profile, (iii) there is an evident difference between imprinted and non-imprinted polymers' adsorption, (iv) the best fit to describe the adsorption profile of the materials was the Langmuir model and

RAMIP was the best polymer to adsorb beta-blockers in an aqueous medium. It was also possible to conclude that both hydrophilic monomers and BSA coating presence on the polymer surface are very important to obtain materials able to completely eliminate the macromolecules, during an extraction procedure. Thus, we believe that the RAMIP-BSA is the best polymer model to be used for the solid phase extraction of these analytes from untreated biological samples.

Acknowledgements

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5 ARTIGO 2

ANÁLISE *ONLINE* DE FÁRMACOS BETA-BLOQUEADORES EM URINA COM A FINALIDADE DE MONITORAMENTO ANTI-*DOPING* EMPREGANDO EXTRAÇÃO EM FASE SÓLIDA MOLECULARMENTE IMPRESSA SEGUIDA DE DETERMINAÇÃO POR CROMATOGRAFIA LÍQUIDA/ESPECTROMETRIA DE MASSAS

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Direct doping analysis of beta-blocker drugs from urinary samples by on-line molecularly imprinted solid-phase extraction coupled to liquid chromatography/mass spectrometry

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The use of beta-blockers to enhance performance in some sports is forbidden. Based on this regulation, there is a demand for dynamic analytical procedures for analyzing these compounds quickly and without manual sample preparation. Therefore, the use of a molecularly imprinted polymer (MIP) in a multidimensional liquid chromatographic system coupled to a mass spectrometer provides a good alternative for improving the selectivity and practicality of the beta-blocker analyses, as described in this paper. A water-compatible MIP for oxprenolol was synthesized by the precipitation method, using methacrylic acid as a functional monomer and 2-hydroxyethyl methacrylate and glycerol dimethacrylate as hydrophilic monomers. A column filled with MIP was coupled to an LC-MS/MS instrument under the multidimensional configuration, with 10.0 mmol L^{-1} ammonium formate buffer (pH 5.0) as the loading and reconditioning mobile phase and a 0.01% formic acid aqueous solution–methanol (30 : 70 v : v) as the elution mobile phase. The system was used for on-line extraction and quantization of oxprenolol (from 1.0 to 75.0 μg L^{-1}), atenolol, propranolol, nadolol, pindolol, labetalol and metoprolol (all from 3.0 to 50 μg L^{-1}) simultaneously, from urine samples. The correlation coefficient was higher than 0.99 for all the analytes. Suitable precision and accuracy were obtained.

Introduction

Beta-blockers are drugs commonly used in the treatment of angina pectoris and hypertension.[1] The drugs are classified as β1 or β2 adrenergic antagonists and act by relaxing muscles and reducing the heart rate.[2] For these reasons, these compounds can be used to improve performance in sports that require accurate steadiness, equilibrium and deftness, such as archery, shooting, gymnastics, golf, darts, automobile racing and billiards.[2–4] Fig. 1 shows the beta-blockers used for some sports that are forbidden by the World Anti-Doping Agency (WADA).

Doping control for these compounds is commonly carried out by analyzing their presence in urine samples by gas chromatography/mass spectrometry or liquid chromatography/mass spectrometry.[1] However, due to the complexity of urine, sample preparation strategies involving solid-phase extraction,[5,6] liquid–liquid extraction[7–9] or pre-column clean-up[10,11] must be used to eliminate interferents and to concentrate the analytes. However, due to the low selectivity of these conventional sample preparation techniques, the use of selective sorbents based on molecular imprinting technology has been prominent in recent years[12–15] and some applications for beta-blockers can also be found.[12,13]

Molecularly imprinted polymers (MIPs) are synthetic materials obtained *via* the copolymerization of a functional monomer with a cross-linker in the presence of a template molecule. After polymerization, the polymer is washed to eliminate the template molecules, and the obtained binding sites are able to recognize the template in terms of size, shape and chemical functionality.[16–20] For the extraction of analytes from aqueous samples, molecular recognition can be highly perturbed by the presence of water molecules, because water can establish non-selective bonds with MIP recognition sites, thus decreasing the method's selectivity. One strategy that can be used to avoid this behavior is to coat MIPs with hydrophilic groups using hydrophilic monomers added at the end of the

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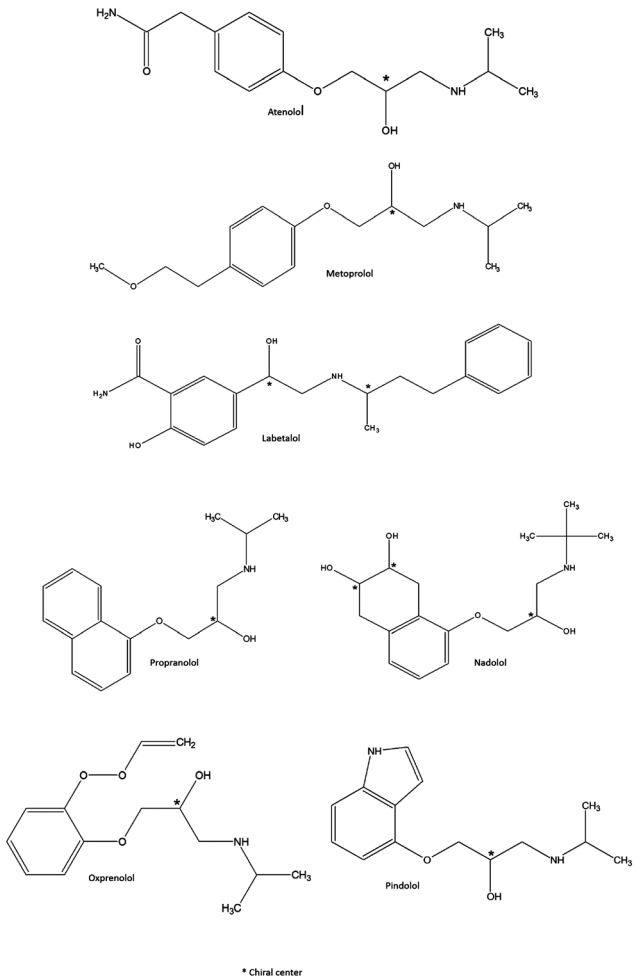

Fig. 1 Molecular structure of common beta-blockers that are forbidden in some sports by the World Anti-Doping Agency (WADA).

polymerization.[13,19,21,22] This hydrophilic layer forms hydrogen bonds with water, thus minimizing the interference of this solvent in the analyte–polymer complex.

Due to the importance of doping analysis of beta-blockers and the relevant characteristics of MIPs, such as selectivity, sensitivity, high stability, longer lifetime, low cost and appropriate recognition in aqueous matrices, we proposed a new hydrophilic MIP selective for beta-blockers and that can be used in a multidimensional, molecularly imprinted, solid-phase extraction system coupled to a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument.

Experimental

Chemicals and solutions

The organic solvents used in this study, namely acetonitrile and methanol, were obtained from Vetec (Rio de Janeiro, Brazil). The solutions were prepared with deionized water (18.2 MΩ cm) obtained from a Milli-Q water purification system (Millipore, Bedford, USA). For the MIP synthesis, oxprenolol, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and 2,2′-azobisisobutyronitrile (AIBN) were used as the template, functional monomer, crosslinking reagent and initiator, respectively (all from Sigma-Aldrich, Steinheim, Germany). HPLC-grade acetonitrile was used as the solvent. Glycerol dimethacrylate (GDMA) and hydroxymethyl methacrylate (HEMA) (both from Sigma-Aldrich, Steinheim, Germany) were used as hydrophilic co-monomers. Methanol and acetic acid (Merck, Darmstadt, Germany) were used during the polymer washing steps.

Stock solutions of oxprenolol (OXP), atenolol (ATE), metoprolol (MET), labetalol (LAB), propranolol (PROP), nadolol (NAD) and pindolol (PIN) (all from Sigma-Aldrich, Steinheim, Germany) were prepared at a concentration of 1.0 mg L^{-1} in HPLC-grade methanol, placed in amber flasks and stored at −18.0 °C for up to 30 days. Working solutions of 0.1 to 500.0 μg L^{-1} were prepared daily by diluting stock solutions in methanol, drying the standard solutions under nitrogen flow and resuspending the standards in a corresponding volume of a blank sample of human urine. This urine was obtained from volunteers who agreed to participate in this study. In order to confirm the absence of beta-blockers, the urine was previously tested by the developed method.

Methanol, formic acid (Biotec, Londrina, Brazil), ammonium formate (Fluka, Seelze, Germany), ammonium hydroxide (Isofar, Jacaré, Brazil) and ammonium chloride (Vetec, Rio de Janeiro, Brazil) were used to prepare the mobile phases for LC-MS/MS analysis.

MIP synthesis and characterization

The synthesis of the OXP molecularly imprinted polymer was based on non-covalent interactions between the template and the functional monomer. The synthesis was carried out by the precipitation method (using a large volume of solvent). In a 250.0 mL glass flask, 1.0 mmol of OXP and 4.0 mmol of MAA were dissolved in 24.0 mL of acetonitrile. Then, 7.0 mmol of EGDMA and 25.0 mg of AIBN were added, and the mixture was purged with nitrogen for 20 min. The flask was closed and immersed in a glycerin bath with agitation at 65.0 °C. After 1 h of synthesis, a mixture of 7.5 mmol of HEMA, 0.5 mmol of GDMA and 24.0 mL of acetonitrile (previously purged with nitrogen for 20.0 min) was added into the synthesis flask, and the polymerization reaction was carried out for more than 23 h.

MIP particles ranging from 75.0 to 106.0 μm in size were selected using steel sieves, and approximately 500.0 mg was washed in an ultrasonic bath with 10.0 mL of a 9 : 1 (v : v) methanol–acetic acid solution for 1 h. The washing procedure was repeated 10 times, and the washing solution was renewed for each repetition. After drying at 70.0 °C for 24 h, approximately 70.0 mg of MIP was packed in a steel column (empty HPLC pre-column) and coupled to the column switching system. The non-imprinted polymer (NIP) was synthesized similarly to the MIP but in the absence of the template molecule.

Initially, the materials were characterized by scanning electron microscopy (Zeiss LEO 440, Cambridge, England).

A kinetics study was carried out by adding 10.0 mg of the polymer (MIP or NIP) to test glass tubes containing 1.0 mL of

1.0 mg L^{-1} OXP phosphate buffer solution (0.01 mol L^{-1}, pH 7.0). The tubes were shaken for 0, 15.0, 30.0, 45.0, 60.0, 75.0, 90.0 or 105.0 min at room temperature (approximately 25.0 °C) and centrifuged at 1000*g*. The OXP remaining in the supernatant was quantified using a system equipped with an HPLC pump coupled to a UV detector (λ: 220 nm, flow rate: 1.0 mL min^{-1}, mobile phase: phosphate buffer solution 0.01 mol L^{-1}, pH 7.0).

Adsorption isotherms were constructed to evaluate the extraction capacities of the MIP and NIP. OXP standard solutions (25.0, 50.0, 100.0, 200.0, 300.0, 500.0, 1000.0 and 1500.0 mg L^{-1}) were prepared in a phosphate buffer solution (0.01 mol L^{-1}, pH 7.0). One milliliter of each solution was transferred to a glass tube containing 10.0 mg of MIP or NIP, and the tubes were shaken for 60.0 min at room temperature. Then, each sample was centrifuged at 1000*g* for 10.0 min. The OXP concentration remaining in the supernatant (equilibrium concentration – C_e) was quantified using the above described system. The mass retained by the polymers (adsorption capacity – Q_e) was calculated by subtraction. The results were analyzed using Freundlich and Langmuir models, with the accepted adequacy standard being the linear correlation coefficient (r).

Liquid chromatography and mass spectrometry conditions

LC-MS/MS analyses were performed using an LC-MS 8030 equipment from the Shimadzu® (Kyoto, Japan), equipped with a Shim-Pack XR-ODS C18 (100 × 3 mm, 2.2 μm) chromatographic column and a triple-quadrupole mass analyzer. The positive electrospray ionization mode was selected, and the MRM (multiple reaction monitoring) transitions and optimal collision energies were optimized for each analyte (Table 1). The identification criterion was the simultaneous presence of the three fragments of each molecule, according to Table 1. The quantitative analyses were carried out using the TIC (total ion chromatogram) of the three MRM transitions of each molecule. The oven, interface and heat block temperatures were set to 40.0, 250.0 and 400.0 °C, respectively. The nebulizing and drying gas flow rates were 1.5 and 15.0 mL min^{-1}, respectively. The mobile phase of the chromatographic column was composed of 0.01% formic acid aqueous solution–methanol at 30 : 70 (v/v). The flow rate was 0.4 mL min^{-1}. The volume of the sample loop was 100.0 μL, and data files were acquired using the LabSolutions® software program.

Table 1 Analytes and their precursors, fragments and collision energies

Analyte	Precursor (m/z)	Fragments (m/z)	Collision energy (kV)
ATE	267.2	145.0	−30
		190.1	−20
		116.1	−10
LAB	329.2	91.1	−35
		162.1	−25
		294.1	−20
NAD	310.1	254.2	−20
		74.1	−25
		201.1	−25
PIN	249.2	116.1	−20
		74.1	−25
		72.1	−25
MET	268.2	116.5	−25
		98.1	−25
		133.2	−25
OXP	266.2	72.1	−25
		224.9	−15
		116.1	−20
PROP	260.2	116.1	−20
		98.1	−20
		183.2	−20

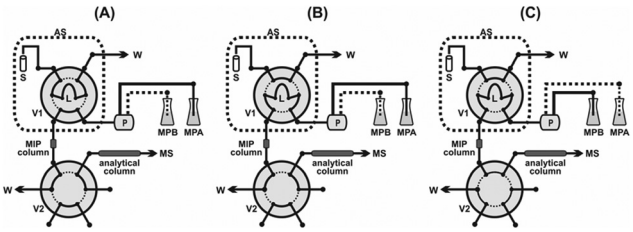

Fig. 2 Chromatographic system composed of pump (P), sample flask (S), autosampler (AS), injection valve (V1), electronic six-port valve (V2), loop (L), MIP and analytical columns, detector (MS), waste (W). MPA and MPB are the extraction and elution mobile phases, respectively.

Column switching

The column switching system (Fig. 2) consisted of a pump (P) delivering either 10 mmol ammonium formate buffer at pH 5.0 as mobile phase A (MPA) or a 0.01% formic acid aqueous solution–methanol at 30 : 70 (v/v) as mobile phase B (MPB), both at 0.4 mL min^{-1}. The pump was connected to an autosampler (AS) equipped with an injection valve (V1) with a 100.0 μL loop. The autosampler was connected to an electronic six-port switching valve (V2), and the MIP column (kept at 45.0 °C) was positioned between V1 and V2. A C18 analytical column (kept at 25.0 °C) was positioned after V2 and before the mass spectrometer (MS). Each cycle of the analysis was composed of three steps. First, 100.0 μL of the sample was collected in the loop (Fig. 2A), where the MPA flows through the MIP column and no mobile phase flows through the analytical column. Next, valve V1 was switched, and the sample was conducted through the MIP column by MPA for 3.0 min (Fig. 2B), where no mobile phase flows through the analytical column. The beta-blockers were retained in the MIP column, and the interferents were eliminated. In the third step, V2 was switched, and MPB eluted the beta-blockers from the MIP and led them to the analytical column and mass spectrometer (Fig. 2C). After 9.5 min, the system returned to the first stage (Fig. 2A), and reconditioning was carried out for 3.5 min. The total time required for each analysis, including the extraction and chromatography/mass spectrometer analysis, was 13.0 min.

Sample preparation

The human urine sample handling procedure was approved by the ethics committee of the Federal University of Alfenas (registration number 193.678). The blank samples were collected from volunteers who agreed to participate in this study. In order to check the absence of beta-blockers, the samples were previously tested by the developed method.

The pH of the samples was previously adjusted to 5.0 using a 1.0 mol L^{-1} formic acid solution. The samples were then centrifuged for 10 min at 1000*g*, the supernatant was collected and directly analyzed using the column switching system.

Validation study

The linearity, sensitivity, selectivity, precision, accuracy, stability, detection and quantification limits and matrix effect were evaluated. These studies were performed using a pool of blank human urine samples (free of the analytes) spiked with ATE, MET, LAB, PROP, NAD and PIN at concentrations ranging from 1.0 to 50.0 μg L^{-1} and with OXP at concentrations ranging from 0.1 to 75.0 μg L^{-1}. The linearity and sensitivity, which are expressed as the correlation coefficient (*r*) and the slope of the calibration curve, respectively, were established using three calibration curves (in six replicates) of all the analytes at six different concentration levels (3.0, 10.0, 20.0, 30.0, 40.0, and 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD, and PIN and 1.0, 5.0, 10.0, 25.0, 50.0 and 75.0 μg L^{-1} for OXP). The selectivity was evaluated by observing the presence or absence of peaks at the same retention times of analytes in a blank sample. The intra-assay precision and accuracy were assessed with six replicates at three concentration levels (3.0, 20.0 and 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 1.0, 25.0 and 50.0 μg L^{-1} for OXP) on the same day. The inter-assay precision and accuracy were evaluated using six replicates analyzed at three concentration levels (3.0, 20.0 and 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 1.0, 25.0 and 50.0 μg L^{-1} for OXP) on three different days. The results were expressed as relative standard deviation percentages and relative errors for precision and accuracy, respectively. The precision and accuracy tests were appraised under the same conditions: equipment, analyst and laboratory. Only the day was the variable focused upon. The limits of detection (LOD) and quantification (LOQ) were established based on the signal/noise ratio (three times for LOD and 10 times for LOQ). Stability studies (expressed as a percentage of relative standard deviations) were conducted by analyzing samples at three concentration levels (3.0, 20.0 and 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 1.0, 25.0 and 50.0 μg L^{-1} for OXP) after 30 days in a freezer (−18.0 °C) and after cycles of freezing and thawing (four cycles of 24 h for each one). The matrix effect was evaluated by analyzing six blank samples from six different individuals (volunteers), fortified with the analytes at a concentration of 10.0 μg L^{-1}, and the result was expressed as the percentage of relative standard deviation. The absence of matrix effect was considered for a relative standard deviation lower than 15%.

Results and discussion

Polymer characterization

Scanning electron microscopy images of the materials (Fig. 3) revealed a MIP morphological structure presenting microsphere agglomerates due to the precipitation method used for the synthesis.

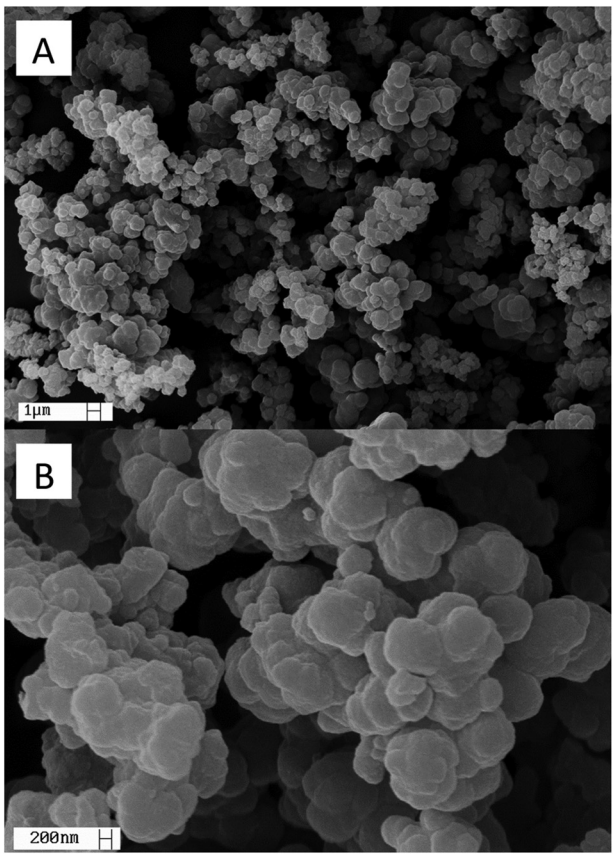

Fig. 3 Scanning electron micrograph of MIP. (A) Magnification of ×6000 and (B) magnification of ×25 000.

Adsorption studies were performed for the MIP and NIP. The first investigation examined the MIP adsorption kinetics. It was observed that adsorption equilibrium was reached in 60.0 min (Fig. 4). Then, adsorption isotherms were constructed for both materials, as described in the section "MIP synthesis and characterization". The MIP and NIP were combined with different concentrations of OXP for 60.0 min because this was

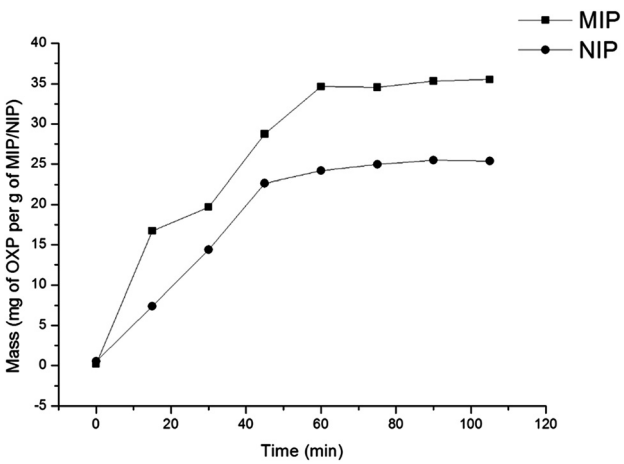

Fig. 4 Absorption kinetics for the MIP and the NIP.

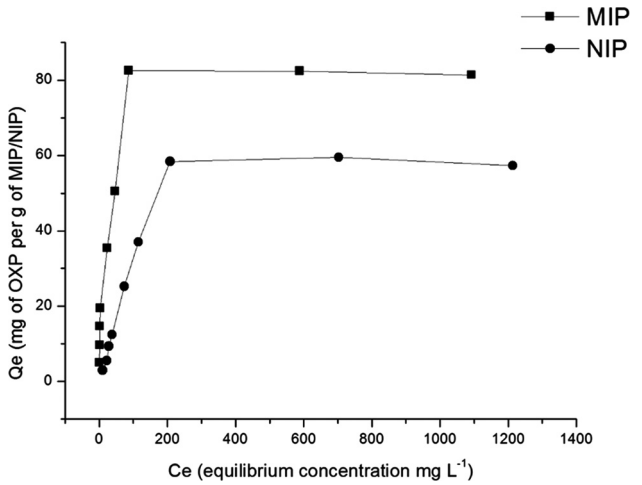

Fig. 5 Adsorption isotherms of OXP for the MIP and the NIP.

the time required to reach equilibrium. As shown in Fig. 5, the adsorption showed a linear relationship until equilibrium was reached; the concentrations were approximately 100.0 and 200.0 mg L^{-1}, respectively, for the MIP and NIP. Based on molecular recognition, the MIP presented the highest adsorption capacity, likely due to OXP interactions, whereas only non-specific interactions prevailed between the NIP and OXP.[23,24]

Then, the Freundlich and Langmuir models were tested for the MIP and NIP. Fig. 6 shows that the Langmuir model was the best fit for the MIP and NIP, with correlation coefficients of 0.99 and 0.98, respectively, whereas the Freundlich model's correlation coefficients were 0.96 and 0.92, respectively. The maximum adsorption capacities for the materials were calculated as the inverse of the slope; for the MIP, the capacity was 82.6 mg g^{-1}, and for the NIP, the capacity was 67.1 mg g^{-1}. The Langmuir model indicates that molecules have a uniform distribution in the binding sites around the polymer and that each binding site is able to receive only one molecule. Furthermore, according to the model, the analytes are retained in a monolayer on the MIP surface, and the energy involved in this process is the same for all binding sites surrounding the polymer.[25–27]

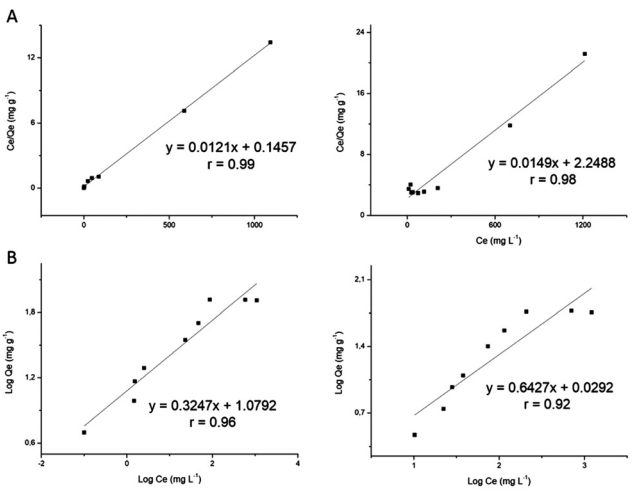

Fig. 6 Langmuir (A) and Freundlich (B) adsorption isotherms of OXP for the MIP and the NIP.

Method optimization

System optimization was performed using a pool of blank human urine samples spiked with ATE, MET, LAB, PROP, NAD, PIN and OXP at a concentration of 100.0 μg L^{-1}. The following variables were evaluated in a univariate manner: standard/sample pH, extraction time, mobile phase flow rate and MPA and MPB compositions. The initial conditions were as follows: standard/sample without pH adjustment, extraction time of 1.0 min, mobile phase flow rate of 0.1 mL min^{-1} and water and methanol as MPA and MPB, respectively.

Initially, the standard/sample pH was evaluated from 3.5 to 10.0 using 0.1 mol L^{-1} formic acid or 0.1 mol L^{-1} ammonium hydroxide aqueous solutions for adjustment. The best results, expressed as the absolute response of the analytical signal and lower RSD%, were achieved at a pH value of 5.0. At this pH, the analytes and the functional monomer (pK_a of approx. 9.0 and 4.0, respectively) were completely ionized, favoring electrostatic interactions between the two.[28]

The extraction time can be defined as the time necessary to extract the analytes and to eliminate the interferents (Fig. 2B) by passing MPA. In this work, the extraction time ranged from 1.0 to 5.0 min. The best result evaluated for sensitivity and precision was obtained at 3.0 min. Extraction for less than 3.0 min resulted in low sensitivity, which was likely due to insufficient time to prepare the sorbent, to receive the sample and to eliminate interferents. A duration longer than 3.0 min resulted in low sensitivity due to the removal of the analytes by the conditioning solution.

The mobile phase flow rate is correlated with the peak symmetry and time retention, and for this reason, this parameter was evaluated from 0.1 to 0.4 mL min^{-1}. The best result was obtained at a flow rate of 0.4 mL min^{-1}, with a decrease in peak broadening and an increase in the analytical frequency. Higher flow rates were tested, but the results were not applicable due to problems with the system pressure.

The properties of MPA are fundamental to improving the extraction of the analytes and eliminating interferents. Thus, water, 10.0 mmol L^{-1} ammonium formate buffer (pH 5.0), 10.0 mmol L^{-1} ammonium acetate buffer (pH 3.0) and 10.0 mmol L^{-1} ammonia/ammonium buffer (pH 10.0) were evaluated. The best results, expressed as the response of the analytical signal, were obtained using a 10.0 mmol L^{-1} formate buffer. Peak broadening was also observed for the ammonia/ammonium buffer because beta-blockers are not ionized at this pH. This fact complicates the interaction between the polymer and the analytes. The selected pH was 5.0 because electrostatic interactions between the analytes and the MIP prevailed under this condition, considering their pK_a values of approximately 9.0 and 4.0, respectively.[28] Finally, the ammonium formate buffer concentration was studied from 2.0 to 10.0 mmol L^{-1} to determine the concentration at which the ionic strength can affect the analyte ionization processes.[29,30]

Higher analytical signals were reached at 10.0 mmol L^{-1} because higher ionic strength improves analyte ionization when the electrospray mode is employed.[29] Higher concentrations were not tested because crystallization in the ion source could occur.

MPB has at least three important functions: to elute the analytes from the MIP column, to separate them in the analytical column and to improve their ionization efficiency in the electrospray source.[31] In addition, this solution needs to be effective to clean the system after the extraction procedure, thus eliminating the memory effect (carry over). Pure methanol and 0.01% formic acid aqueous solution–methanol combined in proportions of 10 : 90, 20 : 80, 30 : 70 and 40 : 60 (v : v) were tested as MPB. Employing pure methanol and 40% of 0.01% formic acid aqueous solution, the analytes were not completely eluted, and the carry over effect was observed. Quantitative elutions were obtained using solutions containing 30.0, 20.0 and 10.0% of 0.01% formic acid aqueous solution, the first of which was selected as the working solution to save the reagents.

After the optimization process, the total time per analysis was 13.0 min. Moreover, the analytical signals were increased in about 884.0, 534.0, 234.0, 235.0, 818.0, 535.0 and 406.0% for ATE, MET, LAB, PROP, NAD, PIN and OXP, respectively, relative to the initial conditions. Fig. 7 shows the chromatograms (TIC) obtained for (A) blank urine sample, (B) after optimization for 1.0 μg L^{-1} of 1-ATE, 2-MET, 3-LAB, 4-PROP, 5-NAD and 6-PIN and for 0.1 μg L^{-1} of 7-OXP and (C) after optimization for 50.0 μg L^{-1} of 1-ATE, 2-MET, 3-LAB, 4-PROP, 5-NAD, 6-PIN and 7-OXP.

Validation assays and method application

The developed method was linear from 3.0 to 50.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and from 1.0 to 75.0 μg L^{-1} for OXP. The intra-assay and inter-assay precisions (obtained as a relative standard deviation) were lower than 20.0% for concentrations near the LOQ and 15.0% for the others, according to the validation guidelines of the United States Food and Drug Administration,[32] as shown in Table 2. Good results with respect to accuracy, stability and the matrix effect can be verified in Table 2 as well. The matrix effect was evaluated using the relative standard deviation between the samples, considering as acceptation criteria values lower than 15.0%. The LODs were 1.0 μg L^{-1} for ATE, MET, LAB, PROP, NAD and PIN and 0.1 μg L^{-1} for OXP, which are appropriate for doping analysis considering the MRPL (minimum required performance levels) for detection and identification of non-threshold substances.[36] Furthermore, the LODs established in this paper are lower or closer than other values described in

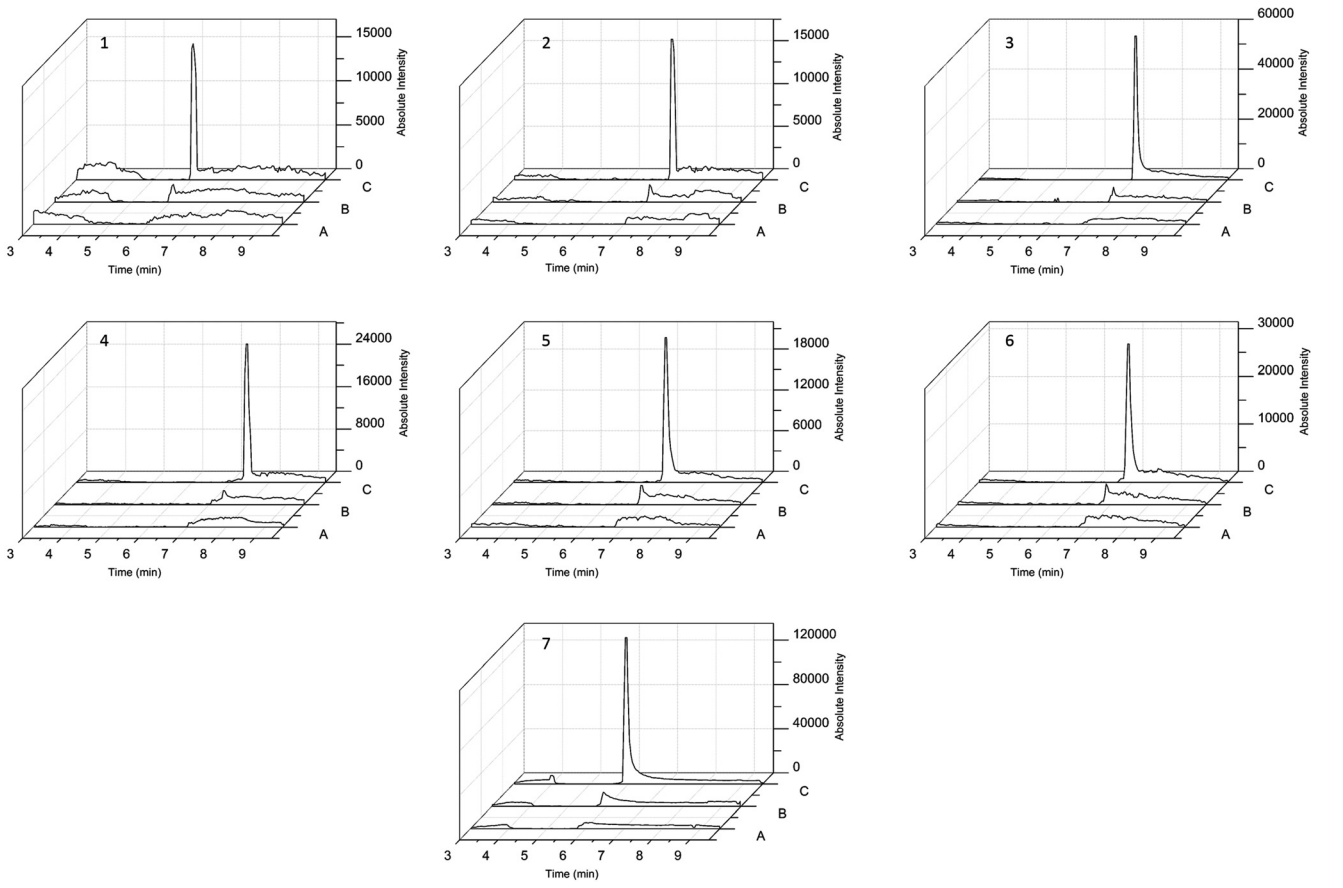

Fig. 7 Chromatograms (TIC) obtained for (A) blank urine sample, (B) after optimization for 1.0 μg L^{-1} of 1-ATE, 2-MET, 3-LAB, 4-PROP, 5-NAD and 6-PIN and for 0.1 μg L^{-1} of 7-OXP and (C) after optimization for 50.0 μg L^{-1} of 1-ATE, 2-MET, 3-LAB, 4-PROP, 5-NAD, 6-PIN and 7-OXP.

Table 2 Validation parameters of online extraction applied in the LC-MS/MS method for the determination of ATE, MET, LAB, PROP, NAD, PIN and OXP in urine

Validation parameters	ATE	MET	LAB	PROP	NAD	OXP	PIN
Linear range (μg L^{-1})	3.0–50.0	3.0–50.0	3.0–50.0	3.0–50.0	3.0–50.0	1.0–75.0	3.0–50.0
Linearity (r^2) ($\bar{x}$, n = 3)	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Slope (a) ($\bar{x}$, n = 3)	3197.8	3316.9	10 233.6	3736.4	3739.2	29 580.5	7729.4
Intercept (b)	6738.3	1615.7	13 674.7	718.4	2953.7	18 371.3	8454.0
LOD (μg L^{-1})	1.0	1.0	1.0	1.0	1.0	0.1	1.0
LOQ (μg L^{-1})	3.0	3.0	3.0	3.0	3.0	1.0	3.0
Matrix effect %RSD (10 μg L^{-1}, n = 6)	12.9	11.2	13.5	12.5	9.5	11.1	11.7
Intra-assay precision %RSD (n = 6)	7.6[a]	14.1[a]	13.3[a]	14.2[a]	19.6[a]	13.0[b]	11.4[a]
	14.4[c]	14.9[c]	7.6[c]	10.8[c]	9.7[c]	11.0[d]	13.2[c]
	8.7[e]	11.9[e]	5.0[e]	10.6[e]	5.2[e]	6.5[e]	13.3[e]
Inter-assay precision %RSD (n = 6, three days)	12.2[a]	13.3[a]	10.7[a]	4.6[a]	17.4[a]	16.9[b]	6.5[a]
	5.6[c]	8.5[c]	9.9[c]	8.5[c]	5.9[c]	7.2[d]	11.7[c]
	4.7[e]	7.8[e]	9.4[e]	11.2[e]	8.0[e]	11.4[e]	5.2[e]
Accuracy E% (n = 6)	9.4[a]	2.8[a]	17.0[a]	8.0[a]	−12.6[a]	−4.5[b]	−9.4[a]
	12.5[c]	12.5[c]	2.5[c]	−2.6[c]	9.8[c]	−7.3[d]	−4.7[c]
	−0.8[e]	−0.6[e]	−1.9[e]	2.5[e]	−4.8[e]	4.2[e]	−4.1[e]
Accuracy E% (n = 6, three days)	−18.0[a]	9.5[a]	15.3[a]	−7.5[a]	14.1[a]	15.0[b]	16.2[a]
	7.5[c]	4.9[c]	−2.7[c]	−10.0[c]	8.6[c]	−11.4[d]	6.1[c]
	−9.8[e]	−3.3[e]	−8.7[e]	−9.3[e]	7.9[e]	5.6[e]	8.0[e]
Stability[f] %RSD (n = 6)	9.3[a]	9.7[a]	11.7[a]	13.2[a]	11.3[a]	6.2[b]	13.7[a]
	10.0[c]	12.3[c]	13.9[c]	8.9[c]	13.6[c]	5.7[d]	10.8[c]
	12.5[e]	11.9[e]	12.2[e]	10.4[e]	13.3[e]	6.0[e]	14.1[e]
Stability[g] %RSD (n = 6)	10.9[a]	14.6[a]	14.3[a]	10.9[a]	7.6[a]	5.9[e]	10.9[a]
	8.8[c]	8.1[c]	14.5[c]	14.7[c]	9.0[c]	11.6[d]	10.8[c]
	9.7[e]	4.8[e]	14.8[e]	12.5[e]	11.5[e]	9.7[e]	11.1[e]

[a] 3.0 μg L^{-1}. [b] 1.0 μg L^{-1}. [c] 20.0 μg L^{-1}. [d] 25.0 μg L^{-1}. [e] 50.0 μg L^{-1}. [f] After 30 days in freezer. [g] After four cycles of freeze and thawing.

Table 3 Comparison between LOD values found in the literature and those obtained in this paper

Ref.	LOD
33: P. V. Eenoo, W. V. Gansbeke, N. De Brabanter, K. Deventer and F. T. Delbeke, *J. Chromatogr., A*, 2011, **1218**, 3306–3316	Values between 25.0 and 500.0 μg L^{-1}
34: M. Kolmonen, A. Leinonen, A. Pelander and I. Ojanperä, *Anal. Chim. Acta*, 2007, **585**, 94–102	500.0 μg L^{-1}
2: G. J. Murray and J. P. Danaceau, *J. Chromatogr., B: Biomed. Appl.*, 2009, **877**, 3857–3864	Values between 8.0 and 62.0 μg L^{-1}
35: M.-J. Paik, J. Leeb and K.-R. Kimb, *Anal. Chim. Acta*, 2007, **601**, 230–233	Values between 0.03 and 2.7 μg L^{-1}
This paper	Values between 0.1 and 1.0 μg L^{-1}

the literature as can be seen in Table 3. The method was selective since was not observed picks in the blank sample at same retention time of analytes. It should be emphasized that the same MIP column was used during all the validation assays, and non-significant differences were observed in the analytical signal after approximately 200 cycles. In addition, the LOD and LOQ for OXP (template) were lower than those of other beta-blockers, likely due to the more favorable interactions between this molecule and the MIP binding sites.

Fortified human urine samples (at 5.0, 15.0, 35.0 and 45.0 μg L^{-1}) were analyzed by the proposed method, and the results showed a low variation between the nominal concentration and the analyzed concentration, with the relative error ranging from −9.5 to 5.9%.

Conclusions

The developed on-line system for direct extraction and analysis of beta-blocker drugs in human urine samples was linear for ATE, MET, LAB, PROP, NAD, PIN and OXP and appropriate for doping analysis. Good figures of merit were attained, such as low LOD, wide linear range, good precision and accuracy, high analytical frequency and minimal sample manipulation. In addition, other interesting system characteristics, such as high selectivity, longer MIP column lifetime, use of small sample and solvent volumes, and ease of operation, among others, should be emphasized. Furthermore, it is important to note that the present system could be used to analyze other classes of drugs from biological samples.

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6 ARTIGO 3

ANÁLISE *ONLINE* DE FÁRMACOS ANTIDEPRESSIVOS TRICÍCLICOS EM PLASMA HUMANO EMPREGANDO EXTRAÇÃO EM FASE SÓLIDA MOLECULARMENTE IMPRESSA DE ACESSO RESTRITO SEGUIDA DE DETERMINAÇÃO POR ESPECTROMETRIA DE MASSAS

ANALYSIS OF TRICYCLIC ANTIDEPRESSANTS IN HUMAN PLASMA USING ONLINE RESTRICTED ACCESS MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION FOLLOWED BY DIRECT MASS SPECTROMETRY IDENTIFICATION/QUANTIFICATION

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Abstract

Tricyclic antidepressants (TCAs) have been used to treat depression disorder symptoms. Many sample preparation strategies have been used for the analysis of TCA in human plasma, however they are either poor in selectivity or require protein elimination pre-treatment. A new class of hybrid materials, called restricted access molecularly imprinted polymers (RAMIPs) seem to be an alternative to circumvent these problems. These materials joined the positive qualities of good protein elimination capacity and high selectivity. As for the analyses techniques, the TCAs' determination has demanded the use of separation systems, as gas or liquid chromatography, which although are very effective and take long time. Mass spectrometry (MS), equipped with electrospray ionization, at atmospheric pressure (ESI), and triple quadrupole mass analyzer have been successfully used for polar drugs. In this context, in order to combine the advantages of both RAMIPs and mass spectrometry, we proposed a study that joins these properties in a single system, where we could analyze TCAs from human plasma, without previous extraction or chromatographic separation. A RAMIP for amitriptyline was synthesized by the bulk method, using methacrylic acid as a functional monomer and glycidilmethacrylate as hydrophilic co-monomer. Then, the epoxide rings' openings were made and the polymer was covered with bovine serum albumin (BSA). A column filled with RAMIP-BSA was coupled to a MS/MS instrument under the online configuration, using water as loading and reconditioning mobile phase and a 0.01% acetic acid aqueous solution: acetonitrile at 30:70 as the elution mobile phase. The system was used for on-line extraction and quantization of nortriptyline, desipramine, amitriptyline, imipramine, clomipramine and clomipramine-d3 (PI) (from 15.0 to 500.0 μg L^{-1}) simultaneously, from plasma samples. The correlation coefficient was higher than 0.99 for all analytes. The RSD (relative standard deviation) values ranged from 1.34% to 19.13% for intra assay precision and 1.32% to 19.77% for inter assay precision. The E% (relative error) values ranged from -19.15% to 19.51% for intra assay accuracy and from -9.04% to 16.22% for inter assay accuracy.

Key words: Tricyclic antidepressants, RAMIP-BSA, direct determination, mass spectrometry.

Introduction

For a long time, tricyclic antidepressants (TCAs) have been used to treat and relieve depression disorder symptoms as a support in the treatment of chronic pain and in cases of nocturnal enuresis in children [1-6]. These drugs are classified into two classes: First, the secondary amines that preferably prevent norepinephrine reuptake and second, the tertiary amines that preferably prevent serotonin reuptake. Usually, their therapeutic levels range from 50.0 to 300.0 μg L^{-1} [7-9]. Despite all the benefits achieved, using these drugs, some side effects can occur frequently, due to its action in other receptors (such as muscarinic receptors). Their main side effects are dry mouth, blurred vision, constipation, tremor, cardiovascular effects, urinary retention and confusion. Moreover, TCAs have low therapeutic index, which require its plasma levels monitoring [3,10].

From the chemical point of view, these molecules present a common chemical structure; which includes the presence of three rings, where in the central ring there are seven or eight carbon atoms, a side chain and a terminal amine group (Fig. 1) [11]. The analysis of these drugs in plasma samples is required in order to monitor the therapy and pharmacokinetic studies, and they have demanded the use of separation systems, like gas or liquid chromatography and capillary electrophoresis, coupled to UV or MS detectors [12]. The most commonly used sample preparation strategies, reported in literature, are conventional liquid-liquid extraction (LLE), solid phase extraction (SPE), solid phase micro extraction (SPME) [12] and, more recently, the use of molecularly imprinted polymers [13]. However, the use of these techniques necessarily need a preliminary protein precipitation to guarantee a reliable analysis. On the other hand, restricted access materials (RAM) have been used to access the direct analysis of TCAs without previous sample preparation and proteins precipitation [14] steps. Despite being a good alternative for the analysis of these drugs in blood samples, RAMs are poor in selectivity, which sometimes can affect the analysis efficiency. A new class of hybrid materials, called restrict access molecularly imprinted polymers (RAMIPs) seems to be an alternative to circumvent these problems. These materials joined both characteristics from RAMs (proteins elimination capacity) and from MIPs (high selectivity) and were successfully used in the analysis of drugs in complex matrices. [15].

Haginaka and co-workers obtained good results using RAMIPs for the extraction of (S)-naproxen [16] (S)-ibuprofen [17] and propranolol [18] from biological samples. Their materials were obtained by coating conventional MIPs with hydrophilic co-monomers. The same strategy

was used to obtain polymers selective for non-steroidal anti-inflammatory [19] antiepileptics [20] and bisphenol-A [21] from environmental samples. Puoci and co-workers [22] obtained a RAMIP selective for caffeine by in bulk polymerization. A hydrophilic layer was created by glycidil methacrylate (GMA) epoxide ring opening. Similar studies were carried out for the analysis of p-aminofenol [23] and sulfonamides [24]. Recently, our group conduced some studies using RAMIPs. Moraes and co-workers [25] synthesized a RAMIP coated with bovine serum albumin (BSA) (restricted access molecularly imprinted polymer covered by bovine serum albumin – RAMIP-BSA) and obtained good results for chlorpromazine analysis from human plasma. Santos and co-authors synthesized different materials selective for beta-blockers and evaluated the consequences of their surface modifications (using hydrophilic comonomers and/or BSA) [26]. They also conducted a study that uses a RAMIP (obtained with hydrophilic comonomers surface recovering) for the extraction of beta blockers from human urine [27].

Mass spectrometry has advanced a lot in terms of instrumentation, including the development of much more efficient ionization techniques and high-resolution analyzers. For the studies of polar drugs, electrospray ionization at atmospheric pressure (ESI) and triple quadrupole mass analyzers have been successfully used [14, 27]. On the other hand, in spite of these advances, previous chromatographic separations are always needed in order to prevent the ionic suppression effect, which can cause imprecision and inaccuracy in the analysis. This effect can be observed when the analyte arrives to the ionization source together with an interferent in high concentration, causing competitive ionization. However, we believe that if a selective sample preparation is used (e.g. molecularly imprinted solid phase extraction), we can avoid the presence of interferents in high concentrations, decreasing the ionic suppression of the analytes.

In this context, in order to combine the advantages of both RAMIPs (such as high selectivity and macromolecule elimination capacity) and mass spectrometry (simplicity and good ability to identify and quantify organic compounds), we proposed a study that joins these properties in a single system, in which it is possible to perform TCAs analysis from human plasma, without previous extraction and without chromatographic separation.

Experimental

Chemicals and solutions

The organic solvents (chloroform, acetonitrile and methanol) were obtained from Vetec (Rio de Janeiro, Brazil). Deionized water (18.2 MΩcm) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA). For the RAMIP synthesis, amitriptyline, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), 4,4′-azobis (4-cyanovaleric acid) (A4-CA) and GMA were used as template, functional monomer, crosslinking reagent, initiator and hydrophilic co-monomer, respectively (all from Sigma-Aldrich, Steinheim, Germany). HPLC-grade chloroform was used as porogenic solvent. Methanol and acetic acid (Merck, Darmstadt, Germany) were used during the polymer washing to remove the template. Perclhoric acid (J. T. Baker, Phillipsburg, New Jersey) was used to perform the epoxide ring opening.

Stock solutions of nortriptyline (NOR), desipramine (DES), amitriptyline (AMI), imipramine (IMI), clomipramine (CLO) and clomipramine-d3 (CLO-d3) (all from Sigma-Aldrich, Steinheim, Germany) were prepared at a concentration of 1.0 mg L^{-1} in HPLC-grade acetonitrile, placed in amber flasks and stored at -22.0 °C for up to 30 days. Working solutions ranging from 1.0 to 500.0 μg L^{-1} were prepared daily by diluting stock solutions in acetonitrile. Acetonitrile, ammonium formate (Fluka, Seelze, Germany), ammonium hydroxide (Isofar, Jacaré, Brazil) and formic acid (Vetec, Rio de Janeiro, Brazil) were used to prepare the mobile phases for the analysis. Ammonium formate was also used in sample pHs evaluation.

RAMIP synthesis and coating

The restrict access molecularly imprinted polymer synthesis was carried out according to the methodology proposed by Puoci and co-authors [23]. The synthesis was carried out by the bulk method. One mmol of AMI and 4.0 mmol of MAA were dissolved in 10.0 mL of chloroform in a 25.0 mL glass flask. Then, 16.0 mmol of EGDMA, 8.0 mmol of GMA and 50.0 mg of A4-CA were added, and the mixture was purged with nitrogen for 20 min. The flask was

closed and immersed into a water bath at 80.0°C for 24 h. After polymerization, the obtained monolith was mechanically ground and particles ranging from 75.0 to 106.0 µm in size were selected using steel sieves. For further studies, the non-imprinted polymer (RANIP) was also synthetized in the same way as the RAMIP but in the absence of the template molecule.

For the cleansing process, the material was transferred into glass tubes (approximately 500.0 mg of material for each tube) and 10.0 mL of a 9:1 (v:v) methanol:acetic acid solution was added. The tubes were placed into an ultrasonic bath for 1 h. The washing procedure was repeated 10 times, and the washing solution was renewed for each repetition. Then, the polymer was dried at 70.0 °C for 24 h. The epoxide ring opening process was made according to PUOCI and co-authors [19]. For this, 400mg of RAMIP was placed into a 100.0 mL glass flask with 25.0 mL of perchloric acid water solution (10% v/v). Then, the flask was agitated at 200 rpm for 24 h at room temperature. At the end of the reaction, we filtered and washed the particles with water until the complete removal of the acid solution.

Following the process described above, about 1.0 g of the obtained polymers were coated with a layer of bovine serum albumin (BSA), according to the methodology proposed by Moraes and co-authors [25]. In order to achieve this, 500.0 mg of either RAMIP or RANIP were placed in 5 mL polypropylene cartridges separately (one cartridge for each polymer). Then, 20 mL of 1% albumin solution (prepared in 0.05 mol L^{-1} phosphate buffer pH 6.0) was percolated by the materials. Afterwards, 25 mL of 5% (w/v) glutaraldehyde aqueous solution was placed in contact with the polymers for 5h, to promote the binding of the albumin molecules. Finally, 10 mL of sodium borohydride 1% (w/v) aqueous solution was percolated by the cartridges, in order to stabilize the protein network. The coated polymers (RAMIP-BSA and RANIP-BSA) were washed with water to remove any reagent residues.

To carry out the ADT extraction from plasma, about 70 mg of RAMIP-BSA was packed in a steel column (empty HPLC pre-column 10×4.6 mm i.d.), which was used to perform the *on line* extraction.

Characterization of polymers

First, the materials were characterized by transmission electron microscopy (TEM) (JEOL JEM 2100, Peabody, USA), equipped with Gatan ES 500W and high resolution transmission electron microscopy (HRTEM), Gatan GIF Tridiem 2kx2k CCD cameras.

To assess the macromolecules' elimination capacity, 25 µL of 44 mg mL^{-1} BSA standard in phosphate buffer 0.01 mol L^{-1} pH 7.0 (approx. the same concentration found in human plasma) were injected in a HPLC system (Shimadzu, Kyoto, Japan), equipped with a pump, a manual sampler with a loop of 50 µL and a UV detector operating at 254 nm. The flow rate was 1.0 mL min^{-1} and the mobile phase was the same buffer previously cited. Then, 70 mg of each polymer were individually packed into a HPLC pre-column (10×4.6 mm i.d.). Each column was placed in the analytical pathway and 25 µL of 44 mg mL^{-1} BSA standard in phosphate buffer 0.01 mol L^{-1} pH 7.0, was injected again. The macromolecules' elimination percentage was calculated by comparison between the areas obtained when the albumin solution was injected without the columns (100% of the albumin has come into detector) and when the same solution was percolated through the columns (100% of the albumin has come into detector).

To perform the selectivity test, the same equipment as well as system with the same conditions, described below, were used (see sections *Equipment and direct analysis conditions* and *Analysis system*).

Initially, an AMI calibration curve was constructed. To do this, AMI methanol solutions, at concentrations of 50; 100; 250, 500, 1000 and 2000 µg L^{-1} were used. Then, 100 µL of each solution were injected directly into the system, in the absence of polymer columns. MPB, at a flow rate of 0.3 mL min^{-1}, was used as a mobile phase. The mass of AMI was calculated using the injection volume and concentration of the solutions. Subsequently, about 70 mg of RAMIP-BSA, RAMIP (imprinted polymers), RANIP-BSA and RANIP (non-imprinted polymers) were packed, individually, in a steel column (empty HPLC pre-column 10×4.6 mm i.d.). The column was placed in the analytical course (see section *Analysis system*) and used to perform the *on line* extraction of the methanol solutions of AMI, at concentrations of 500, 1000 and 2000 µg L^{-1}. The mass of AMI, retained by the polymers was calculated using the previously constructed calibration curve.

Equipment and direct analysis conditions

A LC-MS 8030 (Shimadzu, Kyoto, Japan), equipped with a triple-quadrupole mass analyzer was used to perform the analyses. The positive electrospray ionization mode was selected to ionize NOR, DES, AMI, IMI, CLO and CLO-d3. The SRM transitions and optimal

collision energies were optimized for each analyte (Table 1). The identification criterion was the simultaneous presence of the three fragments of each molecule (Table 1), the ratio between these fragments when compared to beta-blockers standards' analyses and the fragments' relative abundance. The quantitative analyses were carried out using the most intense SRM transition. The oven, interface and heat block temperatures were set to 40.0, 250.0 and 400.0°C, respectively. The nebulizing and drying gas flow rates were 1.5 and 15.0 mL min^{-1}, respectively. The sample loop volume was 100.0 µL. In order to monitor the elimination of proteins, an UV detector (operating at 254 nm) was positioned after the RAMIP-BSA column and before V2. The data files were acquired using LabSolutions® software program.

All the analyses were done in the absence of a chromatography column. After extraction, the ADT identification and quantification were made by the triple quadrupole mass spectrometer.

Analysis system

The used system (Fig. 2) consisted of a pump (P) delivering either water as mobile phase A (MPA) or a 0.01% acetic acid aqueous solution:acetonitrile at 30:70 (v/v): as mobile phase B (MPB), both at 0.3 mL min^{-1}. The pump was connected to an autosampler (AS) equipped with an injection valve (V1) with a 100.0 µL loop. The autosampler was connected to an electronic six-port switching valve (V2), and the RAMIP-BSA column (kept at 45.0°C) was positioned between V1 and V2. An UV detector (operating at 254 nm) was placed after the extraction column to verify the elimination of proteins.

Each analysis cycle was composed of three steps. First, 100.0 µL of the sample was collected in the loop (Fig. 2A), while the MPA flows through the RAMIP-BSA column. Then, valve V1 was switched and the sample was conducted through the RAMIP-BSA column by MPA for 2.5 min (Fig. 2B). During this step ADT were retained in the RAMIP-BSA column and the macromolecules were excluded. After this time, pump B delivered MFB through the RAMIP-BSA column, and in 3.5 min V2 was switched and MPB eluted the analytes from the RAMIP-BSA for 2.4 min and led them to the mass spectrometer (Fig. 2C). For 2.0 min more, pump B delivered MPB through the RAMIP-BSA column to clean the system. Afterwards, the system returned to the first stage (Fig. 2A), and the reconditioning was carried out for 2.0 min.

Samples

The ethics committee of the Federal University of Alfenas (registration number 193.678) approved the human blood plasma sample handling procedure. The blank samples were obtained from volunteers who agreed to participate in this study. In order to confirm the absence of ADT, the samples were previously tested by developed method. All samples were collected using vacuum tubes and heparin as anticoagulant.

The blood samples were first centrifuged for 15 min at 2000x*g* to promote the separation between plasma and red components. Plasma was collected, diluted at a proportion of 1 part of plasma to 4 parts of water, filtered and directly analyzed by the system previously described.

Validation study

The following confidence parameters were evaluated: linearity, sensitivity, selectivity, precision, accuracy, stability, detection and quantification limits as well as matrix effect. The validation studies were performed using a pool of blank human blood plasma samples (free of the analytes) spiked with NOR, DES, AMI, IMI and CLO at concentrations ranging from 1.0 to 500.0 μg L^{-1}. CLO-d3 was used as internal standard at concentration of 100.0 μg L^{-1}. The linearity and sensitivity, which are expressed as the correlation coefficient (r) and the slope of the calibration curve, respectively, were established using three calibration curves (in six replicates) of all the analytes at six different concentration levels (15.0, 30.0, 80.0, 130.0, 250.0 and 500.0 μg L^{-1}). The selectivity was evaluated observing either the presence or absence of peaks at the same retention times of the analytes in a blank sample. The intra-assay precision and accuracy were assessed with six replicates at three concentration levels (15.0, 130.0 and 500.0 μg L^{-1}) on the same day. The inter-assay precision and accuracy were evaluated using six replicates analyzed at three concentration levels (15.0, 130.0 and 500.0 μg L^{-1}) on three different days. The results were expressed as relative standard deviations and relative errors for precision and accuracy, respectively. The precision and accuracy tests were appraised using the same equipment, conditions, analyst and laboratory. Only the day was the variable focused. The limit of quantification (LOQ) was established based on the lowest analyzed concentration that presented satisfactory precision and accuracy. The limit of detection (LOD) was assessed based on the lowest analyzed concentration in which it was possible to detect the interest analytes in these experimental conditions. The stability studies (expressed as relative standard deviations)

were conducted by analyzing the samples at three concentration levels (15.0, 130.0 and 500.0 μg L^{-1}) after 7 days in a freezer (-22.0°C) and after cycles of freezing and thawing (three cycles of 24 h for each one). The matrix effect was evaluated by analyzing six blank samples from six different individuals (volunteers), fortified with the analytes at a concentration of 50.0 μg L^{-1}. The results were expressed as relative standard deviations and relative errors. The absence of matrix effect was considered when relative standard deviations and relative errors were lower than 15%.

Results and discussion

Polymer characterization

Transmission electron micrographies (Fig. 3 a-h and *Supplementary Material*) revealed that the RAMIP-BSA (Fig. 3 c) and RANIP-BSA (Fig. 3 d) are degradated by the incidence of electron beam (Fig. 3 e and f) while the RAMIP (Fig. 3 a) and RANIP (Fig. 3 b) remain unchanged. This degradation occurs in the BSA coating of polymers because of the low concentration of glutaraldehyde used in the polymerization between BSA amine groups [28]. Fig. 3 g and h show the subtraction of the polymers' images after the incidence of the electron beam, t = 0 s and t = 31 s. The images show the BSA coating on the RAMIP-BSA and RANIP-BSA with ratios in area of 31.3% and 26.8%, respectively.

In order to evaluate the capacity of macromolecule elimination and understand the influence of GMA and BSA layers in the exclusion, tests were carried out with RAMIP, RANIP, RAMIP-BSA AND RANIP-BSA. From the results, it is possible to see that only the addition of GMA (hydrophilic comonomers) is not enough to promote total albumin exclusion. However, when the results obtained in this work (about 87% of BSA) are compared with the results obtained by Puoci and cols. [23] (about 55%), it can be seen that better results were obtained in this paper. One probable explanation can be the addition of perclhoric acid, acetic acid was being used during the washing process and it could have collaborated in the epoxide opening rings' procedure. On the other hand, when both use of GMA and BSA layer covering were combined, the BSA, present in the sample, was almost completely excluded. This fact occurs because when both techniques are used and when a buffer with pH (7.0) higher than the

albumin isoelectric point (4.7) [29] is used as mobile phase, there is a higher density of negative charges on the polymer surface, resulting in an electrostatic repulsion, preventing adsorption [25].

The selectivity test showed that the RAMIP-BSA presented higher adsorption of AMI compared to the RANIP-BSA in all tested concentrations (Fig. 4). This fact can be attributed to the different kinds of interaction between the imprinted (RAMIP-BSA) and non-imprinted (RANIP-BSA) polymers and template (AMI). Whereas the interaction between the RAMIP-BSA and AMI is based on selective interactions, the interaction between this molecule and the RANIP-BSA is ruled by nonspecific interactions.

Method optimization

All the tests performed to optimize the analysis system and method were done using a pool of blank human plasma samples spiked with NOR, DES, AMI, IMI, CLO and CLO-d3 at a concentration of 100.0 μg L^{-1}. The optimized variables were extraction time, dilution factor, sample pH, MPA and MPB compositions and mobile phase flow rate. The initial conditions were as follows: extraction time of 1.0 min, sample without pH adjustment, mobile phase flow rate of 0.1 mL min^{-1}, water and acetonitrile as MPA and MPB, respectively and plasma without dilution.

Initially, the extraction time was evaluated from 1.0 to 3.0 min, with increments of 0.5 min. The best result evaluated in terms of sensitivity and precision was obtained at 2.5min. Extraction times less than 2.5 min resulted in low sensitivity, probably because this time was not enough to prepare the sorbent that received the sample and eliminated the interferents as well as plasma proteins, resulting in a dirt accumulation in the ionization chamber. Fig. 5 shows that the time required to eliminate all the proteins from human plasma was approximately 2 min. Periods longer than 2.5 min resulted in low sensitivity due to the removal of the analytes by the conditioning solution.

Human plasma is a viscous solution that can difficult the filtration process and increase the system pressure. Thus, in order to solve this kind of problem, dilution factors were tested ranging from 1:1 to 1:5 (one part of sample and 1, 2, 3, 4 and 5 parts of diluent). 100 μL of diluted samples were injected into the system. Water was used as a solvent diluent. The best results, in terms of sensitivity and precision, were obtained when 1 part of plasma and 4 parts

of water were applied. This can be explained because when a lower proportion of water is used, the ionic suppression occurs in a stronger form, due to the presence of interferents in plasma. Using a higher proportion of water there was a decrease in absolute signal, probably because a smaller amount of analytes were extracted and the ionic suppression decrement was insufficient to compensate this smaller amount of analytes. So, the 1:4 dilution factor was chosen as best condition.

Then the sample's pH was evaluated ranging from 6.0 to 8.0. Lower or higher pH values were not tested because the albumin in RAMIP-BSA surface could be precipitated. An 0.01 mol L^{-1} ammonium formate buffer solution was used in different pHs to perform sample pH adjustment. The buffer pH adjustment was done using 0.01mol L^{-1} formic acid aqueous solution or 0.1mol L^{-1} ammonium hydroxide aqueous solution. No significant differences were observed in sensitivity and precision. Thus, samples without pH adjustment were selected as working conditions. That can be explained because water pH (around 7.0) contributes for electrostatic interaction between analytes and functional monomer, since in this pH value, MAA and TCA are ionized (MAA pKa approx. 4.0 and TCA pKa approx. 9.0) [30].

MPA nature is an important parameter to improve extraction, since MPA is responsible of preparing the sorbent to receive the analytes, carry them by analytical course, eliminating some interferents and reconditioning the sorbent for the next analysis. Therefore, water and 10.0 mmol L^{-1} ammonium formate buffer pH 6.0, 7.0 and 8.0 were tested. Lower or higher pH values were not evaluated due to the risk of precipitating the albumin on the RAMIP-BSA surface. No significant differences were observed between the evaluated MPA compositions, probably because water pH (around 7.0) and 10.0 mmol L^{-1} ammonium formate buffer pHs (6.0, 7.0 and 8.0) are capable of promoting TCA and MAA ionizations, favoring the electrostatic interaction between them. Thus, in order to maintain this method as simple and as easy to perform, water was chosen as the MPA composition.

The choice of MPB is an important parameter and should be optimized since MPB has the function of: eluting the analytes from the RAMIP-BSA, cleanibg the system after extraction procedure, eliminating the memory effect (carry over) and improving the analytes' ionization in an electrospray source. Pure acetonitrile and solutions of acetonitrile combined with 0.01% acetic acid aqueous solutions in proportions of 90:10, 80:20, 70:30, 60:40 and 50:50 (v:v) were tested as MPB. Using pure acetonitrile and 0.01% acetic acid aqueous solutions in proportions of 90:10 and 80:20, the peaks were more asymmetrical (tail appearing), there was an increase in the RSD% and the carry over effect was observed. When a higher proportion of 0.01% acetic acid aqueous solution was used (acetonitrile and 0.01% acetic acid aqueous solution in

proportions of 60:40 and 50:50) there was an increase in the system pressure and in RSD%. The best results were realized when acetonitrile and 0.01% acetic acid aqueous solution were used in the proportion of 70:30.

The mobile phase flow rates correlated with the peak symmetries, analysis time and analyte retention/elution. This parameter was evaluated from 0.1 to 0.6 mL min^{-1}. When the flow rate was higher than 0.3 mL min^{-1} there was a decrease in absolute analytical signal, probably because the TCA were not retained properly due to the short time of their contact with the RAMIP-BSA. Using flow rates below 0.3 mL min^{-1}, the system was not completely cleansed before V2 was switched, so a decrease in analytical signal was observed because there was an ionic suppression due to the accumulation of dirt in the electrospray source. The best result was obtained at a flow rate of 0.3 mL min^{-1}.

Validation assays and method application

For the validation assays, CLO-d3 was used as internal standard at the concentration of 100.0 µg L^{-1}. The developed method was linear from 15.0 to 500.0 µg L^{-1} for NOR, DES, AMI, IMI and CLO. The validation limits were established considering the concentration levels of TCA found in the blood of TCA users [7-8]. The method selectivity was proven by the absence of peaks in the blank samples at the same retention time of the analytes. The intra-assay and inter-assay precisions (obtained as a relative standard deviation, see Table 4) were lower than 20.0% for concentrations near the LOQ and 15.0% for the other concentrations, according to the validation guidelines of the United States Food and Drug Administration [31], as shown in Table 2. The LODs and LOQs were 5.0 µg L^{-1} and 15.0 µg L^{-1}, respectively, for all analyzed TCAs. Accuracy, stability and the matrix effect presented good results (table 2). Matrix effect was not observed since the relative standard deviations and relative errors, between the analyzed six different samples, were lower than 15.0%. The method was selective since there were not observed any picks in the blank sample at the same retention time of the analytes. The same RAMIP-BSA column was used during all the validation assays and non-significant differences were observed in the analytical signals after approximately 200 cycles, which prove the long service time of this kind of sorbent.

Three real samples, obtained from volunteers, who agreed to participate in this study, were analyzed by the proposed method. The volunteers related the use of AMI. AMI was found

at concentrations of 18.63; 75.98 and 22.59 μg L^{-1}, corresponding to samples 1, 2 and 3 respectively. NOR (active metabolite of AMI) was also found, however, for samples 1 and 2, the NOR concentrations were less than LOQ, whereas for sample 3 the concentration was 16.51 μg L^{-1}.

Fig. 6 shows the signal graphics obtained with the optimized system for a blank plasma sample, blank plasma sample fortified with 15.0 μg L^{-1} of AMI(A), CLO(B), DES(C), IMI(D) and NOR(E) and blank plasma sample fortified with 130.0 μg L^{-1} of AMI(A), CLO(B), DES(C), IMI(D) and NOR(E). CLO-d3 (internal standard - PI) was used at a concentration of 100.0 μg L^{-1}.

Conclusions

The developed on-line system for direct extraction and analysis of tricyclic antidepressant drugs in human plasma samples was linear for AMI, CLO, DES, IMI and NOR. Good figures of merit were attained, such as low LOQ, wide linear range, good precision and accuracy, high analytical frequency and sample preparation without previous protein elimination. Moreover, analytes' identification/quantification were performed without a chromatography separation, which makes the analyses cheaper. Moreover, there are other characteristics, as longer RAMIP-BSA column lifetime, good selectivity, use of small solvent volumes, and ease of operation, among others, that should be emphasized.

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Tables

Table 1: Analytes and their precursors, fragments and collision energies.

Analyte	Precursor (*m/z*)	Fragments (*m/z*)	Collision Energy (kV)
NOR	264.4	233.00	-15
		90.95	-20
		105.00	-20
DES	267.4	72.00	-15
		207.95	-30
		192.95	-35
AMI	278.4	90.95	-25
		232.95	-20
		105.05	-25
IMI	281.4	86.00	-20
		193.00	-40
		207.95	-25
CLO	315.0	86.00	-20
		242.05	-25
		226.90	-40
CLO-d3	318.1	89.10	-20
		227.00	-40
		241.90	-30

Table 2: Validation parameters of online extraction applied in the MS/MS method for the determination of NOR, DES, AMI, IMI and CLO in human plasma.

Validation parameters	NOR	DES	AMI	IMI	CLO
Linear range (μg L^{-1})	15.0 - 500.0	15.0 - 500.0	15.0 - 500.0	15.0 - 500.0	15.0 - 500.0
Linearity (r^2) ($\bar{x}$, n=3)	0.9991	0.9996	0.9977	0.9983	0.9991
Slope (a) ($\bar{x}$, n=3)	0.00037	0.00367	0.00133	0.00727	0.00843
Intercept (b)	0.00297	0.02551	0.01032	0.07167	0.06523
LOD μg L^{-1}	5.0	5.0	5.0	5.0	5.0
LOQ μg L^{-1}	15.0	15.0	15.0	15.0	15.0
Matrix effect % RSD (50 μg L^{-1}, n=6)	6.14	5.83	4.06	2.50	5.01
Matrix effect E% (50 μg L^{-1}, n=6)	9.88	14.62	13.37	14.86	-0.20
Intra-assay precision % RSD (n=6)	7.38[a] 9.07[b] 2.66[c]	19.13[a] 5.23[b] 2.84[c]	9.35[a] 4.00[b] 3.58[c]	11.33[a] 2.23[b] 4.68[c]	7.99[a] 1.34[b] 1.93[c]
Inter-assay precision % RSD (n=6, three days)	3.17[a] 3.91[b] 1.53[c]	7.41[a] 7.64[b] 5.88[c]	8.89[a] 2.51[b] 6.55[c]	19.77[a] 9.88[b] 3.62[c]	9.80[a] 2.34[b] 1.32[c]
Accuracy E% (n=6)	12.07[a] -13.14[b] -4.54[c]	7.79[a] 6.30[b] 1.91[c]	0.67[a] -3.83[b] 5.55[c]	19.51[a] 5.39[b] 3.80[c]	-19.15[a] 0.27[b] 0.85[c]
Accuracy E% (n=6, three days)	16.22[a] -9.04[b] -6.20[c]	2.47[a] -0.48[b] -1.58[c]	3.30[a] -5.52[b] -0.57[c]	4.65[a] -4.54[b] 0,78[c]	-8.84[a] -2.11[b] -0.02[c]
Stability[x] %RSD (n=6)	16.68[a] 6.48[b] 12.51[c]	12.48[a] 7.62[b] 9.07[c]	9.55[a] 10.93[b] 11.74[c]	18.05[a] 10.18[b] 11.83[c]	12.12[a] -7.98[b] -9.62[c]
Stability[y] %RSD (n=6)	12.07[a] -13.14[b] -4.54[c]	7.79[a] 6.29[b] 1.91[c]	0.67[a] -3.83[b] 5.55[c]	19.51[a] 5.39[b] 3.80[c]	-19.14[a] 0.27[b] 0.85[c]

[a]15.0 μg L^{-1}, [b]130.0 μg L^{-1}, [c]500.0 μg L^{-1}. [x]after 7 days in freezer and [y]after 3 cycles of freeze and thawing.

Figure captions

Fig.1. Chemical structures of some tricyclic antidepressants commonly used in depression treatment.

Fig.2. The used system (Fig. 2) consisted of a pump (P) delivering either water as mobile phase A (MPA) or a 0.1% acetic acid aqueous solution:acetonitrile at 30:70 (v/v): as mobile phase B (MPB), both at 0.3 mL min^{-1}. The pump was connected to an autosampler (AS) equipped with an injection valve (V1) with a 100.0 µL loop. The autosampler was connected to an electronic six-port switching valve (V2), and the RAMIP-BSA column (kept at 45.0°C) was positioned between V1 and V2. An UV detector (operating at 254 nm) was placed after extraction column to verify proteins elimination.

Fig.3. Transmission electron microscopy image of the: a) RAMIP; b) RANIP; c) RAMIP-BSA (t = 0); d) RANIP-BSA (t = 0); e) RAMIP-BSA (t = 31); f) RANIP-BSA (t = 31); h) RAMIP-BSA (subtract of Fig. 3e of Fig. 3c); d) RANIP-BSA (subtract of Fig. 3f of Fig. 3d). Where t is the time of incidence of the electron beam in the sample.

Fig.4. Retention graph of AMI by RAMIP-BSA. RANIP-BSA, RAMIP and RANIP at concentration of 500 (A), 1000 (B) and 2000 (C) µg L^{-1}.

Fig.5. Graphic of the time required to eliminate all proteins from human plasma.

Fig.6. Graphics obtained with the optimized system for a blank plasma sample, blank plasma sample fortified with 15.0 µg L^{-1} of AMI(A), CLO(B), DES(C), IMI(D) and NOR(E) and blank plasma sample fortified with 130.0 µg L^{-1} of AMI(A), CLO(B), DES(C), IMI(D) and NOR(E). CLO-d3 (internal standard - PI) was used at concentration of 100.0 µg L^{-1}.

Figure 1

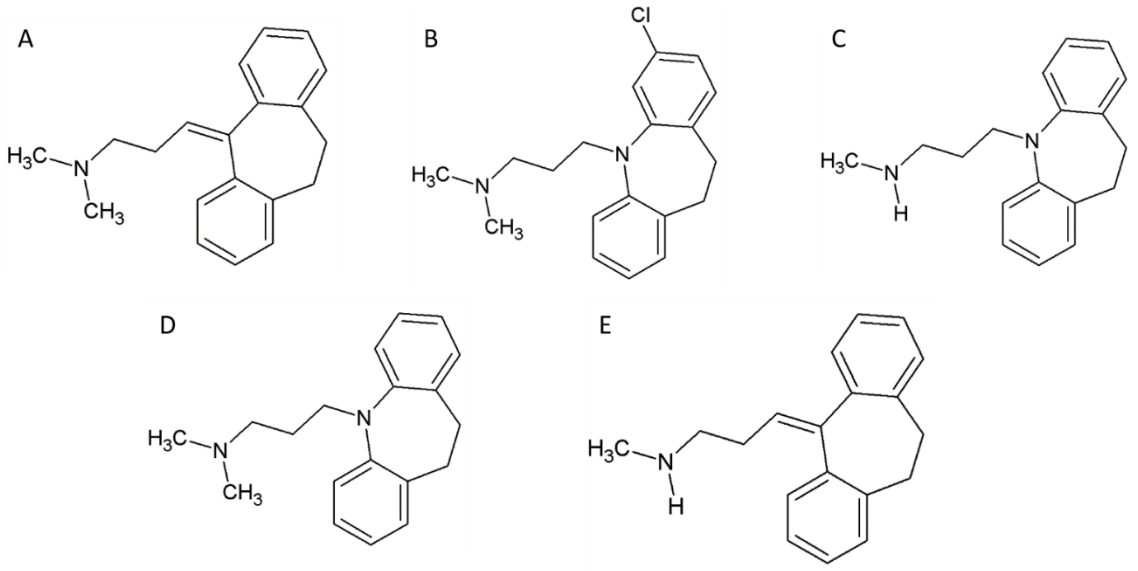


Figure 2

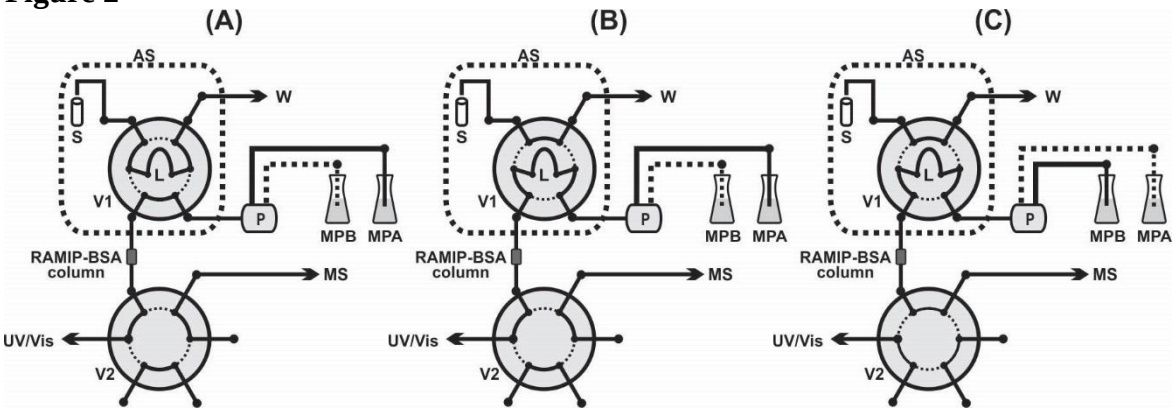


Figure 3

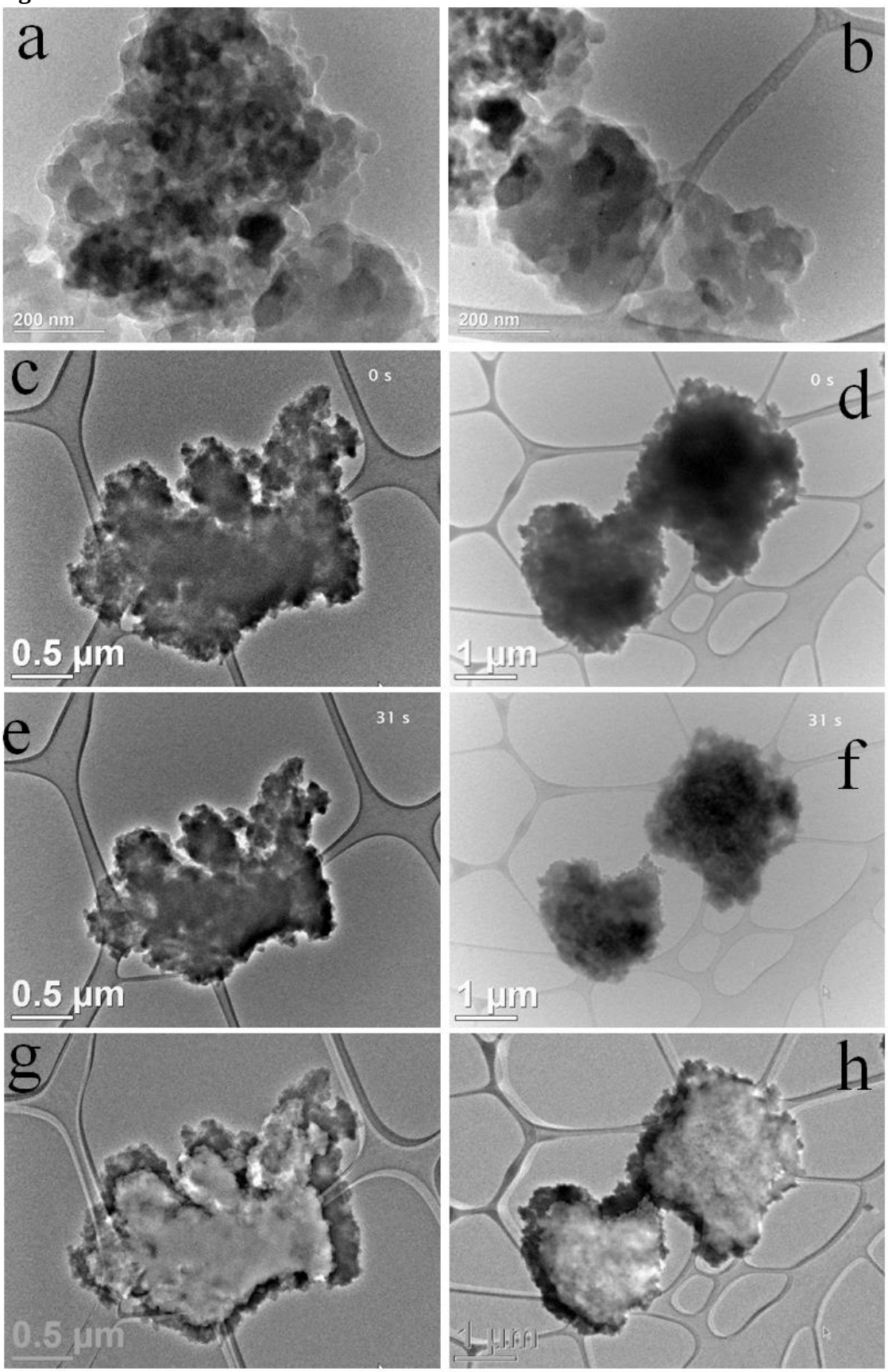


Figure 4

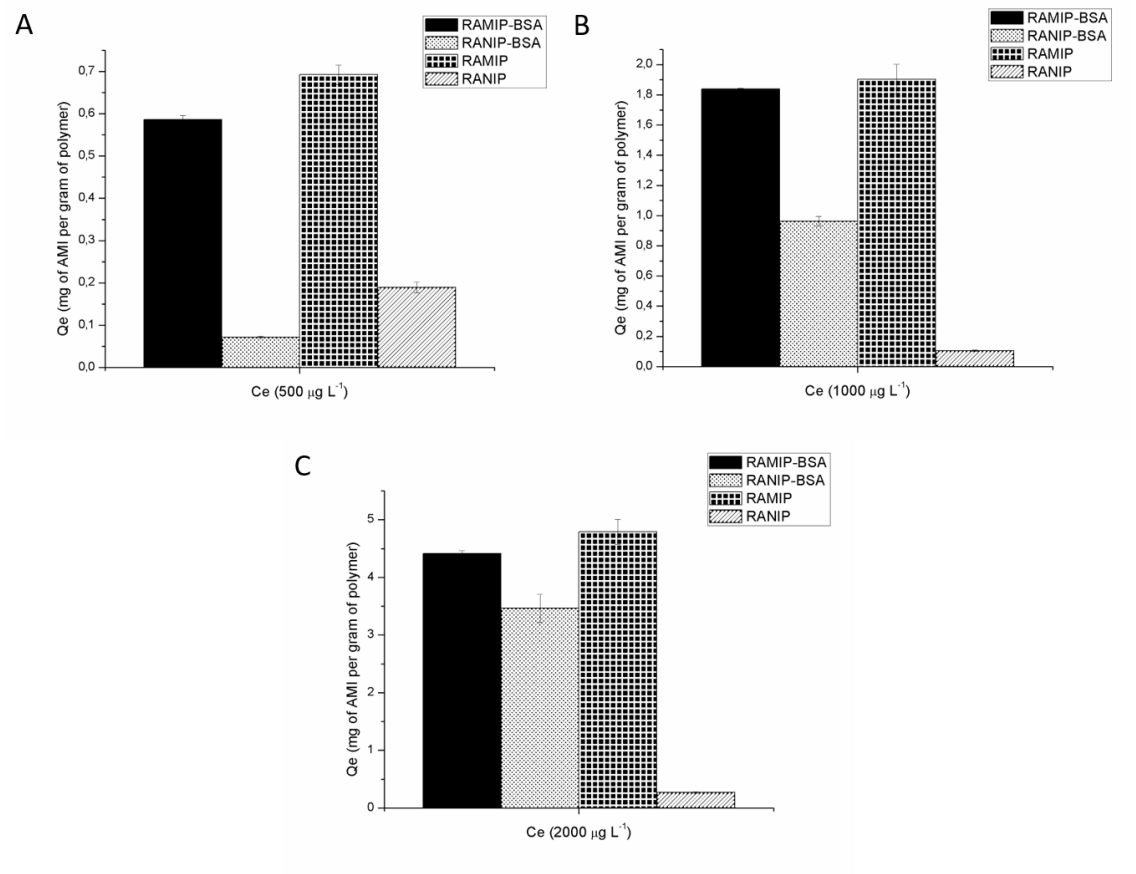

A
RAMIP-BSA
RANIP-BSA
RAMIP
RANIP
Qe (mg of AMI per gram of polymer)
Ce (500 μg L-1)
B
RAMIP-BSA
RANIP-BSA
RAMIP
RANIP
Qe (mg of AMI per gram of polymer)
Ce (1000 μg L-1)
C
RAMIP-BSA
RANIP-BSA
RAMIP
RANIP
Qe (mg of AMI per gram of polymer)
Ce (2000 μg L-1)

Figure 5

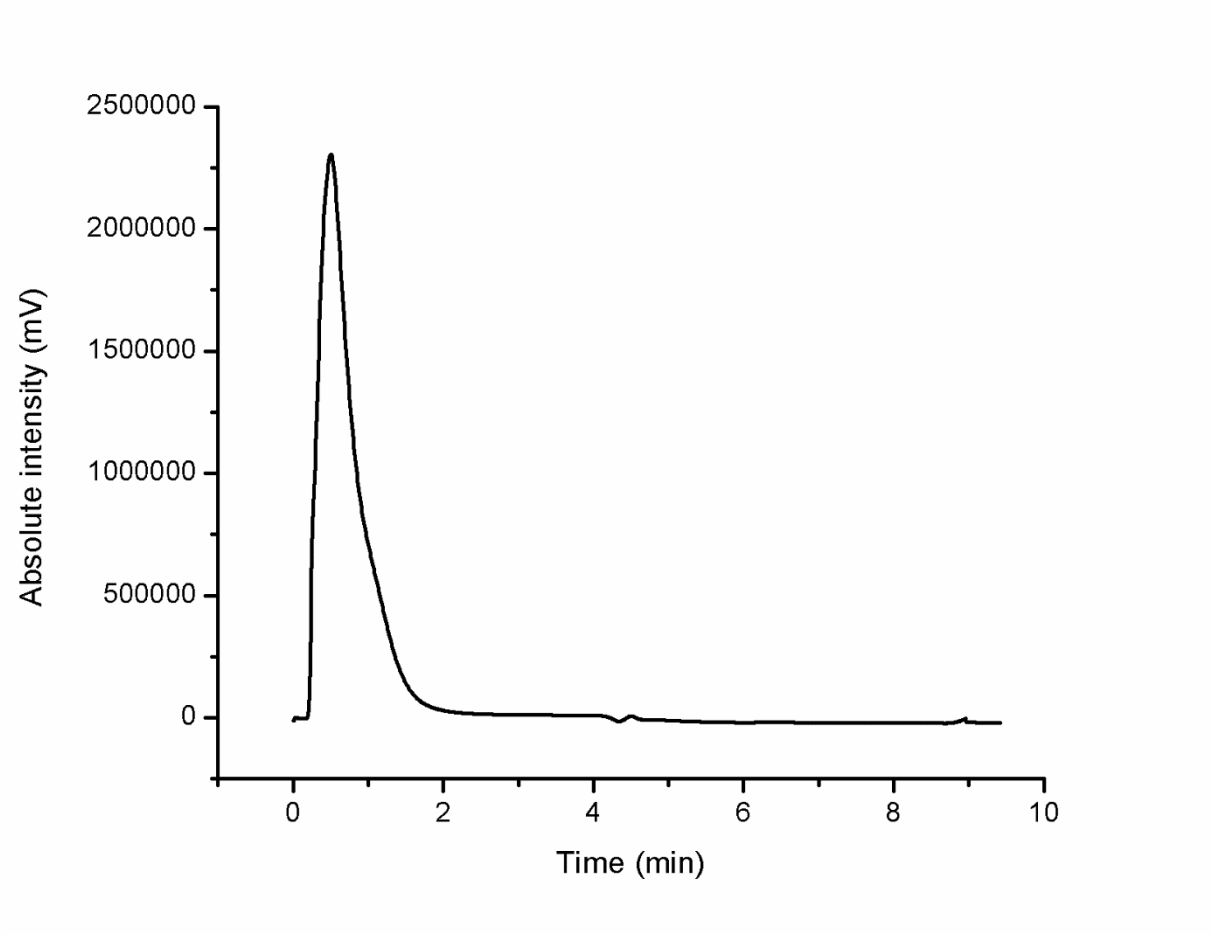
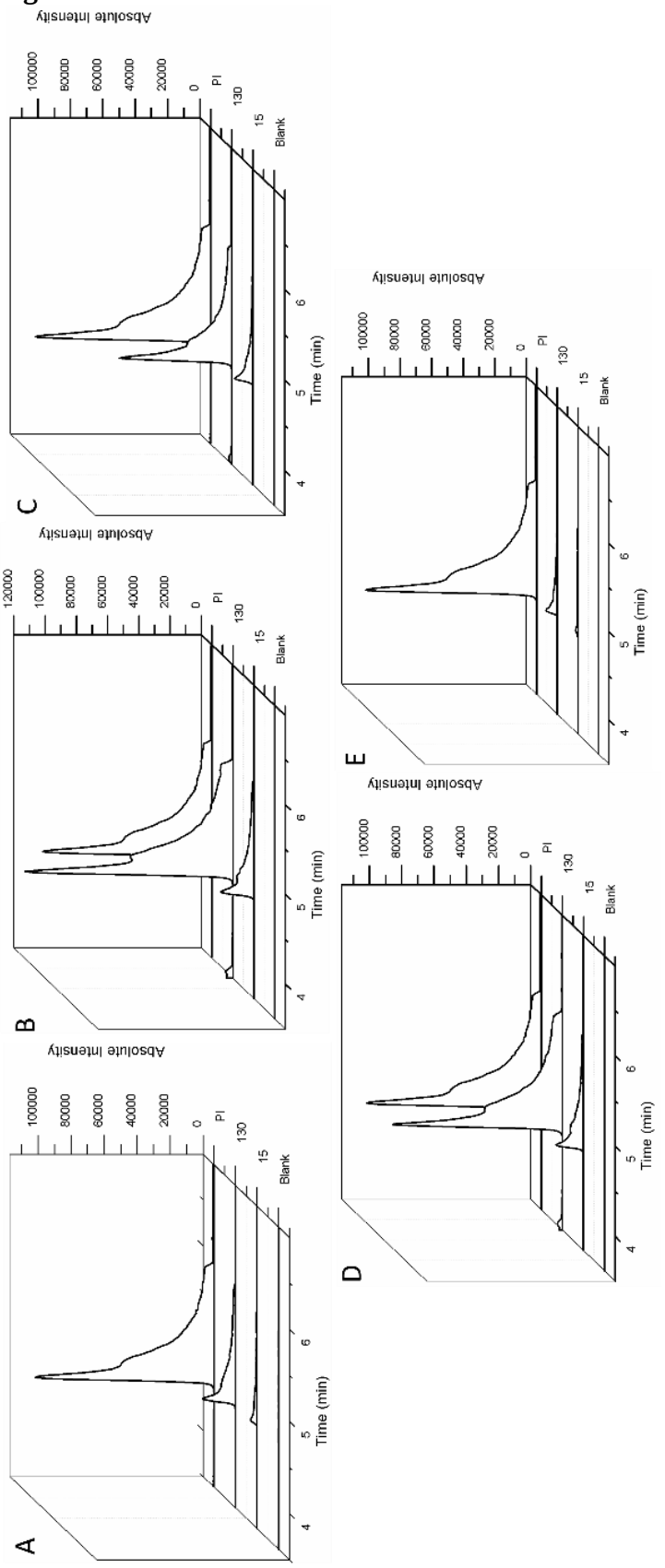

2500000
2000000
1500000
1000000
500000
0
Absolute intensity (mV)
0
2
4
6
8
10
Time (min)

Figure 6


A
B
C
D
E
Absolute Intensity
Time (min)
Blank
15
130
PI
0
20000
40000
60000
80000
100000
120000
4
5
6

7 CONSIDERAÇÕES FINAIS

Na primeira etapa deste trabalho, foi proposto um estudo para avaliar como as modificações na superfície dos MIP poderiam influenciar no comportamento desses materiais e que consequências ocasionariam. Foi possível observar que a maneira pela qual a síntese dos materiais é conduzida, influencia diretamente na forma e tamanho de partículas e que a adição de co-monômeros hidrofílicos, bem como o revestimento com BSA não altera signinificativamente a estrutura química dos mesmos. Os estudos de adsorsão mostraram que os polímeros alcançam o equilíbrio de adsorção em 60 min, que o revestimento com BSA não altera o perfil de adsorção dos mesmos, que é evidente a diferença de adsorção entre polímeros impressos e não impressos e que o modelo que melhor se adequa, a fim de descrever o perfil de adsorção é o modelo de Languimir. Foi possível também perceber que o RAMIP foi o polímero que melhor adsorveu os beta-bloqueadores, sendo esta a razão pela qual ele foi escolhido para o desenvolvimento de um método para análise desses fármacos.

O método desenvolvido para extração *online* e análise de beta-bloqueadores em amostras de urina humana foi linear para ATE, MET, LAB, PROP, NAD, PIN e OXP e apropriado para análise de doping. Boas figuras de mérito foram alcançadas, como o baixo LOD, boa precisão e exatidão, alta freqüência analítica e mínima manipulação das amostras. Além disso, outras características interessantes devem ser enfatizadas, tal como grande vida útil da coluna RAMIP, o uso de pequenos volumes de amostras e de solventes e facilidade de operação do sistema.

A metodologia desenvolvida para extração *online* e análise de antidepressivos tricíclicos em amostras de plasma humano foi linear para AMI, CLO, DES, IMI e NOR. O limite de quantificação foi adequado para o tipo de monitorização proposta, bem como precisão e exatidão. Vale ressaltar que o preparo das amostras ocorreu sem eliminação prévia de proteínas, graças à utilização do RAMIP-BSA, um material inovador capaz de reter seletivamente os analitos e ao mesmo tempo eliminar as macromoléculas presentes na matriz. Além disso, a identificação/quantificação dos antidepressivos foi realizada sem separação cromatográfica, o que torna as análise mais baratas.

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