Thiago Caon

DESENVOLVIMENTO DE ESTRATÉGIAS FARMACOTÉCNICAS PARA A MELHORIA DAS CARACTERÍSTICAS BIOFARMACÊUTICAS DO MESILATO DE SAQUINAVIR
&
AVALIAÇÃO DA PERMEABILIDADE BUCAL DO CLORIDRATO DE DONEPEZILA

Tese submetida ao Programa de Pós-Graduação em Farmácia da Universidade Federal de Santa Catarina como requisito parcial para a obtenção do título de Doutor em Farmácia

Orientadora: Profª. Drª. Cláudia Maria Oliveira Simões
Co-orientadora: Profª. Drª. Letícia Scherer Köester

Florianópolis
2014

Caon, Thiago

Desenvolvimento de estratégias farmacotécnicas para a melhoria das características biofarmacêuticas do mesilato de saquinavir & Avaliação da permeabilidade bucal do cloridrato de donepezila - Florianópolis, SC, 2014. 182 p.

Tese (doutorado) – Universidade Federal de Santa Catarina, Centro de Ciências da Saúde. Programa de Pós-Graduação em Farmácia.

Incluir referências
Farmácia. 2. Dispersões sólidas 3. Permeabilidade bucal. I. Simões, Cláudia Maria Oliveira. II. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Farmácia. III. Teste de doutorado.

"Desenvolvimento de estratégias farmacotécnicas para a melhoria das características biofarmacêuticas do mesilato de saquinavir & Avaliação da permeabilidade bucal do cloridrato de donepezila"

POR

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Florianópolis, **20 de fevereiro de 2014**.

AGRADECIMENTOS

À Deus, por ter me guiado durante toda esta jornada de quatro anos, com *insights* nos momentos de desespero e dúvida, mostrando que tudo é possível quando há esforço e determinação.

Ao meu pai, um herói que sempre lutou pela vida, esbanjando dignidade, caráter, honestidade e compreensão.

À minha mãe, exemplo de bondade, simplicidade, esforço, batalha, que nunca mediu esforços para ajudar os outros.

À minha orientadora Cláudia, por estes quase 10 anos de convívio, que sempre mostrou seu pulso firme, com muita maturidade para compartilhar seu vasto conhecimento, grande incentivadora da minha carreira científica.

À minha co-orientadora Letícia, exemplo de trabalho e competência, pela calorosa recepção na UFRGS e por nunca ter medido esforços para concretizar esta pesquisa.

À professora Silvia, grande conselheira e amante da ciência, pelos momentos prazerosos de discussão do trabalho e por todas as suas idéias que agregaram ainda mais valor a esta pesquisa.

I would like to express my sincere gratitude to my advisor Dr. Joseph Nicolazzo for the continuous support during my Ph.D intership in Australia, for his patience, understanding, motivation, enthusiasm, and immense knowledge.

À professora Simone pela colaboração nos ensaios de estabilidade e por contribuir para a efetivação do meu estágio de Doutorado Sandwich.

Ao professor Carlos por toda sua dedicação nos experimentos de caraterização de estado sólido e pela pronta disponibilidade em continuar com parcerias futuras.

Ao professor Gustavo, inesgotável fonte de idéias, pela colaboração, permintindo-me o acesso a técnica de LC-MS/MS.

À professora Bibiana Araújo, da UFRGS, pelo tratamento dos dados farmacocinéticos.

À Ariadne pelo auxílio nas análises de MEV, à Gislaine e ao Jadel por todo apoio nos experimentos *in vivo* com os cães, e a Melina pela colaboração nas análises de LC-MS/MS.

To Yijun for his collaboration in ITC experiments. 謝謝!

As Julianas, que não só facilitaram meu processo de adaptação no Laboratório de Desenvolvimento Galênico, da Faculdade de Farmácia da UFRGS, em Porto Alegre, mas também por gentilmente terem me oferecido acomodação.

Ao Ricardo, amigo que com sua maneira diferente e peculiar de pensar as coisas, ajudou-me a traçar os caminhos iniciais deste trabalho. Um amigo especial com quem galguei os degraus iniciais desta caminhada. Hoje, ausente do nosso convívio, estendo a ele alegria desta vitória.

To my great friends connoisseurs of the English language who have always been generous in their reviews of my papers.

A mis hermanas latinas Romi y Flor por guiarme a la llegada a Australia y por el apoyo en la preparación de los “Graphical Abstract” en muchos artículos. Cheers!!!

To Patrick and Liang for fun trips to the abattoir in Laverton at 6 am.

À FAPESC/CNPq/MS/SES-SC pelo financiamento da maior parte deste estudo.

À CAPES pelo provimento da bolsa de Doutorado, via Rede de Nanobiotecnologia, e pela concessão da bolsa de estágio na Austrália.

Aos meus amigos do Laboratório de Virologia Aplicada, não apenas pelos nossos momentos prazerosos regados a cafés, mas também àqueles em que discutíamos resultados e todos se empenhavam em achar soluções.

Agradeço também as outras pessoas que contribuíram, de alguma forma, para a realização deste trabalho e que não foram aqui mencionadas.

"Do passado um baú de memórias
Que de brincadeiras faziam história,
Ora puxavam um carrinho de lata e barbante
Ora era pipa de gravetos e sua rabiola

Do passado um baú de memórias
Que esse homem traz história
Outrora menino subia num galho
O fruto engolia lançando bem longe os caroços
Agora tanto mais longe é sua trajetória

Do passado um baú de memórias
Que no presente de saudade o doutor chora
Outrora lata e gravetos foram preciosos brinquedos
Tanto mais são no agora"

(Andréa Granada)

RESUMO

Para fármacos com solubilidade aquosa limitada, estratégias farmacotécnicas visando a melhoria das propriedades biofarmacêuticas pode ser mais interessante do que a investigação de rotas alternativas de administração, o que não acontece para muitos dos fármacos que apresentam alta solubilidade aquosa. Neste estudo, prepararam-se dispersões sólidas de mesilato de saquinavir (MS) com o objetivo de aumentar a sua biodisponibilidade oral e também investigou-se a permeabilidade bucal deste fármaco bem como do cloridrato de donepezila (DPZ). Para o preparo das dispersões sólidas, utilizaram-se dois principais carreadores, o PEG 4000 e o Gelucire^{TM} 44/14 e, uma vez que observaram-se problemas de instabilidade físico-química, o PVP K30 foi também incluído na formulação (um agente bem conhecido por evitar/retardar a cristalização de fármacos). A formulação com PEG 4000 levou a uma precipitação de fármaco após a dissolução tendo em vista sua baixa capacidade de solubilização, aumentou ainda mais o efluxo mediado pela gp-P e, desta forma, baixa biodisponibilidade oral foi observada. Por outro lado, o sistema com Gelucire^{TM} proporcionou menor dissolução em meio gástrico, porém, foi capaz de inibir a glicoproteína-P, demonstrando maior capacidade de solubilização do fármaco quando da sua liberação da formulação, o que consequentemente explicaria sua maior biodisponibilidade oral, similar àquela da formulação comercial (Svir^{TM}). Formulações preparadas com Gelucire^{TM} mostraram ser promissoras para fins comerciais em vista da similar taxa de absorção do fármaco em relação aquela proporcionada pelo Svir^{TM}. Após a administração oral de MS (200 mg) na forma de dispersões sólidas contendo Gelucire^{TM} ou Svir^{TM}, os perfis farmacocinéticos do MS nas duas formulações foram similares e melhor descritos a partir de um modelo de dois compartimentos e com tempo latência. A farmacocinética do MS após administração intravenosa (1 mg/Kg) foi melhor descrita por um modelo de três compartimentos. O MS não foi absorvido pela mucosa bucal devido o seu alto peso molecular, diferentemente do DPZ, que apresentou alta absorção bucal (± 20% da quantidade inicialmente administrada). Os reforçadores químicos de penetração transdérmica selecionados para o ensaio com o DPZ reduziram o seu coeficiente de permeabilidade bucal, o que é desejável tendo em vista a possibilidade de se controlar a liberação do DPZ, prolongando-a, o que reduziria os intervalos entre as dosagens (extremamente relevante para pacientes com Doença de Alzheimer uma vez que são mais suscetíveis a esquecerem da medicação).

Palavras-chaves: dispersões sólidas, dissolução, biodisponibilidade, permeabilidade bucal, mesilato de saquinavir, cloridrato de donepezila.

ABSTRACT

Pharmacotechnical strategies aiming to improve the biopharmaceutical properties can be more interesting than the investigation of alternative administration routes for drugs presenting a limited aqueous solubility, unlike some highly water-soluble drugs. In this study, solid dispersions of saquinavir mesylate (SQVM) were prepared aiming to increase oral bioavailability and it was also investigated buccal permeability of this drug as well as donepezil hydrochloride (DPZ). Two major carriers (PEG 4000 and Gelucire® 44/14) were used to prepare solid dispersions, and given that chemical and physical instability problems had been shown, PVP K30 (a well-known polymer to prevent drug crystallization) was also included in the formulation. PEG 4000-based formulation led to precipitation of the drug its upon dissolution in view of its reduced capacity to solubilize the SQVM. It was also observed a significant efflux of SQVM mediated by P-gp which may account for the poor bioavailability of SQVM. On the other hand, Gelucire-based system provided lower drug dissolution in gastric medium, however, it was able to inhibit P-glycoprotein, showed higher solubilizing capacity of the SQVM upon release, which could explain the greater oral bioavailability of SQVM, similar to that of the commercial formulation (Svir®). Gelucire-based formulation is more promising for commercial applications given the similarity of SQVM absorption rate to that from the Svir®. Furthermore, a similar disposition of SQVM was obtained after oral administration of the lipid delivery systems (Gelucire®-based formulation vs Svir®, 200 mg/dose), which were best described by a two-compartment model with lag time. The pharmacokinetic of SQVM after intravenous administration (1 mg/Kg) were best described by a three-compartment model. SQVM was not absorbed through the buccal mucosa due to its high molecular weight, unlike the DPZ, which showed high buccal absorption (close to 20 % of the initial drug amount). Transdermal penetration enhancers selected for testing with DPZ reduced its buccal permeability coefficient, which is desirable in view of the possibility to control the release of DPZ, extending it, reducing the intervals between doses (this characteristic is extremely important for patients with Alzheimer's disease because they are more susceptible of forgetting to take medication).

Keywords: solid dispersions, dissolution, bioavailability, buccal permeability, saquinavir mesylate, donepezil hydrochloride.

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APRESENTAÇÃO GERAL

Este trabalho teve início na Universidade Federal do Rio Grande do Sul, onde foram preparadas diferentes dispersões sólidas (DS) de mesilato de saquinavir (MS) e realizados os ensaios de dissolução, sob a supervisão da Profa. Dra. Letícia Scherer Köester.

Sequencialmente, na Universidade Federal de Santa Catarina, realizaram-se ensaios de caracterização físico-química com as formulações (espectroscopia Raman e na região do infravermelho, difração de raios X, microscopia óptica e eletrônica de varredura), bem como os ensaios de estabilidade acelerada, ambos realizados em colaboração com outros grupos de pesquisa da Instituição (Laboratório de Difração de Raios X, Departamento de Física; CFM & Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, CCS), o que foi extremamente importante para o meu processo de formação multidisciplinar.

Após a seleção/otimização das formulações mais promissoras considerando-se o incremento da dissolução *in vitro* e sua estabilidade físico-química, foram realizados os ensaios de transporte intestinal *in vitro* a fim de avaliar o efeito dos excipientes na absorção do MS e, sequencialmente, os ensaios de biodisponibilidade *in vivo*, nos quais as formulações desenvolvidas foram comparadas a preparação comercial do MS, denominada Svir^{TM}. Considerando-se que a quantificação de um fármaco no plasma, em muitos casos, exige metodologias analíticas altamente sensíveis, buscou-se parceria com o Laboratório de Eletroforese Capilar (Departamento de Química, UFSC) e, desta forma, as amostras foram quantificadas por Cromatografia Líquida de Alta Eficiência (CLAE) acoplada a Espectrometria de Massas. O perfil farmacocinético da formulação desenvolvida mais promissora foi também analisado por modelos compartimentais, comparativamente àquele da formulação comercial Svir^{TM}.

Todos os resultados das etapas experimentais acima descritas estão apresentados agrupados no capítulo I desta tese. Uma vez que o desenvolvimento de DS para a melhoria das propriedades biofarmacêuticas de fármacos poucos solúveis ainda é uma estratégia pouco explorada no Brasil, primeiramente foi apresentada uma revisão bibliográfica do tema em questão. Com relação a parte experimental, a primeira etapa envolveu o preparo, caracterização físico-química, a determinação dos perfil de liberação *in vitro* e aspectos da estabilidade das diferentes DS preparadas com o MS. Estes resultados foram organizados em um artigo, já publicado, e as formulações com maior estabilidade físico-química e perfis de liberação mais adequados foram selecionadas para os testes subseqüentes. A segunda etapa incluiu ensaios de dissolução em pH intestinal com as formulações selecionadas, avaliação do efeito dos excipientes sobre a

permeabilidade intestinal do MS através do modelo de células Caco-2 e, finalmente, foram realizados os ensaios de biodisponibilidade em cães, também com a formulação comercial Svir^{TM}, a qual foi utilizada como controle e para fins de comparação. Uma vez que poucos trabalhos abordando a farmacocinética de sistemas lipídicos auto-emulsionáveis e do MS podem ser encontrados na literatura, realizou-se uma análise compartimental e não-compartimental comparativa entre estes sistemas, sendo os resultados também agrupados na forma de publicação (último artigo do Capítulo I).

A etapa final desta tese, identificada como capítulo II, incluiu a avaliação da mucosa bucal como rota para administração do MS, a qual foi realizada na *Monash University*, sob supervisão do Dr. Joseph Nicolazzo. Tendo em vista os resultados negativos obtidos nesta etapa, os quais são justificados pelo alto peso molecular do MS, um manuscrito de revisão foi preparado, com o objetivo de discutir as principais estratégias que poderiam ser utilizadas para aumentar absorção bucal de macromoléculas, tais como o fármaco estudado. Paralelamente, iniciou-se o estudo da administração bucal do cloridrato de donepezila, um fármaco utilizado no tratamento da Doença de Alzheimer tendo em vista suas limitações farmacocinéticas (atinge concentrações plasmáticas muito rapidamente e podem ser observadas flutuações plasmáticas) quando administrado por via oral, já que a via bucal ainda não tinha sido explorada.

CAPÍTULO I

DESENVOLVIMENTO DE ESTRATÉGIAS FARMACOTÉCNICAS PARA A MELHORIA DAS CARACTERÍSTICAS BIOFARMACÊUTICAS DO MESILATO DE SAQUINAVIR

Os experimentos desta etapa foram realizados na Universidade Federal de Santa Catarina e Universidade Federal do Rio Grande do Sul sob orientação das professoras Dr. Cláudia Maria Oliveira Simões e Letícia Scherer Koester

Fontes de financiamento:
Projeto: FAPESC/CNPq/MS/SES-SC
(número do projeto: 15.949/2009-2)
Recursos humanos: CAPES/MEC Nanobiotecnologia
(número do projeto: 759/2009)

"O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo, fará coisas admiráveis."
(José de Alencar)

1. INTRODUÇÃO

Atualmente, o percentual de compostos ativos que apresenta relevante valor terapêutico e baixa solubilidade aquosa é estimado em torno de 25-40% do total de fármacos desenvolvidos. Essa baixa solubilidade pode reduzir a absorção gastrointestinal, limitando o emprego terapêutico de tais compostos (BIKIARIS, 2011). Além disto, fármacos com baixa solubilidade podem depositar-se em sítios específicos, formando agregados que aumentam seus efeitos tóxicos (LIPINSKI, 2002). Neste sentido, diferentes abordagens têm sido propostas para a resolução deste problema, tais como redução do tamanho das partículas para aumentar a área de superfície; solubilização em sistemas surfactantes; formação de complexos solúveis em água; utilização de pró-fármacos e outros derivados de fármacos, bem como redução da cristalinidade do fármaco, e preparo de dispersões amorfas através da constituição de dispersões sólidas (BIKIARIS, 2011).

A formação de sais não é recomendada para compostos neutros e apresenta limitações para compostos fracamente ácidos ou básicos. Além disto, a formação de agregados ou a rápida interconversão nas suas formas ácida ou básica pode não resultar nos incrementos esperados de dissolução. A solubilização de fármacos em solventes orgânicos ou em meio aquoso pelo uso de surfactantes e/ou co-solventes, pode ser indesejável dos pontos de vista de aceitabilidade pelo paciente e de futura comercialização. A estratégia de redução do tamanho de partícula apresenta limitações técnicas quanto ao controle da granulometria. O uso de pós muito finos em formulações comerciais apresenta dificuldades de manipulação e baixa molhabilidade. Neste sentido, com o objetivo de contornar as limitações acima citadas, Sekiguchi e Obi propuseram, em 1961, um novo sistema a fim de reforçar a biodisponibilidade de fármacos com baixa solubilidade aquosa, que mais tarde foi denominado de *dispersões sólidas* (DS) (SERAJUDDIN, 1999) e que se refere à mistura de, pelo menos, dois componentes sólidos, geralmente uma matriz hidrofílica e um fármaco hidrofóbico. A matriz pode ser tanto cristalina quanto amorfa, e o fármaco pode encontrar-se molecularmente disperso como partículas amorfas ou cristalinas (CHIOU; RIEGELMAN, 1969, SHARMA, JAIN, 2011).

Os processos de produção das DS podem ser categorizados em dois grupos: métodos de fusão e de evaporação do solvente. O método de fusão consiste na fusão do fármaco dentro de um carreador, seguido de resfriamento ou pulverização do produto obtido. Diferentes taxas de resfriamento podem ser utilizadas para solidificar a mistura (agitação em banho de gelo, imersão em nitrogênio líquido, tratamento com gelo seco, solidificação à temperatura ambiente em dessecador,

entre outros). Neste processo, a mobilidade molecular do carreador deve ser suficientemente alta para permitir a incorporação do fármaco em sua matriz. Uma adaptação comum na fase de fusão consiste em ressuspender o fármaco em um carreador previamente fundido, ao invés de fundir simultaneamente fármaco e carreador. A degradação de fármacos instáveis a altas temperaturas pode ser evitada com esta adaptação. Problemas de miscibilidade entre fármaco e carreador também poderão ocorrer devido a uma alta viscosidade do carreador polimérico, quando fundido. Nestes casos, adaptações do método incluindo extrusão a quente ou aglomeração por fusão poderão ser alternativamente utilizados (VASCONCELOS; SARMENTO; COSTA, 2007).

Tendo em vista que esta estratégia farmacotécnica ainda não foi utilizada para o MS, experimentos foram realizados neste sentido. Para tal, o método de fusão foi selecionado considerando sua simplicidade técnica e sua aplicabilidade em triagens de formulações. Além disto, considerou-se o efeito de algumas variáveis que interferem no processo de produção (temperaturas de resfriamento/solidificação, tempo de armazenagem, monitoramento da relação amorfo-cristalino, entre outros aspectos).

2. OBJETIVOS

2.1 OBJETIVO GERAL

- Desenvolver e caracterizar dispersões sólidas do mesilato de saquinavir como estratégia para a melhoria das características biofarmacêuticas desse fármaco, e realizar a avaliação pré-clínica, *in vitro* e *in vivo*, da absorção intestinal destes sistemas.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar as características físico-químicas do MS, através de difração de raios X (DRX), espectroscopia Raman, e microscopia eletrônica de varredura (MEV);
- Avaliar a compatibilidade, solubilidade aparente e estabilidade do MS, na presença de diferentes adjuvantes;
- Desenvolver e validar um método analítico por CLAE para doseamento do MS;
- Realizar estudos de formulação com vistas à obtenção de cápsulas duras contendo MS veiculado em carreadores de natureza hidrofílica (PEG 4000) e anfifílica (Gelucire^{TM} 44/14);
- Selecionar a formulação com as propriedades físico-químicas e de dissolução *in vitro* mais adequadas para os testes de estabilidade acelerada e biológicos;
- Avaliar o efeito das temperaturas de resfriamento e de armazenagem (25^{o}C vs. -20^{o}C) e do tempo de armazenagem (1 dia vs. 7 dias) sobre a estabilidade das DS obtidas;
- Avaliar a influência da umidade sobre a estabilidade do Gelucire^{TM} 44/14;
- Realizar estudos de estabilidade acelerada, sob diferentes condições de temperatura e umidade, com a DS mais adequada, previamente selecionada;
- Realizar estudos do transporte intestinal *in vitro* com a DS mais estável de cada grupo de carreador utilizado (Gelucire^{TM} 44/14 e PEG 4000);
- Avaliar o efeito dos diferentes excipientes sobre o mecanismo de efluxo do MS mediado pela glicoproteína-P;
- Realizar a análise comparativa da biodisponibilidade *in vivo* da DS mais estável de cada grupo de carreador utilizado (Gelucire^{TM} 44/14 e PEG 4000), e da formulação comercial (Svir^{TM}), produzida pelo Laboratório Cristália, em cães Beagle;
- Caracterizar a farmacocinética da formulação selecionada (aquela preparada com Gelucire^{TM} 44/14) e do Svir® por modelos compartimentais.

3. REVISÃO BIBLIOGRÁFICA

3.1. CONTEXTUALIZAÇÃO/PROBLEMÁTICA

O número de indivíduos infectados pelo HIV no mundo continua em crescimento, atingindo a marca dos 34 milhões de infectados em 2010. Embora a epidemia tenha estabilizado em determinadas regiões, altas taxas de incidência ainda são observadas na Europa Oriental e Ásia Central. A África Sub-Sahariana continua sendo a região mais afetada, fato que pode estar relacionado com a escassez de recursos para tratamento e/ou demanda excessiva por cuidados médicos, sobrecarregando os sistemas de saúde, que já se encontram fragilizados (UNAIDS, 2010). No Brasil, de 1980 a junho de 2011, foram notificados cerca de 608 mil casos de AIDS, com aproximadamente 241 mil óbitos até 2010, sendo a região Sudeste a mais afetada (BRASIL, 2012).

Neste sentido, o Ministério da Saúde vem ampliando, desde 1996, a distribuição gratuita de medicamentos antirretrovirais, através do Programa Nacional DST/AIDS (Programa integrador de medidas de prevenção e promoção da saúde, apontado como modelo internacional), o que tem elevado a expectativa e a qualidade de vida dos pacientes. Adicionalmente, este Programa reduziu as taxas de hospitalização, com uma economia estimada em mais de US$ 2 bilhões até 2006 (OKIE, 2006), bem como a taxa de incidência da doença no país - de 22,2 casos por 100 mil habitantes em 2002 para 17,9 casos por 100 mil habitantes em 2006 (BRASIL, 2012).

Ainda que tal Programa tenha ampliado a cobertura e a sobrevida dos pacientes, observou-se elevação expressiva dos gastos com o mesmo, o que poderia comprometer a continuidade desta política de acesso a terapia antirretroviral (MEINERS, 2008). Entre 1997 e 2007, o número de pacientes submetidos a este tratamento cresceu mais de cinco vezes (GRECO; SIMÃO, 2007). No curto prazo, algumas medidas (tal como a análise custo-efetividade de medicamentos que poderiam ser incluídos nas terapias combinadas) deverão ser consideradas para garantir a permanência deste Programa. Outras intervenções incluem a dispensação racional destes medicamentos, o diagnóstico precoce e a prevenção da patologia, evitando a disseminação do HIV. Estudos farmacotécnicos para fins de melhoria dos aspectos biofarmacêuticos de formulações contendo fármacos antirretrovirais, tal como o aumento da sua biodisponibilidade também devem ser realizados, tendo em vista as vantagens proporcionadas.

Os problemas de adesão e resistência ao tratamento tornam esta problemática do custo da terapia antirretroviral ainda mais crítica, pois geralmente requerem a inclusão de fármacos patenteados (MEINERS, 2008). Em 2000, os antirretrovirais produzidos no Brasil

ocupavam uma posição equivalente aos importados protegidos por direito de propriedade; porém, esta relação tem sido alterada nos últimos anos com a inclusão de novos medicamentos importados (GRECO; SIMÃO, 2007; REIS, 2011). Um estudo que considerou dados de 188 transações realizadas pelo Ministério da Saúde, através do Programa Nacional DST/AIDS, entre 1998 e 2002, revelou que o preço médio dos medicamentos sem proteção patentária é cerca de 73% menor do que os patenteados. Observou-se, ainda, que a participação progressiva de medicamentos patenteados provocou uma ruptura no comportamento de queda do investimento médio anual por paciente para a compra de antirretrovirais, observado até 2004. O gasto médio anual por paciente caiu em mais de US$ 6 mil em 1997 para cerca de US$ 1,3 mil em 2004, mas logo voltou a subir, atingindo quase US$ 2,2 mil em 2005 (MEINERS, 2005). Atualmente, o Ministério da Saúde utiliza 72% do seu orçamento para a compra de antirretrovirais (US$ 400 milhões) com medicamentos importados, sendo que, desse montante, 60% são utilizados para a aquisição de cinco destes medicamentos – lopinavir, tenofovir, darunavir, raltegravir, atazanavir (GRECO, 2011). Desta forma, é visível a relação entre o aumento da proporção de medicamentos patenteados e o custo médio do tratamento por paciente.

Entre 1987 e 1994, encontravam-se disponíveis para uso clínico apenas fármacos de nucleosídeos inibidores da transcriptase reversa do HIV. Entre 1994 e 1995, os conhecimentos adquiridos sobre a patologia e a possibilidade de se quantificar a carga viral promoveram mudanças significativas, que foram acompanhadas pelo desenvolvimento dos primeiros fármacos inibidores da protease do HIV. Recentemente, com o surgimento dos fármacos inibidores da fusão viral e da integrasse do HIV, ampliaram-se as perspectivas para o tratamento dos pacientes HIV+ e/ou com AIDS (DE CLERCQ, 2009; RACHID; SCHECHTER, 2008).

Considerando o mecanismo de ação antiviral, estes compostos podem ser agrupados em seis classes: (1) inibidores da transcriptase reversa análogos de nucleosídeos, (2) inibidores da transcriptase reversa não nucleosídeos, (3) inibidores da transcriptase reversa análogos de nucleotídeos, (4) inibidores da protease, (5) inibidores da integrase e (6) inibidores da entrada dos vírus nas células alvo (inibidores da fusão e inibidores de co-receptores) (DE CLERCQ, 2009; BRODER, 2010). É recomendada a administração concomitante de pelo menos três desses fármacos, o que confere vantagens tais como sinergismo de ação; possibilidade de redução das doses, com concomitante redução dos efeitos adversos; redução da probabilidade de desenvolvimento de resistência viral (RABOUD et al., 2002; PANEL DE EXPERTOS DE GESIDA, 2013).

Atualmente, o Brasil dispõe de vinte e um fármacos antirretrovirais, sendo sete inibidores da transcriptase reversa análogos de nucleosídeos, três inibidores da transcriptase reversa não-análogo de nucleosídeos, nove inibidores da protease, um inibidor da integrase e um inibidor da entrada do HIV nas células. Deste total, apenas dez são produzidos no Brasil: didanosina (1998), lamivudina (1999), zidovudina (1993), estavudina (1997), tenofovir (2011), nevirapina (2001), efavirenz (1999), indinavir (1997), ritonavir (1999) e mesilato de saquinavir (1999) (GRECO; SIMÃO, 2011).

Ainda que existam diferentes opções terapêuticas, fatores como intolerância e/ou má adesão ao tratamento, uso prévio de esquemas inadequados e, mais raramente, resistência primária, têm contribuído para o aparecimento de vírus resistentes, exigindo novos esquemas antirretrovirais, os denominados “esquemas de resgate”, tendo o saquinavir como um dos seus representantes. A eficácia do tratamento com os antirretrovirais está bem estabelecida, levando-se em consideração os benefícios clínicos aos pacientes e a redução dos índices de morbidade e mortalidade (DE CLERCQ, 2009; MEINERS, 2008).

Como citado anteriormente, os inibidores da protease do HIV (IP) estão entre as opções terapêuticas para o tratamento de pacientes HIV+ ou com AIDS, particularmente para evitar problemas associados à resistência viral. Até o momento, há 10 fármacos desta classe licenciados para uso (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir e darunavir). Com exceção do tipranavir (que tem uma estrutura química baseada no esqueleto de uma cumarina), a síntese de todos os outros se baseou no princípio peptidomimético, ou seja, eles têm um arcabouço hidroxietileno, que mimetiza a ligação peptídica (poliproteínas *gag* e *gag-pol*), que é clivada pela protease do HIV. Portanto, inibem a ação da protease do HIV (processo pós-traducional), interferindo no processamento proteolítico das proteínas precursoras virais (DE CLERCQ, 2009).

Dentre estes fármacos inibidores da protease, o saquinavir foi selecionado para este estudo por ser um fármaco constante na Relação Nacional de Medicamentos (BRASIL, 2008b), por fazer parte do esquema terapêutico do Programa Nacional DST/AIDS (BRASIL, 2008), e por apresentar inconveniências relacionadas à sua biodisponibilidade e absorção intestinal.

3.2. CARACTERÍSTICAS DO FÁRMACO ESTUDADO

O mesilato de saquinavir (Fig. 1) caracteriza-se como um pó cristalino branco, com massa molecular de 766,96 g/mol. Este fármaco

é altamente hidrofóbico, com baixa solubilidade aquosa (2,2 mg/mL em água), 0,08 mg/mL em fluido gástrico simulado e praticamente insolúvel em fluido intestinal simulado a 25°C. Assim, pode-se afirmar que o MS apresenta uma solubilidade pH-dependente, limitada solubilidade em fluido gástrico e praticamente insolúvel em fluido intestinal (ALBANO; INFELD, 2009).

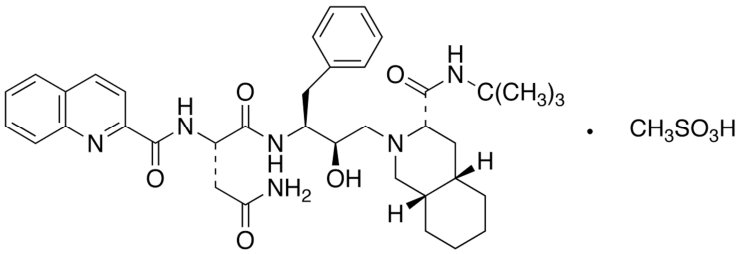

Figura 1. Estrutura química do mesilato de saquinavir.
Fonte: ALBANO, INFELD (2009)

Este inibidor de protease, aprovado em 1995, foi o primeiro desta classe, sendo inicialmente veiculado, tanto na forma de cápsulas duras quanto de comprimidos revestidos (Invirase™, ROCHE). Ambas formas farmacêuticas apresentam uma baixa biodisponibilidade oral, o que pode estar relacionada tanto a problemas de absorção quanto a um extensivo metabolismo de primeira passagem. Sua biodisponibilidade média foi de 4% (CV de 73%, faixa: 1-9%) em voluntários sadios (n=8), que receberam uma única dose de 600 mg (3 cápsulas com 200 mg cada) de saquinavir. Na presença de dieta rica em gorduras, a biodisponibilidade aumentou em aproximadamente duas vezes e, desta forma, é recomendável que o medicamento seja administrado após as refeições (ALBANO; INFELD, 2009). Mais tarde, esta mesma companhia farmacêutica realizou estudos para aumentar a biodisponibilidade oral do saquinavir e, em 1997, um novo produto foi lançado no mercado, o Fortovase™. Esse medicamento, que incluía uma combinação de mono- e diacilgliceróis de cadeia média veiculados em cápsulas moles, aumentou a biodisponibilidade do fármaco em aproximadamente 3X (ALSENZ et al., 1998; STRICKLEY, 2004). No entanto, em fevereiro de 2006, o laboratório fabricante do Fortovase™ anunciou a sua retirada do mercado devido a uma redução da demanda e não por questões relacionadas à sua eficácia e segurança. O Invirase™ continuou a ser utilizado, porém, em combinação com outro inibidor de protease, o ritonavir (que atua inibindo a glicoproteína P, uma enzima envolvida no efluxo de fármacos tal como o saquinavir) (CAMERON, 1998; FDA, 2006). Consequentemente, a biodisponibilidade é aumentada quando do uso desta associação.

Mesmo sendo evidente a necessidade de melhorar a biodisponibilidade/ absorção do saquinavir, e sabendo-se que diferentes estratégias farmacotécnicas poderiam auxiliar nesta tarefa (OJEWOLE et al., 2008), pouco se tem investigado no que se refere ao desenvolvimento de novos produtos, que teria como objetivos concomitantes solucionar problemas relacionados à sua biodisponibilidade/absorção, melhorar a adesão do paciente ao tratamento, e diminuir custos de sua produção. Como já dito anteriormente, o Brasil se destaca no cenário mundial quando se considera o tratamento de pacientes com HIV/AIDS e vem buscando a independência na produção de alguns dos medicamentos que integram o coquetel antirretroviral, dentre esses, o saquinavir. Para alcançar tal objetivo, existe uma parceria oficial entre um laboratório farmacêutico nacional (Cristália) e a FIOCRUZ (Far-Manguinhos), que tem como prioridade a busca de alternativas para a melhoria do sucesso terapêutico dos antirretrovirais por eles produzidos.

A escolha do esquema terapêutico mais adequado inclui avaliações dos riscos de intolerância e de toxicidade, além da capacidade de adesão do paciente ao tratamento. Um dos fatores que conta para o sucesso deste tratamento é a facilidade de ingestão dos medicamentos e, neste sentido, as dimensões físicas têm influência direta neste aspecto. O fato dos medicamentos inibidores da protease do HIV, disponíveis no mercado, possuírem tamanho de cápsula superior ao das tradicionais constitui uma barreira para o sucesso do tratamento. Pensando nisto, os parceiros Laboratório Cristália/Far-Manguinhos desenvolveram minicápsulas gelatinosas moles, comercializadas com o nome de fantasia Svir™, com dimensões aproximadamente 50% menores e maior biodisponibilidade, quando comparadas ao produto de referência (Invirase™, Roche). No entanto, o saquinavir existente nestas minicápsulas é também veiculado em uma emulsão líquida, encapsulada em invólucro mole, o que requer o emprego de tecnologia específica, já que nesta forma farmacêutica, a produção do invólucro ocorre de forma simultânea ao enchimento da cápsula (DE LUCCA et al., 2005; PACHECO et al., 2005).

Os gastos públicos nacionais, somente para a compra dos medicamentos do Programa Nacional DST/AIDS, estão em torno de R$ 800 milhões/ano para 200 mil de pacientes atendidos (GRECO, 2011). Assim, evidencia-se a necessidade constante de reduzir os custos de produção de tais medicamentos, por exemplo, modificando as formas farmacêuticas existentes, buscando tecnologias mais eficazes com custos de produção mais baixos, além de continuar incentivando a produção nacional de novos medicamentos. Para tal, existe uma ampla gama de veículos e tipos de sistemas que podem ser utilizados (adição de surfactantes e/ou ciclodextrinas, lipídeos e reforçadores de absorção, micronização e produção de dispersões sólidas) para modular a

dissolução de fármacos pouco solúveis, como é o caso do MS (ATEF; BELMONTE, 2008).

3.3. CONSIDERAÇÕES GERAIS SOBRE DISPERSÕES SÓLIDAS

As DS são divididas em diferentes tipos, de acordo com suas propriedades físico-químicas. A principal diferenciação é realizada com base no estado físico que se encontra o fármaco ou o carreador no sistema, ou seja, se amorfos ou cristalinos. Em sistema monotéticos e eutéticos, tanto o carreador quanto o fármaco se apresentam como cristalinos. Em sistemas eutéticos, o fármaco e o carreador são completamente miscíveis no estado líquido (fundido) e, após resfriamento, formam uma mistura física com ponto de fusão inferior àquele do fármaco ou carreador. Por outro lado, em sistemas monotéticos, o ponto de fusão do carreador não é alterado na presença do fármaco (VIPPAGUNTA et al., 2007). Em sistemas eutéticos, que incluem carreadores hidrofílicos, a alta solubilidade aquosa do carreador permite uma rápida dissolução em meio aquoso, com a liberação de finos cristais do fármaco. Esta redução do tamanho das partículas tem resultado em um aumento da taxa de dissolução, como ocorreu em DS contendo a mistura de fenofibrato ou loperamida com PEG (LAW et al., 2003; WEUTS et al., 2005).

No segundo tipo de DS, o fármaco é disperso em uma fase cristalina ou amorfa, separados na matriz vítrea, constituindo as denominadas suspensões sólidas (CRAIG 2002). Este tipo de DS é comumente constituído por polímeros amorfos como polivinilpirrolidona (PVP) ou materiais semicristalinos, tais como PEG (VAN DEN MOOTER et al., 1998). Além disto, a formação de complexos pode ser possível com alguns carreadores, tais como PVP (GAREKANI et al., 2003).

O terceiro tipo caracteriza uma solução sólida, sistema no qual fármaco e carreador são totalmente solúveis entre si, como resultado de interações moleculares específicas. Neste sistema, o fármaco está presente como uma dispersão molecular no interior do carreador. Soluções sólidas podem ser ainda classificadas de acordo com sua miscibilidade (soluções sólidas contínuas ou descontínuas) ou de acordo com o modo com que as moléculas de fármaco estão distribuídas na matriz (substitucional, intersticial ou amorfa) (LEUNER; DRESSMAN, 2000). Poucos sistemas de carreador/fármaco têm sido caracterizados como solução sólida contínua, ou seja, a situação em que os componentes são miscíveis entre si em todas as proporções (KONNO; TAYLOR, 2006). Neste caso, a força de ligação entre os componentes é superior à força das moléculas individuais. Por outro lado, soluções descontínuas (situações onde a miscibilidade entre os componentes é limitada) são mais comuns (LEUNER; DRESSMAN, 2000). Por exemplo, a solubilidade sólida da trealose em dextrose é

inferior a 10% (m/m) (VASATHAVADA et al., 2004), enquanto que a solubilidade do nimodipino em PEG 300 a 25°C é aproximadamente 5% (URBANETZ; LIPPOLD, 2005). Considerando-se aspectos práticos, Goldberg et al. (1965) recomendaram que o termo "solução sólida" seja utilizado apenas quando a
solubilidade entre os dois componentes (fármaco e carreador) exceda 5%.

Além destas DS clássicas, foi proposto um quarto grupo, que contém uma mistura de polímero e/ou surfactantes como carreadores (MURA et al., 2005). Nestas DS, as propriedades do carreador são ajustadas de modo que o fármaco seja mantido em um estado molecularmente disperso em altas concentrações (JANSSENS et al., 2008).

Uma vez que o preparo de DS tem contribuído para gerar resultados promissores na biodisponibilidade de muitos fármacos fracamente solúveis em meio aquoso, esta área tem adquirido papel estratégico no setor farmacêutico, desde a obtenção dos primeiros sistemas (entre as décadas de 50 e 60). Ainda que algumas limitações envolvendo o método de preparo dessas DS, a estabilidade física e química do fármaco e do veículo, a reprodutibilidade das propriedades físico-químicas, assim como aspectos relativos ao escalonamento tenham sido observadas para algumas preparações (SERAJUDDIN et al., 1999), esforços a fim de otimizar diferentes sistemas têm sido realizados com sucesso, como pode ser observado no Quadro 1.

Quadro 1. Exemplos de dispersões sólidas disponíveis comercialmente.

Nome comercial	**Fabricante**	**Fármaco**	**Carreador**
Gris-PEG	Pedinol Pharmacal Inc.	Griseofulvina	PEG6000
Cesamet	Valeant Pharmaceuticals	Nabilona	PVP
Kaletra	Abbott	Lopinavir, ritonavir	PVPVA
Sporanox	Janssen Pharmaceutica	Itraconazol	HPMC
Intelence	Tibotec	Etravirina	HPMC
Certican	Novartis	Everolimus	HPMC
Isoptin SR-E	Abbott	Verapamil	HPC/HPMC
Nivadil	Fujisawa Pharmaceutical Co., Ltd	Nivaldipina	HPMC
Prograf	Fujisawa Pharmaceutical Co., Ltd	Tacrolimo	HPMC
Rezulin	Desenvolvido pela Sankyo	Troglitazone	PVP

HPMC, hidroxipropilmetilcelulose; HPC, hidroxipropilcelulose; PVP, polivinilpirrolidona; PVPVA, polivinilpirrolidona vinil acetato.
Fonte: Janssens & Van den Mooter (2009)

3.4. CRITÉRIOS PARA A SELEÇÃO DO CARREADOR

O principal objetivo das soluções vítreas destinadas a uma liberação imediata é o de permitir que o fármaco possa ser molecularmente liberado nos fluidos intestinais e constituir uma solução supersaturada que facilite o acesso do fármaco às paredes do intestino, o que permite sua absorção e, finalmente, o alcance da circulação sistêmica. Por outro lado, a formulação deve ser física- e quimicamente estável durante o período de estocagem. Isto representa um grande desafio para os formuladores e altos estados de energia devem ser utilizados a fim de se obter tanto uma biodisponibilidade adequada quanto uma estabilidade aceitável e compatível com uma aplicação comercial. Assim, a seleção do carreador tem forte impacto sobre a taxa de sucesso da estratégia de DS a ser utilizada (JANSSENS; VAN DEN MOOTER, 2009). Diferentes polímeros vêm sendo utilizados como carreadores em formulações comerciais, tais como hidroximetilcelulose, polietilenoglicol, óxido de polietileno e polivilpirrolidona (Quadro 2).

Quadro 2. Carreadores comumente utilizados em dispersões sólidas

Carreador	Propriedades/Vantagens	Limitações	Referências
Polietilenoglicol (PEG)	PEGs apresentam um peso molecular médio de 1500-20000, sendo semicristalinos e solúveis em água. A parte cristalina pode existir sob diferentes formas, estendida ou dobrada. São compatíveis com métodos de fusão e de evaporação por solvente. O mecanismo pelo qual aumenta a dissolução envolve uma maior solubilização e molhabilidade dos fármacos.	A liberação do fármaco é dependente do peso molecular e da proporção de PEG utilizada. Problemas de estabilidade durante preparo ou armazenagem têm sido observados. Utilização de PEG com baixo peso molecular e/ou um fármaco que tem efeito plastificante pode resultar em um produto indesejável.	Damian et al. (2000), Law et al. (2001), Urbanetz (2006), Verheyen et al. (2002)
Polivinilpirrolidona (PVP)	Polímero amorfo. Pesos moleculares entre 2500 e 50000 (K12 a K30) têm sido utilizados para compor DS. Solúvel em água (aumenta com o PM) e em solventes orgânicos. Ideal para preparo de DS pelo método do solvente. O mecanismo pelo qual aumenta a dissolução envolve uma maior solubilização e molhabilidade dos fármacos. Interações antiplastificantes e ligações de hidrogênio estabilizam DS com PVP.	Sua utilização no método de fusão a quente é limitado devido a alta T_g do polímero. A liberação de fármacos é dependente do PM do PVP uma vez que o aumento da viscosidade com aumento do PM retarda a dissolução de fármacos. Quanto maior proporção de PVP, maior será a amorfização e solubilização.	Konno & Taylor (2006), Matsumoto & Zografi (1999), Van den Mooter et al. (1998), Sethia & Squillante (2004), Vasanthavada et al. (2004).
HPMC	PM varia entre 10000 e 1500000. Compatíveis com métodos de fusão e de evaporação por solvente. Adição de PEG de cadeia curta a HPMC tem melhorado a dissolução de fármacos sem alterações da estabilidade.		Janssens et al. (2008), Konno et al. (2008), Six et al. (2003), Tanno et al. (2004), Won et al. (2005)

Carreador (cont.)	Propriedades/Vantagens (cont.)	Limitações (cont.)	Referências (cont.)
Gelucires	Glicerídeos poliglicolizados saturados consistindo de misturas de mono-, di- e triglicerídeos e ésteres de ácido mono- e digraxos de PEG 1500. Os mais utilizados tem sido o Gelucire^{TM} 44/14 e o 30/13. Compatíveis com métodos de fusão e de evaporação por solvente. Solubiliza fármacos lipofílicos após o contato com meios aquosos, facilitando a absorção.	Efeito de envelhecimento devido a sua natureza lipídica pode ser reduzido pela adição de outros carreadores como PVP.	Damian et al. (2000), Sethia & Squillante (2004), Shimpi et al. (2005), Vippagunta et al. (2002)
Vitamina E TPGS	Preparada pela esterificação do ácido d-α-tocoferol succinato com PEG 1000. Miscível em água. Baixa concentração micelar crítica e ponto de fusão. Compatível com método de fusão e solução.	Pode exigir mistura com outros carreadores para aumentar o ponto de fusão da DS.	Sethia & Squillante (2004), Shin & Kim (2003)
Óxido de polietileno (PEO)	Polímero não-iônico hidrofílico e cristalino. A estrutura química de unidades repetidas é idêntica aquela do PEG, porém, o PM é significativamente maior. Baixa temperatura de processamento no método de fusão representa uma de suas vantagens.	Problemas de estabilidade são possíveis devido à natureza cristalina do PEO.	Li et al. (2006), Schachter et al. (2004)

Para se obter estabilização cinética em soluções vítreas supersaturadas, a alta temperatura de transição vítrea é um critério imprescindível para candidatos a carreador. A presença de grupamentos doadores e aceptores de ligações de hidrogênio conferem benefícios adicionais, uma vez que interações específicas aumentam a solubilidade sólida do fármaco no carreador e também parecem ser importantes na inibição de separação de fase e cristalização de fármacos a partir de uma solução vítrea (MARSAC; SHAMBLIM; TAYLOR, 2006).

É também desejável que os carreadores sejam inertes e reconhecidos como seguros. Com relação à produção de DS, estabilidade térmica e termoplasticidade são desejáveis para sistemas preparados a partir de extrusão com estágio a quente, enquanto que a solubilidade em solventes orgânicos é pré-requisito para carreadores a serem utilizados na produção de DS via método do solvente (JANSSENS; VAN DEN MOOTER, 2009).

A solubilidade do carreador em sistemas aquosos interfere diretamente na taxa de liberação do fármaco a partir de DS preparadas com o mesmo. Caso for desejável uma liberação imediata, carreadores com maior solubilidade aquosa devem ser priorizados. Nas DS em que o fármaco encontra-se molecularmente disperso, a taxa de liberação é controlada pela dissolução do carreador e, nestes casos, é relevante que o carreador tenha alta solubilidade aquosa, sendo que o carreador dissolvido ainda influenciará a solução supersaturada de fármaco formada. Alguns carreadores solubilizam o fármaco liberado, enquanto que outros estabilizam a solução supersaturada formada (JANSSENS; VAN DEN MOOTER, 2009). Neste estágio, deve-se evitar que o fármaco cristalize ou forme precipitado em função de problemas de solubilidade no meio em que é liberado. A combinação com surfactantes naturais, tal como os sais biliares, pode reduzir ainda mais a quantidade de fármaco livre (DRESSMAN et al., 2007). A relação do esvaziamento gástrico com a taxa de liberação do fármaco a partir da formulação ou com a quantidade de fármaco absorvida na parede intestinal representa o grau de supersaturação do fármaco no estômago e intestino, respectivamente (KOSTEWICZ et al., 2004)

3.5. ESTABILIDADE DE DISPERSÕES SÓLIDAS

Formas amorfas são termodinamicamente instáveis em relação às formas cristalinas e, desta forma, sólidos apresentam uma tendência espontânea a se converterem em sua forma cristalina. Esta transição pode ocorrer durante a estocagem, sob diferentes condições de umidade e temperatura (BHUGRA; PIKAL, 2008), bem como durante o contato com o meio de dissolução (ALONZO et al., 2010). Ambos os

casos invalidam as vantagens conferidas pela utilização de um sistema amorfo. Neste sentido, é necessário inibir a cristalização ao longo do tempo de estocagem do produto e manter um nível suficiente de supersaturação na administração oral (NEWMAN; KNIPP; ZOGRAFI, 2012).

Uma estratégia interessante para aumentar a estabilidade física deste sistema é utilizar DS amorfas. Nestes sistemas, fármaco e polímero solúveis em água são combinados a fim de gerar uma mistura amorfa com apenas uma fase (PADDEN et al., 2011). Uma miscibilidade entre ambos os componentes parece ser imprescindível para a manutenção da estabilidade física, em longo prazo, bem como níveis apropriados de supersaturação no sítio de absorção (NEWMAN; KNIPP; ZOGRAFI, 2012).

A capacidade dos polímeros de inibirem a cristalização em amostras estocadas pode estar relacionada com a temperatura de transição vítrea (T_g) dos mesmos em relação àquela do fármaco, bem como a capacidade dos polímeros de aumentarem a T_g da dispersão, o que reduz a mobilidade molecular do fármaco, nas condições de temperatura e umidade comumente encontradas (BHUGRA; PIKAL, 2008). Polímeros que interagem fortemente com fármacos, quando da constituição de DS, geralmente através do estabelecimento de ligações de hidrogênio, inibem a cristalização mesmo em baixas concentrações. Nestes sistemas, o efeito da T_g não é significante, e as ligações de hidrogênio proporcionam estabilidade por interferirem diretamente nos processos de nucleação e crescimento dos cristais, sendo que a forte energia de interação fornece maior resistência à cristalização (HANCOCK; SHAMBLIN; ZOGRAFI, 1995).

A mobilidade molecular é um dos principais fatores determinantes da estabilidade de compostos amorfos. Estudos têm demonstrado que a reatividade química dos mesmos pode estar relacionada com a mobilidade molecular, sendo que uma mobilidade molecular aumentada promoveria maior degradação química. Neste contexto, a redução da mobilidade molecular tem sido proposta como estratégia para melhorar a estabilidade durante a estocagem. No entanto, nem sempre uma redução da mobilidade molecular aumenta a estabilidade durante a estocagem, até porque diferentes fatores podem influenciar este parâmetro. Reações químicas nas quais a mobilidade molecular tem pouca influência não poderão ser inibidas. A compreensão da relação quantitativa entre mobilidade e reatividade química não fornece apenas informações úteis para uma estabilização destes sistemas, como também permite entender se métodos avançados podem ser utilizados na predição da estabilidade durante a estocagem. Caso a reatividade química de um determinado composto seja afetada pela mobilidade molecular relacionada à transição vítrea, sua estabilidade durante a estocagem pode ser prevista baseando-se

neste fator, mas não pode ser prevista por testes de estabilidade acelerada. Se a reatividade química não for afetada pela mobilidade molecular relacionada à transição vítrea, a estabilidade de estocagem pode ser prevista pela extrapolação dos dados obtidos a partir de condições aceleradas (YOSHIOKA; ASO, 2007).

No momento em que as DS encontram o meio de dissolução *in vitro* ou *in vivo*, a supersaturação da solução deve ser mantida ao longo do tempo, o que garantirá uma completa dissolução e um aumento da biodisponibilidade (BROUWERS; BREWSTER; AUGUSTIJNS, 2009). Caso o fármaco e o polímero se dissolvam muito rapidamente, a supersaturação deve ser mantida por fatores que incluam o polímero dissolvido, o qual deve ser capaz de inibir a nucleação bem como o crescimento do cristal em solução (CURATOLO; NIGHTINGALE; HERBIG, 2009).

3.6. FERRAMENTAS ANALÍTICAS PARA CARACTERIZAÇÃO DE DISPERSÕES SÓLIDAS

Ainda que a estratégia de dispersões sólidas tenha sido efetiva para aumentar a solubilidade e a dissolução de vários fármacos, como pode ser observado pelo número elevado de estudos publicados nestes últimos anos, problemas de instabilidade física limitam sua aplicação comercial, onde formas de alta energia se convertem espontaneamente em formas mais estáveis e menos solúveis. Neste sentido, a utilização de métodos analíticos para monitorar esta estabilidade física são de suma importância durante o processo de otimização de novas formulações, pois permite compreender os mecanismos de estabilização. Estas ferramentas analíticas proporcionam evidências diretas das alterações que ocorrem, nos níveis molecular e intermolecular, em determinada amostra, tal como a cristalização que ocorre em uma matriz de dispersão (exemplos, pico de difração do cristal, variações nas características térmicas, alterações nas vibrações de ligações intermoleculares). Nos casos em que métodos quantitativos podem ser empregados, os efeitos de diferentes fatores que interferem na tendência e na taxa de cristalização podem ser estimados, tais como o tipo de polímero e as condições ambientais de estocagem (PALERMO; ANDERSON; DRENNEN, 2012). Cabe ressaltar que nenhum destes métodos é efetivo quando tratado isoladamente com o propósito de caracterizar um sistema sólido e, desta forma, uma análise conjunta dos resultados obtidos com as diferentes técnicas deve ser realizada.

3.6.1. DIFRAÇÃO DE RAIOS X

A combinação entre difração de raios X (DRX) e análise térmica representa o padrão ouro para se determinar diferenças de ordem molecular (periodicidade de átomos ou moléculas em cristais), bem como para diferenciar compostos amorfos de compostos cristalinos. Fracas características de dispersão de materiais não cristalinos resultam em um amplo halo amorfo nos difratogramas. Por outro lado, a repetição de estruturas cristalinas construtivamente difrata os raios X de modo que picos agudos com ângulos específicos poderão ser observados, facilitando a rápida identificação da amostra. Esta informação é compartilhada na análise térmica com uma única temperatura de transição vítrea (T_g). No entanto, com materiais amorfos, a ausência de endoterma de fusão é observada (PALERMO; ANDERSON; DRENNEN, 2012).

A difração de raios X de pós (DRXP) também pode ser utilizada para quantificar determinado componente de uma DS, incluindo o grau/percentual de cristalinidade da amostra, através de técnicas específicas de refinamento (ALEXANDER; KLUG, 1948). Este método é bastante laborioso e exige a inclusão de um padrão interno, a fim de aumentar a confiabilidade dos resultados obtidos. Este padrão interno geralmente é misturado com a amostra e seus picos de difração não devem interferir naqueles da amostra, assim como problemas de orientação preferencial também não deverão ser observados (ZEVIN; KIMMEL, 1995). Recentemente, alternativas, tal como a análise multivariada de dados de padrões de difração, têm se mostrado efetivas para a quantificação de preparações multicomponentes, como é o caso de DS contendo misturas de polímero e fármaco, dispensando o uso de padrão interno (RUMONDOR; TAYLOR, 2010).

O limite de detecção pode variar dependendo do instrumento utilizado (sistema convencional ou síncronton), protocolo de amostragem (tempo de coleta, geometria), e características da amostra. Para misturas com fármaco cristalino e excipientes amorfos, um limite na ordem de 0,2 a 0,5% (w/w) é descrito (SARSFIELD et al., 2006). Quanto maior o grau de cristalinidade do carreador/excipiente, menor será o limite de detecção para o fármaco estudado.

3.6.2. ESPECTROSCOPIA VIBRACIONAL RAMAN

Espectros Raman são obtidos pela irradiação da amostra com uma fonte de laser com captura da radiação dispersa ao feixe incidente, e as análises exigem uma alteração da polarizabilidade das moléculas irradiadas. Moléculas não-cristalinas e excipientes hidrofílicos são fracos dispersores Raman em relação a pequenas moléculas aromáticas e heterocíclicas (PALERMO; ANDERSON; DRENNEN, 2012).

Ainda que existam estas limitações, é relativamente fácil identificar picos de fármacos, mesmo em baixas concentrações de uma matriz (TAYLOR; LANGKILDE, 2000). Outra vantagem é que, na maioria dos casos, formas farmacêuticas sólidas podem ser rapidamente analisadas com pouca ou nenhuma preparação da amostra, na maioria dos casos, o que torna esta técnica bastante versátil e permite sua utilização nas etapas de controle e desenvolvimento de novas formulações. A instrumentação pode incluir tanto a Transformação de Fourier (TF) quanto fontes dispersivas. A coleta do espectro com a TF reforça a relação sinal-ruído, enquanto que a fonte de comprimento de onda com infravermelho próximo evita problemas em amostras fluorescentes (PALERMO; ANDERSON; DRENNEN, 2012).

Métodos de espectroscopia RAMAN têm sido efetivos na diferenciação de formas amorfas e cristalinas de um mesmo fármaco (HU et al. 2007). Além disto, a técnica pode ser utilizada para monitorar e quantificar transições de fase no estado sólido *in situ* que ocorrem durante a dissolução de fármacos amorfos (SAVOLAINEN et al. 2009). A combinação de um desenho experimental bem definido e um tratamento matemático apropriado pode permitir a utilização da técnica para análises quantitativas de misturas no estado sólido (HEINZ et al., 2007).

O deslocamento da frequência Raman é idêntico ao da frequência de absorção no infravermelho (IR), o que torna as técnicas complementares. Ao invés de alterações da polarizabilidade, a absorção no IR requer alterações no momento dipolo (ou seja, na distribuição de carga) durante a vibração ou rotação das moléculas. Uma desvantagem do IR é que a preparação da amostra não é tão trivial quanto na espectroscopia Raman e, em alguns casos, podem não ser representativa. No IR, amostras são geralmente preparadas como *pellets* triturados e diluídos com brometo de potássio. Os processos de trituração ou compactação podem promover certo estresse mecânico, resultando em modificações de fase cristalina (YOSHIOKA; HANCOCK; ZOGRAFI, 1994; OKUMURA; OTSUKA, 2005).

As interações entre polímero e água, bem como entre fármaco e polímero, podem ser visualizadas com esta técnica. Polímeros polares e outros excipientes solúveis em água podem absorver quantidades significativas de água, aumentando a plasticidade e as trocas estruturais que, por sua vez, afetam a mobilidade do fármaco disperso. Além disto, a absorção de água pode potencializar a degradação de fármacos (KARARLI; CATALANO, 1990). Interações entre água e polímeros têm sido confirmadas por deslocamento dos picos da carbonila. Por exemplo, quando os polímeros PVP e PVA são considerados, a cadeia lateral pirrolidona estabelece interações com água mais fortes que sua cadeia acetato (TAYLOR; LANGKILDE; ZOGRAFI, 2001).

As interações químicas intermoleculares entre fármaco e excipientes nas DS são comumente relatadas, porém, pouco compreendidas. A miscibilidade molecular esperada nas DS requer alguma interação, que pode ser específica (ex.: ligações de hidrogênio e interações íon-dipolo) ou não específicas (ex.: forças de Van der Wals). Exemplos de DS estabilizadas por interações químicas intermoleculares incluem fármacos com grupos doadores de hidrogênio e excipientes com um anel pirrolidona (TAYLOR; ZOGRAFI, 1997). Nas análises espectroscópicas, com o propósito de se observar interações entre polímero e fármaco, picos de grupos funcionais específicos e com modos de vibração distintos têm sido selecionados e monitorados quanto aos seus deslocamentos e alterações de intensidade (FORSTER; HEMPENSTALL; RADES, 2001). Quanto menor a proporção de fármaco relativamente à de polímero, mais difícil será a identificação dos sinais espectrais do fármaco e, desta forma, técnicas complementares se mostram necessárias para a validação dos dados obtidos.

3.7. DISSOLUÇÃO DE DISPERSÕES SÓLIDAS

Diferentes fatores podem contribuir para incrementar a solubilidade do fármaco e a taxa de dissolução a partir de DS. A redução do tamanho de partículas, o que aumenta a área de superfície do fármaco na DS, especialmente no caso de soluções sólidas ou eutéticas, permite uma rápida dissolução do fármaco. Além disto, uma melhoria da molhabilidade de partículas hidrofóbicas, a ausência de agregação das partículas do fármaco, bem como a maior solubilização pelo carreador representam outros fatores que aumentam a dissolução (CRAIG, 2002; SETHIA; SQUILLANTE 2003; KAUSHAL; GUPTA; BANSAL, 2004). Um aumento na solubilidade também pode ser alcançado com a obtenção/conversão de fármacos em seu estado amorfo, pois pouca ou nenhuma energia é requerida para modificar esta estrutura durante o estágio de dissolução. Após a dissolução, o fármaco encontra-se disperso em uma solução supersaturada, que pode precipitar com o tempo (KAUSHAL; GUPTA; BANSAL, 2004). Adicionalmente, interações intermoleculares entre fármaco e carreador promovem dissolução do fármaco a partir da DS, uma vez que elas controlam o estado físico e o tamanho de partículas do fármaco na DS. Nos casos em que os efeitos de redução do tamanho de partículas, hidrofilicidade do polímero e molhabilidade são semelhantes, sendo que a principal contribuição para o aumento da dissolução é a ocorrência de interações intermoleculares entre fármaco e polímero (BANSAL; KAUSHAL; BANSAL, 2007). A intensidade destas interações também parece influenciar o mecanismo de dissolução (KARAVAS et al., 2006) e o aumento da dissolução pela estratégia de DS tem aumentado a

biodisponibilidade de vários fármacos (KHOO; PORTER; CHARMAN, 2000; SHIMPI et al., 2005, AMBIKE; MAHADIK; PARADKAR, 2005) e mostrou-se mais efetiva que a micronização (DANNENFELSER et al. 2004).

3.8. BIODISPONIBILIDADE DE DISPERSÕES SÓLIDAS

Conforme comentado anteriormente, o desenvolvimento de DS com fármacos amorfos é uma estratégia amplamente utilizada para melhorar a dissolução e a biodisponibilidade de alguns fármacos (SHIMPI et al., 2005; JANSSENS et al., 2010). Adicionalmente, sistemas com PEG vêm sendo comumente utilizados na preparação de DS microcristalinas. O PEG pode se desintegrar em uma mistura física, reduzindo assim a interação eletrostática e a agregação das partículas dos fármacos, resultando em aumento da sua dissolução (VEIGA; ESCOBAR; BERNAD, 1993). Várias DS foram formuladas com PEGs de diferentes pesos moleculares, tais como aquelas com nifedipino (LAW et al., 1992), norfloxacino (FAWAZ et al., 1996), piroxicam (BHATTACHARYYA et al., 1993), oxodipino (VEIGA; ESCOBAR; BERNAD, 1993), griseofulvina (VEIGA; ESCOBAR; BERNAD, 1993) e ibuprofeno (GHOSH et al., 1998). A maior parte dos fármacos tende a formar cristais quando formulados com PEG para constituir uma DS e o mecanismo de aumento da dissolução nestes casos envolve aumento da área de superfície, redução da interação eletrostática e agregação entre partículas do fármaco (VEIGA; ESCOBAR; BERNAD, 1993; BHATTACHARYYA et al., 1993).

No entanto, há também exemplos onde a utilização de DS não promoveu melhorias no grau de absorção de fármacos, como foi o caso da indometacina formulada com hidroxipropilcelulose, de modo a constituir uma DS amorfa, onde se observou um aumento de 30x na taxa de dissolução em relação ao fármaco. O preparo da DS fez com que a taxa de absorção oral fosse mais rápida, porém, o(a) grau/extensão da absorção foi equivalente ao(à) da preparação contendo apenas o fármaco. Este comportamento poderia ser explicado devido à alta permeabilidade e à baixa solubilidade do composto, uma vez que a indometacina é um fármaco pertencente à classe II, de acordo com o Sistema de Classificação Biofarmacêutico (SCB), no qual sua absorção é limitada pela dissolução (CHOWDARY; SURESH BABU, 1994). Desta forma, ainda que melhorias da taxa de dissolução proporcionada por uma DS amorfa possam resultar uma absorção mais rápida, deve-se também observar um aumento no grau/extensão da mesma. Uma análise conjunta das diferentes variáveis que interferem na biodisponibilidade de DS (Quadro 3) também é recomendável no sentido de se otimizar o sistema proposto.

Quadro 3. Fatores que interferem na absorção e biodisponibilidade (BD) de dispersões sólidas.

Propriedades	Fatores	Observações	Efeito sobre a biodisponibilidade (BD)
Físico-químicas	Lipofilicidade	Relação entre aumento da lipofilicidade e absorção é parabólica.	Alta lipofilicidade – fármaco interage com membranas ou deposita gordura com pequeno aumento na BD. Baixa lipofilicidade – depende da polaridade, fraco aumento na BD
	Estabilidade	Se estabilidade física for baixa, composto pode sofrer nucleação e constituir uma estrutura cristalina.	Reduz BD
		Estabilidade química é geralmente fraca na formulação ou em solução como uma função do pH; absorção do fármaco dependerá dos pHs locais.	Depende do local de liberação. Pode aumentar a BD se a liberação ocorrer em um local com uma estabilidade ótima ou diminuir quando fármaco é instável.
	Ionização	Solubilidade aumenta para fármacos ionizáveis dependendo do pH – bases fracas apresentam problemas de solubilidade em pH elevados e, contrariamente, ácido fracos em pHs reduzidos.	Redução na BD para a via transcelular passiva quando solubilidade é aumentada para fármacos ionizáveis. A liberação de formas amorfas necessita ser localmente direcionada no TGI.
	Tamanho	Tamanho e permeabilidade são inversamente proporcionais	Em geral, moléculas maiores têm menor BD
	Solubilidade limitada	Dissolução e permeabilidade são normalmente rápidas	Quando a solubilidade controlar a BD, o intestino pode saturar com o fármaco e um aumento da dose não afetará a BD. Para uma forma amorfa onde o composto pode manter um estado supersaturado é possível que a absorção seja limitada pela solubilidade.

Propriedades	Fatores (cont.)	Observações (cont.)	Efeito sobre a biodisponibilidade (cont.)
Formulação	Aumento da área de superfície (redução do tamanho de partícula)	Aumenta a solubilidade	Compostos com baixa solubilidade demonstraram aumento da BD, dependente da lipofilicidade
	Excipientes	Excipientes lipofílicos e desintegrantes	Podem aumentar a dissolução e BD
		Ligantes tem efeito contrário ao dos desintegrantes	Podem reduzir BD por restringir a BD
		Tensoativos podem aumentar a solubilidade e permeabilidade	Podem reduzir a tensão superficial, formar micelas e aumentar a BD; podem também interagir com transportadores de MDR para aumentar a BD
		Polímeros podem aumentar solubilidade, ou ainda, serem utilizados para gerar co-cristais	Podem aumentar BD, porém, interações entre polímero e fármaco podem também reduzir BD. Altas concentrações de polímeros podem reduzir a taxa de liberação e, conseqüentemente, a BD.
	Dissolução limitada	Tempo de dissolução é superior ao tempo de residência no intestino. Permeabilidade e solubilidade podem ser rápidas	BD pode aumentar com aumento da dose; alterações nos excipientes podem aumentar a dissolução e BD
Fisiológica	Conteúdo do lúmen	Os fluidos do TGI podem sofrer alterações assim como o pH, flora e componentes tais como os sais biliares. A presença de nutrientes/alimento pode alterar a composição do lúmen, bem como a presença de restos celulares e muco.	Dependendo do local, fármacos lipofílicos podem ser incorporados por micelas lipídicas ou biliares alterando a BD de forma imprevisível. Absorção pela flora reduz BD. Diferenças nos efeitos de BD são dependentes do fármaco. Meio de dissolução biorelevante é limitado na modelagem de conteúdo luminal.

Propriedades	Fatores (cont.)	Observações (cont.)	Efeito sobre a biodisponibilidade (cont.)
Fisiológica	Epitélio	A complexa camada de muco e glicocálix não é efetivamente modelada através das linhagens tradicionais *in vitro*	A natureza do muco e do glicocálix pode limitar a BD de solutos lipofílicos. Pode também alterar o pH na superfície da membrana devido a efeitos tamponantes.
	Bicamada lipídica	Composição da bicamada lipídica pode variar significativamente com a dieta. Ensaio de permeabilidade em membranas artificiais paralelas (PAMPA) pode contribuir na avaliação do transporte transcelular passivo	Este efeito é freqüentemente negligenciado. A bicamada lipídica é composta de ácidos graxo incorporados nos fosfolipídios. Há também uma distribuição polarizada de fosfolipídios, com alguns tais como a fosfatidilserina e fosfatidiletanolamina aparecendo apenas na parte interna da bicamada. Efeitos de carga podem alterar BD.
	Transportadores de efluxo	Podem resultar em resistência múltipla a fármacos e limitar a permeabilidade quando presentes em um dos lados da membrana (apical ou basolateral). Podem agir em conjunto dependendo da afinidade do substrato. Caso representar um processo saturável de Michaelis-Menten, um aumento da solubilidade resultará em aumento da permeabilidade. Excipientes também podem interferir na funcionalidade de algumas isoformas.	Bombas de efluxo no lado apical (voltado para o lúmen intestinal) limitam absorção/permeação de fármacos, reduzindo BD. O foco primário é nas bombas de efluxo da família ABC (PGP, BCRP, MRP1 e 2). Há também um número expressivo de transportadores de soluto (SLC) que estão envolvidos no efluxo de fármacos do citoplasma para o espaço extracelular.

Propriedades	Fatores (cont.)	Observações (cont.)	Efeito sobre a biodisponibilidade (cont.)
Fisiológica	Transportadores de influxo	Podem aumentar a permeabilidade de uma variedade de substratos, normalmente compostos polares	Aumenta BD de fármacos polares. Substratos para transportadores de influxo raramente requerer dispersões amorfas
	Metabolismo	Geralmente resultam na formação de metabólitos quimicamente modificados que são mais polares a fim de facilitar a excreção. Há duas principais vias metabólicas: fase I que resulta em modificação química do fármaco através de diferentes reações (ex.: oxidação, deaminação...) e fase II que resulta em conjugados químicos (ex.: glicuronidação).	Reduz BD por remover o fármaco e torná-lo mais polar para excreção. Dispersões amorfas podem aumentar a absorção de compostos lipofílicos pela via transcelular passiva e aumentar o potencial de saturação de enzimas metabolizadoras no epitélio do TGI e no fígado, aumentando a BD.
	Permeabilidade limitada	A permeabilidade através da barreira epitelial permanecerá baixa, independente do grau de solubilidade. A dissolução poderá ser rápida, particularmente para dispersões.	Quantidade de fármaco absorvido aumenta com aumento da dose através da via passiva transcelular. Dispersões podem aumentar a BD.

Adaptada de Newman, Knipp, Zografi (2011).

Algumas DS têm também sido formuladas como sistemas ternários, a fim de reforçar a biodisponibilidade. Esses sistemas incluem a adição de outro excipiente ao carreador, tais como tensoativos ou modificadores de pH (TRAN et al., 2010), os quais podem reforçar a taxa de liberação do fármaco por reduzirem o ângulo de contato entre fármaco e solvente do sistema disperso. Diferentes compostos de superfície vêm sendo utilizados, tais como os Tween 20 e 80, a fosfatidilcolina e o lauril sulfato de sódio (JOSHI et al., 2004; GOHEL; PATEL, 2003). Estudos *in vivo* subsequentes com algumas destas formulações mostraram melhorias da biodisponibilidade (JOSHI et al., 2004).

3.9. IDENTIFICAÇÃO DE PONTOS CRÍTICOS DE CONTROLE AO LONGO DAS DIFERENTES ETAPAS EXPERIMENTAIS

A partir do trabalho de Newman, Knipp e Zograffi (2012), foi possível identificar os diferentes pontos críticos a serem controlados nas diferentes etapas experimentais (Quadro 4). O objetivo desta abordagem é apresentar os critérios que devem ser considerados durante o desenvolvimento de DS, ou seja, os limites a serem respeitados para a otimização desta forma farmacêutica. Tais limites críticos foram definidos qualitativamente, com base na experiência acumulada por pesquisadores da área, ao longo dos anos, em relação aos tópicos citados. Este Quadro pode servir como um guia para formuladores que desenvolvem este tipo de formulação (DS) e cujo objetivo seja melhorar as propriedades biofarmacêuticas de um determinado fármaco (solubilidade/ permeabilidade).

Quadro 4. Considerações e recomendações relativas ao desenvolvimento de dispersões sólidas do mesilato de saquinavir (adaptado de NEWMAN; KNIPP; ZOGRAFI, 2012).

Aspecto	Considerações	Recomendações
Físico	Seleção do polímero	- Considerar características físico-químicas do polímero (solubilidade, ponto de fusão, molhabilidade, higrospicidade, efeito do pH, taxa de dissolução,...); - Avaliar possibilidade de utilização em larga escala (disponibilidade de matéria-prima vs. custo); - Observar compatibilidade com o método de produção selecionado; - Considerar o desempenho em testes de dissolução com outros fármacos de baixa solubilidade; - Observar a existência de relatos sobre efluxo do fármaco em estudo.
	Proporção polímero-fármaco	- Realizar testes de solubilidade com concentrações crescentes de fármaco; - Evitar separação de fase; - Priorizar proporção que otimiza a estabilidade física.
	Miscibilidade	- Observar se um sistema monofásico miscível é formado (geralmente confere maior estabilidade) - Avaliar se há necessidade de inclusão de tensoativos (para evitar a formação de precipitados).
	Processo de produção	- Deve ser compatível com os polímeros selecionados; - Deve considerar as características físicas do fármaco (considerar efeito de moagem, trituração ou outros processos envolvidos no preparo das DS) - DS amorfas produzidas em altas temperaturas podem ser miscíveis, porém, pode ocorrer separação de fase após resfriamento a temperatura ambiente ou em outras condições específicas.
	Higrospicidade	- Avaliar o efeito sobre a cristalização; - Considerar a possibilidade de degradação dos polímeros ou, até mesmo, do fármaco. - Avaliar o efeito sobre a temperatura de transição vítrea (T_g).
Dissolução (*in vitro*)	Aparato e condições do meio	- Deve mimetizar o trato gastrointestinal (estas condições podem não serem aplicáveis para discriminar diferenças entre os lotes); - Utilizar método/aparato que permita uma maior correlação *in vitro*/*in vivo*; - É desejável a utilização de método de dissolução de duas fases (gástrica e intestinal).

Aspecto	Considerações	Recomendações
Dissolução (*in vitro*)	Condições *sink versus* não *sink*	- É desejável que ambas as condições sejam testadas e comparadas com dados a partir de estudos *in vivo*; - Para fármacos com baixa solubilidade aquosa, deve-se considerar a possibilidade de cristalização no TGI quando condições não *sink* são utilizadas.
	Dissolução em presença de altas proporções de polímero	- Considerar se propriedades do polímero são compatíveis com método de dissolução selecionado (evitar flutuação das cápsulas; formação de gel do polímero, adesão do sólido no vidro, pás e eixos); - Verificar se polímeros tem solubilidade dependente do pH.
	Teste de supersaturação	- Avaliar em meio biorrelevante se fármaco está em solução ou se sofre cristalização ou precipitação.
Ensaio Biológico (*in vivo*)	Jejum *versus* estado alimentado	- Considerar efeito de alimentos sobre a absorção de fármacos.
	Condições *sink versus* não *sink*	- A condição sink não deve ser definida apenas com base na concentração de fármaco na formulação/DS, mas também deve considerar variáveis como pH, volume e tempo de residência gastrintestinal.
	Efeito local dos polímeros sobre o pH	- Considerar efeito dos polímeros sobre o pH, especialmente aqueles utilizados em revestimento entérico.
	Diferenças interespécies	- Investigar o uso de modelos animais alternativos (porcos e *minipigs* são os modelos animais não primativas mais representativos).
	Transportadores e metabolismo	- Determinar se o fármaco é substrato para transportadores humanos (ou modelo animal selecionado p/ estudo) e/ou enzimas metabolizadoras e o efeito que isto apresentará sobre a absorção.
	Fisiologia gastrointestinal	- Para moléculas com alta lipofilicidade, absorção linfática e não apenas a sanguínea necessitam ser determinadas previamente; - Considerar estratégias *in silico* para predizer a absorção e refinar/otimizar formulações.

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4. ARTIGO PUBLICADO

Arch. Pharm. Res. (2013) 36:1113–1125
DOI 10.1007/s12272-013-0142-2

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

Development and physicochemical characterization of saquinavir mesylate solid dispersions using Gelucire 44/14 or PEG 4000 as carrier

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Received: 20 January 2013 / Accepted: 26 April 2013 / Published online: 23 May 2013

Abstract Solid dispersions of saquinavir mesylate containing either Gelucire® 44/14 or poly(ethylene glycol) (PEG) 4000, or mixtures of each carrier with Tween 80 or polyvinyl pyrrolidone (PVP) K30 were prepared in order to enhance the drug dissolution rate. These systems were prepared by the melting method and characterized by X-ray powder diffraction, microscopical techniques, and Raman spectroscopy aiming to establish a relationship between physicochemical and dissolution properties under different cooling conditions. Modifications in degree of crystalline order/disorder over time were observed in preparations with both carriers. Overall, formulations cooled and stored at −20 °C showed less variation in dissolution rates than those at 25 °C. Although Tween 80 has enhanced the known self-emulsifying properties of Gelucire® 44/14, its combination with PEG 4000 displayed miscibility problems. The addition of PVP K30 was not the most effective approach in enhancing the dissolution in early steps; however, the drug dissolution was stable after 7 days of storage at 25 °C. The combination of PEG 4000 and PVP K30 maintained the dissolution properties for 60 and 90 days at 25 °C/95 % relative humidity and 40 °C/75 % (f_2 values >50), respectively.

Keywords Solid dispersion · Saquinavir mesylate · In vitro dissolution · X-ray powder diffraction · Raman spectroscopy

Electronic supplementary material The online version of this article (doi:10.1007/s12272-013-0142-2) contains supplementary material, which is available to authorized users.

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Introduction

Saquinavir (SQV), a potent inhibitor of HIV protease, has been approved by the EMA and FDA to treat HIV infection in combination with other antiretroviral agents (Knechten et al. 2010). This drug has low bioavailability (<4 %), which could be the result of both low solubility and permeability since it is a class IV drug (Lindenberg et al. 2004). For this reason, SQV requires frequent and large medication dosing for a satisfactory efficacy during treatment, consequently reducing patient adherence to the therapeutic regimen (Pathak et al. 2010). Therefore, the development of new SQV formulations has been studied as an alternative to solve these problems (Buchanan et al. 2008; Pathak et al. 2010).

Solid dispersions (SDs) afford an approach to formulation of drugs with poor aqueous solubility (Serajuddin 1999), with both economic and technological advantages. On the other

hand, the physical and chemical stability of drugs and vehicles, the scale-up of manufacturing processes, formulation into dosage forms and the reproducibility of their physicochemical properties limit the commercial application of this system, and thus must be overcome (Serajuddin 1999).

Poly(ethylene glycol) (PEG) and Gelucires are carriers widely used to prepare SDs. PEG is a semi-crystalline hydrophilic polymer often used to prepare SDs by using the melting/fusion method (Heo et al. 2005), in which molecules are present in the helical structure of PEG (Dhirendra et al. 2009). Furthermore, this polymer can either facilitate or retard processing-induced transformation since it can act as a solvent once converted into a liquid phase upon melting (Mirza et al. 2006). The molecular weight of PEG polymers used in the SDs ranges from 1,000 to 20,000 and they present several advantages such as low melting point, wide drug compatibility and hydrophilicity, high viscosity and low toxicity (Damian et al. 2000). Gelucire® 44/14, an amphiphilic pharmaceutical carrier characterized by a melting point of 44 °C and a hydrophilic–lipophilic balance of 14, is one of the main representatives of the Gelucires group. This material is composed of surfactants (mono- and diesters of PEG), co-surfactants (monoglycerides), and an oily phase (di- and triglycerides) (Liu et al. 2011). In contact with aqueous fluids, it forms a fine emulsion inducing a pseudo-solubilization of poorly water-soluble drug (Chambin and Jannin 2005).

Although the development of SDs has been investigated over the last decades in view to improve low aqueous-soluble drugs dissolution rate and bioavailability, to our knowledge, this is the first report regarding the preparation of SDs for saquinavir mesylate (SQVM). Therefore, we produced SDs by melting/fusion using well-known carriers under different cooling conditions in order to investigate the aging behavior of these SDs. The solid state properties of such systems were assessed by X-ray powder diffraction (XRPD), microscopical techniques and Raman spectroscopy and the data were correlated to those of in vitro dissolution.

Experimental section

Preparation of SDs

The SDs were prepared by the melting method. SQVM (50 mg per capsule) was added to the molten carrier only (PEG 4000 or Gelucire® 44/14, 450 mg per capsule), and to the mixture of each carrier with Tween 80 or polyvinyl pyrrolidone (PVP) K30 in a 8:1 (w/w) ratio, as can be observed in Table 1. The mixtures were heated at 10 °C above the melting point of each carrier (54 °C—Gelucire 44/14® and 63–69 °C—PEG 4000) for 10 min with continuous stirring. According to preliminary tests performed in our laboratory, the use of Ultra-turrax® provided SDs with higher content homogeneity. Thus, three agitation cycles (13,500 rpm, 1 min each) were used to prepare these systems. Each hard gelatin capsule was filled (500 mg of this formulation) and stored at −20 or 25 °C for 1 and 7 days until the tests have been performed.

HPLC analysis

A Shimadzu LC-10A system (Kyoto, Japan) was employed with an LC-10AD pump and SPD-10AV ultraviolet detector (set at 240 nm), with a C18 reverse phase column (Perkin Elmer, 5 µm particle size, 250 × 4.6 mm) as stationary phase. The mobile phase consisted of acetonitrile and 30 mM potassium dihydrogen phosphate (pH adjusted to 3.2 with orthophosphoric acid) in the ratio of 60:40 (v/v). Flow rate was 1 ml min^{-1} and retention time was 4.5 min. This method was previously developed and validated according to ICH Guidelines and it was linear ($r > 0.999$), precise (intra- and interday relative standard deviations <2.15 and 3.07 %, respectively), accurate (recoveries ranged from 99 to 101 %) and specific.

Determination of apparent solubility

Solubility studies were performed by adding an excess of SQVM (approximately 4×) in 1.5 ml of gastric media (pH = 1.2) with or without surfactants and carriers. These excipients were added in the same proportions as the SDs were manufactured (Table 1). The solutions were vortexed for 1 min and kept in a benchtop shaker incubator (Quimis®, Brazil) shaking at ~140 rpm for 24 h at 37 °C. Once removed from the incubator, the solutions were vortexed for 2 min, centrifugated (14,000 rpm, 37 °C) and a sample was taken from the supernatant. After appropriate dilution in gastric media, the samples were analyzed by HPLC as described in "HPLC analysis" section. Every combination was prepared in triplicate.

In vitro dissolutions studies

These tests were carried out before the physicochemical characterization in order to verify if the proposed system has an enhancing effect on dissolution properties. Release profiles from SDs were determined in triplicate by the USP rotating paddle method with minor modifications. Capsules containing only 50 mg of SQVM (pure drug) or SDs were added to 900 ml of dissolution medium (0.01 M HCl) at 37 ± 0.5 °C and stirred at 75 rpm on standard dissolution equipment (Nova Ética, Brazil). Samples of 5 ml were withdrawn at 10, 15, 20, 30, 45, 60, 90 and 120 min and the amount taken was immediately replaced with fresh dissolution medium maintained at the same temperature. The SQVM concentrations were determined by HPLC (as

Table 1 Apparent solubility and thermodynamic parameters of the saquinavir mesylate and different solid dispersions prepared with Gelucire® 44/14 or PEG 4000 in acid aqueous media (pH = 1.2) at 37 °C

Samples	Ratios	Concentration (μg ml^{-1})	$\Delta\Delta G^{\circ}_{tr}$ (kJ mol^{-1})
Saquinavir mesylate	–	78.85 ± 11.47	–
Gelucire® 44/14 + saquinavir mesylate	(9:1)	328.60 ± 20.36	−3.68
Gelucire® 44/14 + Tween 80 + saquinavir mesylate	(8:1:1)	373.32 ± 7.57	−4.01
Gelucire® 44/14 + PVP K30 + saquinavir mesylate	(8:1:1)	296.11 ± 6.26	−3.41
PEG 4000 + saquinavir mesylate	(9:1)	101.18 ± 10.29	−0.64
PEG 4000 + Tween 80 + saquinavir mesylate	(8:1:1)	116.78 ± 4.05	−1.01
PEG 4000 + PVP K30 + saquinavir mesylate	(8:1:1)	89.35 ± 1.41	−0.32

The proportion of carrier and excipient used in this experiment was equivalent to that of solid dispersions

described in "HPLC analysis" section). Dissolution profiles of SDs were individually compared to that of pure SQVM using the similarity factor f_2 according to the equation described by Moore and Flanner (1996) (Eq. 1). This test was also used to evaluate the effect of storage and manufacturing conditions on the dissolution performance.

$$f2 = 50 \times \log\left\{\left[1 + \frac{1}{n}\sum_{j=1}^{n} w_j\left(R_j - T_j\right)^2\right]^{-0.5} \times 100\right\}, \quad (1)$$

where R_j is the mean of the dissolved drug from reference data at time point j, T_j is the mean of the dissolved drug from test data at time point j, n is the number of time points, w_j is an optional weight factor, and for current purpose, $w_j = 1$. The factor is 100 when the profiles are identical and approaches 0 as the dissimilarity increases. Generally similarity factor in the range of 50–100 is acceptable according to US FDA (1997).

Physicochemical characterization

Microscopic evaluation

The drug distribution in different excipient combinations were observed under an optical microscope (Nikon, Japan) connected to a digital camera.

The surface and cross-section morphological features of different SDs were examined using SEM (Jeol JSM-6390LV, Japan). Prior to this evaluation, the samples were fixed by mutual conductive adhesive tape on aluminum stubs and covered with a thin layer of gold in vacuum. The SEM was operated at an acceleration voltage of 15 kV.

Powder X-ray diffractometry (XRPD)

The diffraction patterns of SQVM, the used excipients and the prepared SDs were obtained using a PANalytical X'Pert PRO Multipurpose Diffractometer. Measurements were performed with Cu Kα radiation, which was generated at an accelerating voltage of 45 kV and a current of 40 mA. The samples were scanned over an angular range of $3° < 2\theta < 60°$, with a step size of 0.33° and a counting time of 40 s per step. A comparison of high intensity peaks associated to the polymer (PEG 4000 or Gelucire® 44/14) and SQVM was performed and the results were expressed as relative intensity units.

Raman spectroscopy

The Raman spectra were recorded at room temperature in backscattering geometry using a PeakSeeker 785 PRO Raman system, with a diode laser of 785 nm and 300 mW at the source. The Peltier-cooled charge couple device detector was employed to detect the dispersed Raman signals (spectral resolution of 6 cm^{-1}). The spectra were collected from different parts of the samples surface in the scanning range of 200–1,800 cm^{-1}, with an acquisition time of 30 s.

Stability study

The formulation able to maintain the dissolution of SQVM over time was selected for long-term stability tests under well-controlled conditions. In view of the previous results, only SDs containing PEG 4000, PVP K30 and SQVM (8:1:1, w/w/w) were placed in chambers equilibrated with saturated salt solutions of NaCl to 75 % relative humidity (RH) and K_2SO_4 to 95 % RH at 40 and 25 °C, respectively (conditions 1 and 2). Periodically (initial, 1, 2 and 3 months), samples were removed, characterized by in vitro dissolution studies (f_2 values between 50 and 100 indicate that the two dissolution profiles are similar), X-ray diffractometry, Raman spectroscopy and the drug content from SDs was also monitored.

Results

Solubility studies and microscopical analysis

A drug-excipient compatibility screening was carried out to identify suitable excipients for the blended formulation.

The initial selection considered the results of microscopical analysis and solubility tests. Natural surfactants as bile salts are expected to enhance the intestinal absorption of some poorly soluble drug (Meaney and O'Driscoll 2000). In preliminary tests, deoxycholic and colic acid were added to the carriers and the formation of a yellow precipitated was observed in these cases and for this reason such excipients were discarded. Given that SQVM presents higher solubility in acid aqueous media compared to alkaline conditions (Albano et al. 2009; Pathak et al. 2010), citric acid was incorporated in order to reduce the microenvironment pH and thus enhance drug dissolution. Additionally, this approach might contribute to reduce the pH-dependent proteins functionality which are involved in drug efflux. However, citric acid was also eliminated since a yellow precipitate appeared after 5 h of agitation. In these tests, Tween 80 was the most effective excipient in increasing drug solubility in the carrier. An indication of the transfer process of SQVM from acid aqueous solution to the other containing different excipients and/or carrier was obtained from the values of Gibbs free energy change. The Gibbs free energy of transfer (ΔG°_{tr}) was calculated by using Eq. 2

$$\left(\Delta G^{\circ}_{tr}\right) = -2.303\, RT \log \frac{S_o}{S_s} - 1, \quad (2)$$

where S_o/S_s is the ratio of molar solubility of SQVM in solutions containing different excipients to that of the acid aqueous solution. The Gibbs free energy values provide the information whether the reaction condition is favorable or unfavorable for drug solubilization (Potluri et al. 2011). ΔG°_{tr} values were all negative indicating the spontaneous nature of SQVM solubilization (Table 1).

SDs presented a homogeneous distribution of the drug in both tested carriers under microscopic analysis (Fig. 1). SEM observations did not allow to distinguish any differences between the formulations prepared under different cooling conditions (−20 and 25 °C) after 1 or 7 days of storage (data not shown). Formulations prepared with Gelucire® 44/14 presented a more homogeneous surface distribution than those prepared with PEG 4000 (Fig. 2). For the formulations prepared with PEG 4000 and PVP K30, pieces of spherical particles were observed and it may be related to the pore-forming properties of the PVP K30 in erodible materials as PEG (Tuntikulwattana et al. 2010).

Dissolution studies

The drug dissolution rate was significantly improved by formulation of SQVM in a SD (Fig. 3). These results can be attributed to improved wettability and dispersibility, a particle size reduction, a crystalline/amorphous or amorphous/crystalline conversion and the presence of an intermediate state with nanocrystalline disorder. Micronized SQVM showed slow dissolution rate which is likely to be due to its tendency to agglomerate (Albano et al. 2009), and so reducing the surface area in contact with the dissolution medium.

In preliminary tests, formulations were pulverized after cooling in order to verify the effect of surface area on drug dissolution and similar dissolution profiles were obtained (data not shown). Based on these findings, the hot melt extrusion method was disregarded for the preparation of the SDs. The dissolution method used in this study had a discriminatory potential to detect changes in manufacturing process (for instance, changes in cooling rate) (Tables 2, 3). The formulations cooled and stored at 25 °C during 7 days showed slower dissolution rates than those underwent at −20 °C (except for the formulation prepared with Gelucire® 44/14 + PVP K30; Table 3). Although the incorporation of surfactants as Tween 80 in formulations have commonly been use to increase dissolution rate of drugs (Onyeji et al. 1999), SDs with Tween 80 did not provide a considerable improvement in this parameter in our studies (Fig. 3). On the other hand, the drug dissolution profiles from SDs containing Tween 80 were more homogeneous (appointed by the lower standard deviation) than those without this surfactant. The addition of PVP K30, aiming the inhibition of drug recrystallization, ensured stable drug release from SDs over time for both carriers (except for the SDs prepared with Gelucire® 44/14 and PVP K30 stored at −20 °C). Even though the combination of Gelucire® 44/14 and PVP K30 stored at 25 °C has maintained the released percentage of SQVM over time, these SDs showed slower drug release than the other formulations prepared with this carrier whatever storage conditions. SDs prepared only with PEG 4000 or with PEG 4000 and PVP K30 presented similar drug release after 1 day of preparation, however; only those containing PVP K30 preserved the initial dissolution profile after 7 days (particularly the formulation cooled at −20 °C).

In view of the fact that substantial changes in the in vitro dissolution properties of products during storage may alter the bioavailability, the physicochemical characterization of these materials is essential in order to detect and understand the main causes of these changes.

Physicochemical characterization

Firstly, it is important to mention that XRPD patterns were analyzed according to the order–disorder effects in crystal structure and how they impact on X-ray diffraction line profile. Typically, the crystalline and amorphous forms can be distinguished by XRPD due to the diffraction peak and halo patterns, respectively. The crystalline system is defined by three long-range order (LRO) symmetry

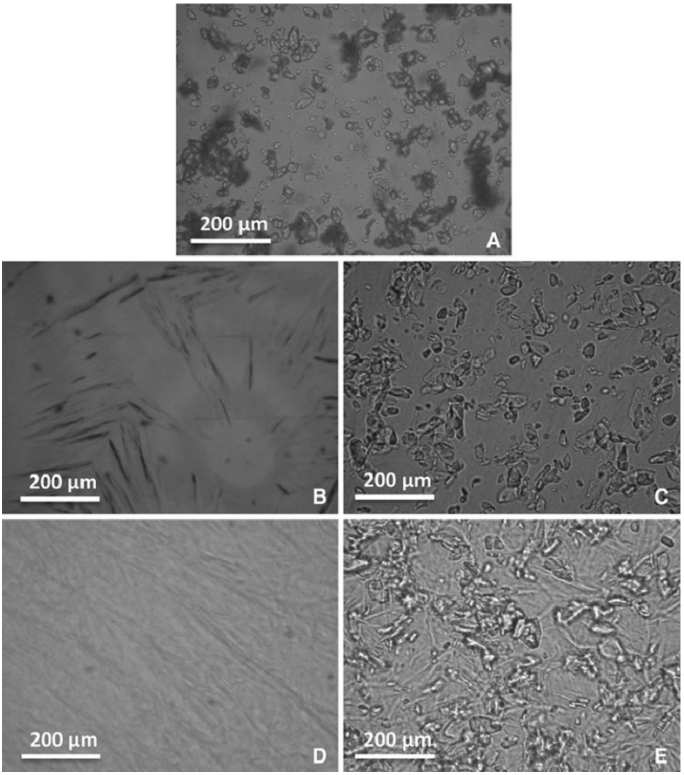

Fig. 1 Microscopical analysis showing that saquinavir mesylate (**a**) was uniformly distributed in solid dispersions containing PEG 4000 and Gelucire® 44/14 (**c** and **e**, respectively; drug-to-carrier ratio of 1:9). PEG 4000 (**b**) and Gelucire® 44/14 (**d**) alone were analyzed separately and used as control

operators (translational, orientational, and conformational) while an ideal amorphous state is characterized by the absence of all symmetry operators. Moreover, there is an intermediate state between these two forms in that the loss of these intrinsic LRO symmetry operators can lead to disordering of the crystalline phase (Bates et al. 2006). This effect should not be misunderstood with a decreasing of the "degree of crystallinity". According to the USP (2006), solids can be classified as being crystalline, non-crystalline, or a mixture of the two forms. In this sense, the degree of crystallinity depends on the fraction of crystalline material in the mixture (two-state model).

SQVM was characterized by diffraction peaks in the 2θ range from 5° to 17°. The diffraction peaks of the pure SQVM (Fig. 4) and in the SDs prepared with Gelucire® 44/14 (Fig. 5) were similar, pointing that the SQVM crystal structure did not change. A lower intensity of SQVM peaks could be associated to the high proportion of the carrier used to prepare these SDs (80 or 90 % w/w). Diffraction peaks of SQVM strongly reduced were found for the formulations prepared with PEG 4000 after 7 days storage (Fig. 6) probably due to highly ordered structure of excipient (Kumar et al. 2010).

Given that the tested formulations presented crystalline, amorphous and semi-crystalline phase mixtures, the relative intensities were dependent on the crystalline order–disorder degree of the formulation components. If the carrier has preserved amorphous-like characteristics, the drug reflections were easily detected. On the other hand, if the carrier has preserved a highly ordered structure, the drug crystalline peaks can be slightly detected; especially when low drug proportion is considered and it does not exhibit a well-defined crystalline phase (for these situations, XRPD intensity of drug peaks are extremely low since the XRPD pattern from PEG 4000 is strong). Another fact is that the drug may be solubilized by PEG matrix,

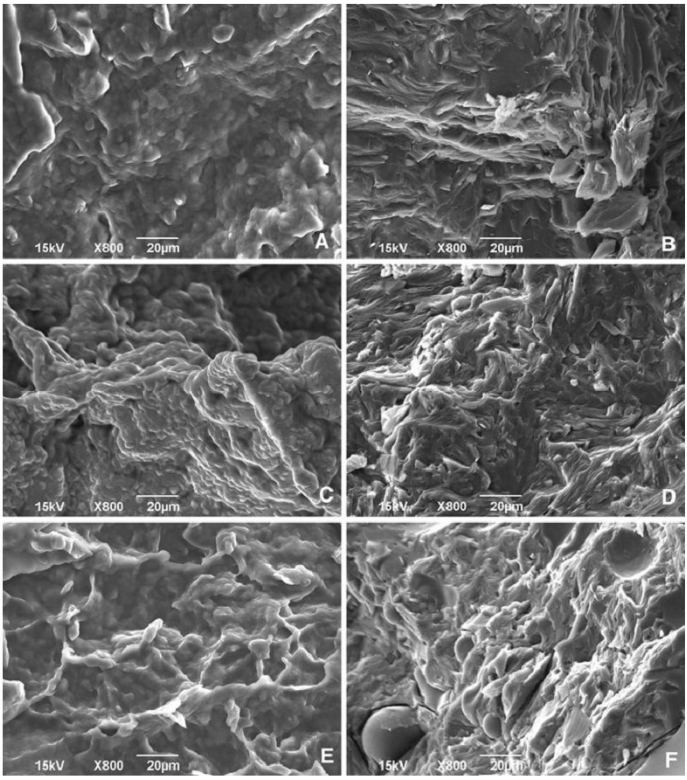

Fig. 2 SEM images of solid dispersions prepared with Gelucire® 44/14 (**a**, **c**, **e**) or PEG 4000 (**b**, **d**, **f**) and Tween 80 (**c**, **d**) or PVP K30 (**e**, **f**). The proportion of carrier and excipient was equivalent to that used in the "Preparation of SDs" section

which also would result in reduction of XRPD intensity. Thus, hot stage analyses were carried out in two stages (slow and fast cooling rates; data not shown), confirming the presence of a crystalline phase in PEG 4000-containing SD.

SDs prepared with Gelucire® 44/14 showed an increase in peak intensities of drug after storage (apart from the formulation prepared with Gelucire® 44/14/PVP K30 stored at 25 °C; Fig. 5) while those prepared with PEG 4000 showed an increased crystal structure disorder for the carrier (aside from the SDs prepared with PEG 4000 stored at 25 °C and with PEG 4000 + PVP K30 stored at −20 °C, which retained the crystalline characteristics of carrier; Fig. 6). Gelucire® 44/14 showed minor modifications during the storage, retaining an amorphous character. The formulations prepared with Gelucire® 44/14 and Tween 80 showed higher peak intensities for the drug than other formulations (Fig. 5).

Raman spectroscopy was sensitive to detect some drug bands in presence of excipients (Fig. 7). SDs with PEG 4000 did not show spectral differences over cooling time (day 1 vs. 7). Conversely, the spectral changes observed at 750–1,000 and 1,250–1,500 cm^{-1} for the formulations prepared with Gelucire® 44/14 after storage suggested the occurrence of molecular structure modification. SDs stored in different cooling temperatures (25 vs. −20 °C) had Raman spectra without modifications.

Stability studies

The SQVM content in each capsule did not change after a 3-month storage (ranged from 99 to 103 %). Similar dissolution profiles were observed for SDs after being stored for 30 days under different conditions (condition 1—45 °C/75 % RH and condition 2—25 °C/95 % RH; Fig. 8). A slight increase in this parameter was observed

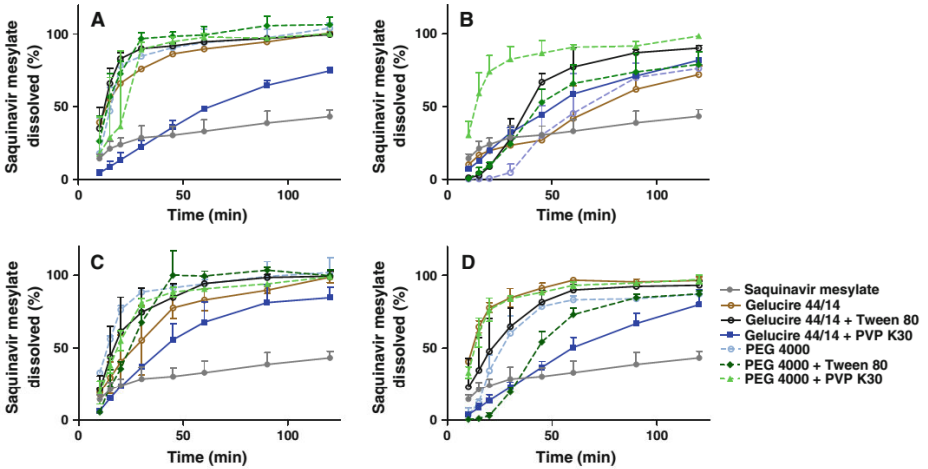

Fig. 3 Release profiles from different solid dispersions of saquinavir mesylate after 1 (**a**, **c**) and 7 days (**b**, **d**) of preparation, stored at 25 °C (**a**, **b**) and −20 °C (**c**, **d**). Results are shown as mean ± SD ($n = 3$)

Table 2 Comparative dissolution profiles between each solid dispersion and pure drug using f_2 factor

Saquinavir preparation	Storage conditions (°C)	f_2 values (day 1)^A	f_2 values (day 7)^B	Difference |A − B|
Gelucire® 44/14	−20	28.5	24.2	4.3
	25	20.5	43.0	22.5
Gelucire® 44/14 + Tween 80	−20	21.8	25.0	3.2
	25	19.4	27.6	8.2
Gelucire® 44/14 + PVP K30	−20	29.0	36.0	7.0
	25	38.3	34.0	4.3
PEG 4000	−20	18.0	22.5	4.5
	25	18.8	32.6	13.8
PEG 4000 + Tween 80	−20	27.1	29.3	2.2
	25	19.7	30.6	10.9
PEG 4000 + PVP K30	−20	19.1	18.4	0.7
	25	24.9	19.3	5.6

Only one measurement was considered after 85 % dissolution of both samples according to the FDA recommendations (FDA 1997)

after a 60-day storage (100 % of SQVM released after 30 min, differently from the previous period, which was 45 min). After 90 days, a substantial reduction of the dissolution profile was observed in the SDs stored at 45 °C/75 % RH (condition 1, f_2 value = 0.31) and small alterations for those stored at 25 °C/95 % RH (condition 2, f_2 value >50).

SQVM and carriers presented physicochemical modifications after storage under different conditions (Fig. 9). For condition 1, an increase of peak intensities of drug and carrier was observed after a 90-day storage. Therefore, high temperature and humidity increased crystalline order in SDs components. Substantial Raman spectra changes were observed only for SDs after a 60-day storage with the displacement of the base line and appearance of an additional spectral band between 1,200 and 1,250 cm^{-1}.

For condition 2, modifications of the relative intensities of drug and carriers were observed. The drug presented increased peak intensities, while the PEG 4000 seemed to acquire more disorder characteristics after a 60-day storage since the peak intensities were lower and border than those observed at initial period. No modifications were observed in Raman spectra. Moreover, these

Table 3 Comparative dissolution profiles of the same formulations stored at different periods (1 vs. 7 days) using f_2 factor

Saquinavir preparation	Cooling and storage temperature	
	25 °C	−20 °C
Gelucire® 44/14	18.2	31.6
Gelucire® 44/14 + Tween 80	17.7	55.5
Gelucire® 44/14 + PVP K30	57.0	45.3
PEG 4000	13.7	27.9
PEG 4000 + Tween 80	16.4	42.0
PEG 4000 + PVP K30	37.0	69.7

Only one measurement was considered after 85 % dissolution of both samples according to the FDA recommendations (FDA 1997)

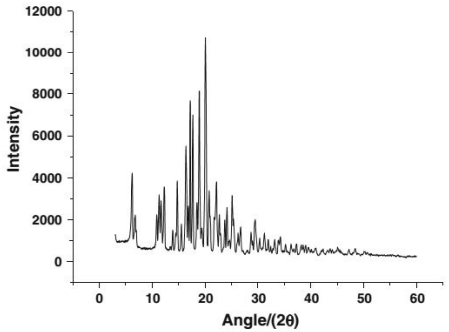

Fig. 4 X-ray patterns from saquinavir mesylate

SDs presented a viscosity reduction throughout time, with a gel-like characteristic.

Discussion

In the present study, formulations cooled and stored at −20 °C displayed less variation of dissolution properties than those underwent at 25 °C probably due to the differences in molecular mobility which modify the amorphous–crystalline ratio as well as the interactions between drug and polymer.

Although the apparent solubility of SQVM in Gelucire® 44/14 was found higher than that in PEG 4000 (by comparing SDs with similar excipients, except carrier), an opposite effect on in vitro dissolution enhancement was observed. Gelucire® 44/14-containing SDs could present a reduced ability to release SQVM in acid medium (pH = 1.2) compared to those SDs prepared with PEG 4000. Mohsin et al. (2009) found that Gelucire-based systems have different release behavior depending on pH. Gelucire at basic pH displayed relatively higher erosion as compared to acid pH, probably due to partial hydrolysis of Gelucire matrices, particularly in ester bonds. Moreover, differences in experimental variables as time (2 h—dissolution and 24 h—apparent solubility) and sample processing (SD vs. physical mixture) may contribute to explain these differences between apparent solubility and dissolution results.

Tween 80 provided a greater increase in SQVM dissolution compared to the other conditions (only carrier or carrier–PVP K30) for SDs prepared with Gelucire® 44/14. This surfactant increased the apparent solubility of the drug in gastric medium (Table 1), reinforcing the known self-emulsifying properties of Gelucire® 44/14. Formulations prepared only with Gelucire® 44/14 at −20 °C and those containing this carrier combined to the PVP K30 at 25 °C were the only ones that did not show reduction in the dissolution rate at 7-day storage period. These findings may be related to the lower crystalline order of the drug in both conditions (see Fig. 5). The water incorporation in Gelucire-containing SDs can be critical in order to reduce the physicochemical stability of these systems and could explain the changes in the dissolution profiles over time.

All formulations prepared with Gelucire® 44/14 showed substantial spectral changes after 7 days. From data analysis, it was hypothesized that chemical changes in this carrier could explain this behavior, and thus a new experiment considering the effect of temperature and humidity on this carrier was carried out. Gelucire® 44/14 in the absence or presence of water (25 % w/w) was processed similarly to the formulations, and stored at 25 or 37 °C during 1 week. Subsequently, Raman spectroscopy and XRPD were carried out.

Samples treated with water showed greater disorder characteristics than those untreated, which presented XRPD reflections of a typical crystalline sample (Fig. 10). Temperature (25 vs. 37 °C) increased crystalline order of carrier; however, high water content increased the crystalline disorder and molecular mobility of the system. More significant spectral changes (peak broadening, reduced intensity) occurred in water treated samples, and the temperature caused minor modifications. In summary, humidity provided more disorder in the system than temperature and thus formulations prepared with this carrier should be stored under controlled humidity condition. In increased water content, amphiphilic excipients as Gelucire® 44/14 are able to form mesophases that hinder its complete dissolution at body temperature (Svensson et al. 2004). Since various SDs typically have a carrier-controlled dissolution, this fact could contribute to explain the differences found for samples stored during 1 and 7 days. Sutananta et al. (1996) also observed differences in theophylline dissolution rate from samples prepared with Gelucire® 50/13 and -50/02 upon storage under elevated humidity.

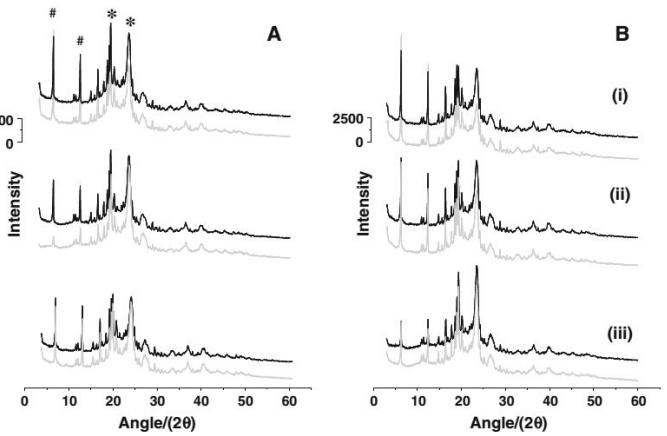

Fig. 5 X-ray patterns from solid dispersions of saquinavir mesylate prepared with Gelucire® 44/14 after 1 (*black line*) and 7 days (*gray line*) of preparation, stored at 25 °C (**a**) or −20 °C (**b**). (*i*) Gelucire® 44/14 + Tween 80 + SQVM, (*ii*) Gelucire® 44/14 + PVP K30 + SQVM, (*iii*) Gelucire® 44/14 + SQVM. (#) and (*) represent crystalline regions for the drug and carrier, respectively

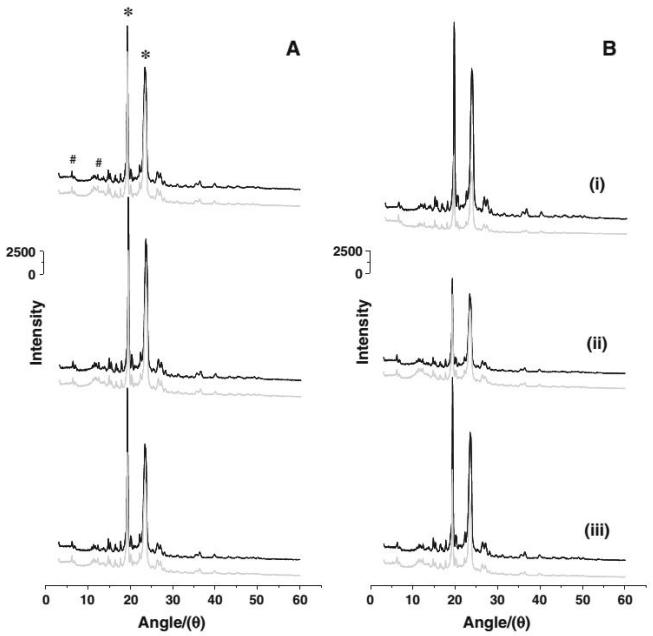

Fig. 6 X-ray patterns from solid dispersions of saquinavir mesylate prepared with PEG 4000 after 1 (*black line*) and 7 days (*gray line*) of preparation, stored at 25 °C (**a**) or −20 °C (**b**). (*i*) PEG 4000 + SQVM, (*ii*) PEG 4000 + PVP K30 + SQVM, (*iii*) PEG 4000 + Tween 80 + SQVM. (#) and (*) represent crystalline regions for the drug and carrier, respectively

Although the addition of Tween 80 in gastric medium has been the most effective approach to improve the apparent solubility of SQVM as far as PEG preparations are concerned, this condition was not the most effective at enhancing the drug dissolution. If the cooling temperature of these formulations with Tween 80 is decreased (−20 °C), interactions between polyethylene oxide chains of the surfactant and PEG may become even more

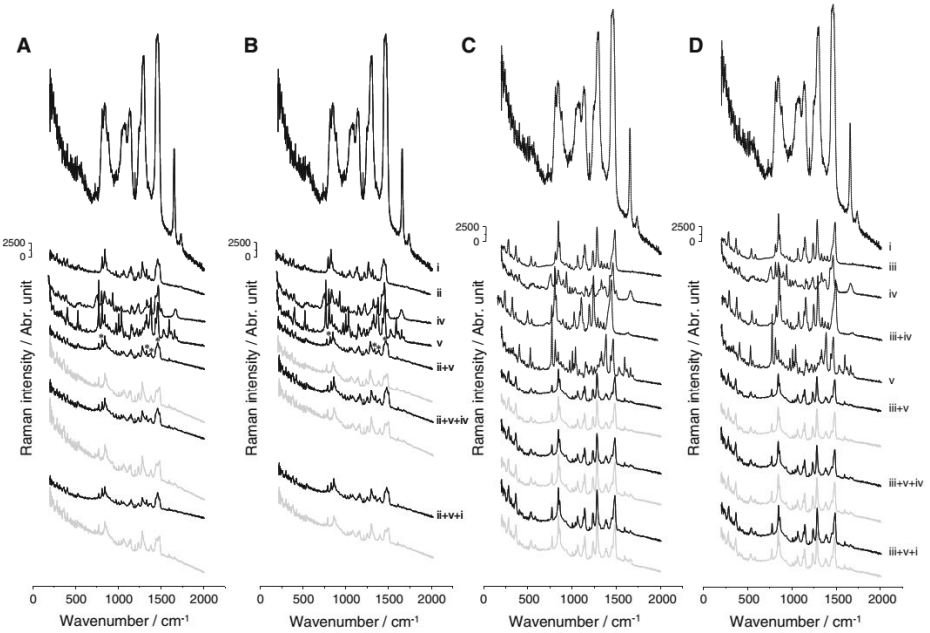

Fig. 7 Raman spectra obtained from solid dispersions of saquinavir mesylate after 1 (*black line*) and 7 days (*gray line*) of preparation, stored at 25 °C (**a**, **c**) and −20 °C (**b**, **d**). **a**, **b** were prepared with Gelucire® 44/14 while **c**, **d** with PEG 4000. Individual spectra for the drug and excipients were obtained in order to understand which material could present chemical changes. (*i*) Tween 80, (*ii*) Gelucire® 44/14, (*iii*) PEG 4000, (*iv*) PVP K30, (*v*) saquinavir mesylate. (*) indicates regions of spectral changes

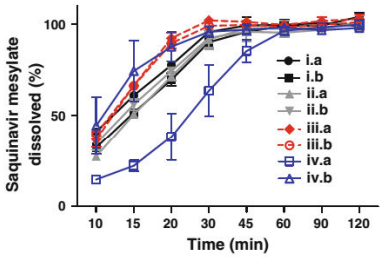

Fig. 8 Transition in dissolution profiles of solid dispersions prepared with saquinavir mesylate, PEG 4000 and PVP K30 (1:8:1) after storage for 1 (*i*), 30 (*ii*), 60 (*iii*) and 90 (*iv*) days at 45 °C/75 % RH (*a*) and 25 °C/95 % RH (*b*)

complicated, increasing the immiscibility between both, as previously reported (Morris et al. 1992) and further reducing drug dissolution. Physical changes were more evident for SDs prepared with Tween 80 over time, with a considerable increase of disorder after 7 days. The water absorption by PEG 4000 would provide greater disorder of PEG crystals, explaining the intensity reduction of the X-ray after a 7-day storage. At the same time, water might affect PEG–Tween or PEG–drug interactions as well as it would increase the drug crystallinity, which may justify the differences found for the dissolution profiles over time. The surfactant addition in PEG-containing SDs did not modify the crystalline order of carrier at the initial period (1 day after the preparation) corroborating the hypothesis that Tween 80 might be incorporated into the amorphous domains of the polymer (Unga et al. 2010).

SDs containing PEG 4000 and PVP K30 were not the most effective at enhancing the dissolution in early steps, however the formulation maintained the drug dissolution behavior stable after a 7-day storage at both temperatures (−20 and 25 °C). These findings can be explained by minor changes in the crystalline order of the carrier in such preparation. Aso and Yoshioka (2005) reported that small amounts of PVP increase the enthalpy relaxation time of amorphous drugs, decreasing their molecular mobility. Labuschagne et al. (2001) evaluated the interaction between PEG and PVP and

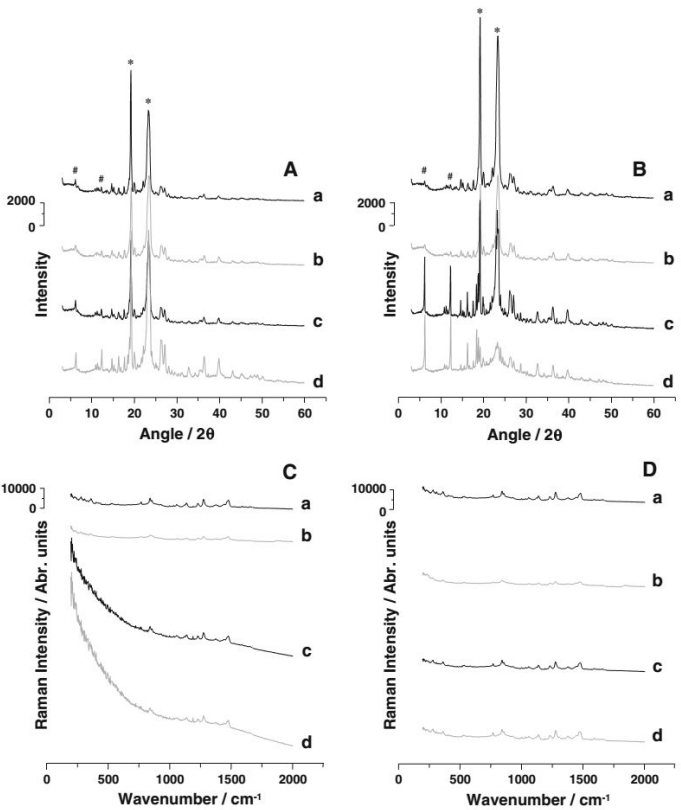

Fig. 9 Physical (**A**, **B**) and chemical (**C**, **D**) stability of saquinavir mesylate solid dispersions evaluated by X-ray diffraction and Raman spectroscopy, respectively. PEG 4000- and PVP K30-containing formulations were stored for 1 (*a*), 30 (*b*), 60 (*c*) and 90 (*d*) days at 45 °C/75 % RH (**A**, **C**) and 25 °C/95 % RH (**B**, **D**). (#) and (*) represent crystalline regions for the drug and carrier, respectively

proposed an interaction between the hydroxyl groups of PEG and the carbonyl groups of PVP. In the presence of water molecules, this interaction is not affected since PEG molecules can interact with PVP carbonyl groups through water molecules bonded to this group. Thus, the formation of this complex would minimize the water effects, which would justify the higher physicochemical stability of SDs prepared with PVP. The pore-forming ability by PVP K30 observed during SEM analysis (Fig. 2) might also increase interactions of dissolution medium with the polymer matrix, which would explain the higher amount of released drug in this condition. The same polymer combination was previously used to enhance the dissolution rate of nimodipine from SDs (prepared by fusion method), a poorly water-soluble drug with low bioavailability and limited clinical efficacy (Gorajana et al. 2010). Suhagia et al. (2006) also used this approach to enhance the dissolution rate of etoricoxib.

Given that the SD prepared with PEG 4000 and PVP K30 was the only one able to increase and maintain the dissolution of SQVM over time at the initial screening stage, this formulation was selected for long-term stability tests under well-controlled conditions. Changes in drug dissolution profiles after storage are not acceptable from a stability standpoint and demand further investigation for the development of stable robust formulations. The formulation maintained the SQVM dissolution properties after 60 and 90 days of storage at 25 °C/95 % RH and 40 °C/75 % (f_2 values >50), respectively, nonetheless it presented a physical–chemical stability limited to 30 days. Dispersions with higher proportion of PVP K30 in PEG or Gelucire®-containing preparations should be tested in future studies. Thybo et al. (2007) also observed that SDs with higher content of PVP K30 retained the tolfenamic acid in amorphous state throughout the stability study

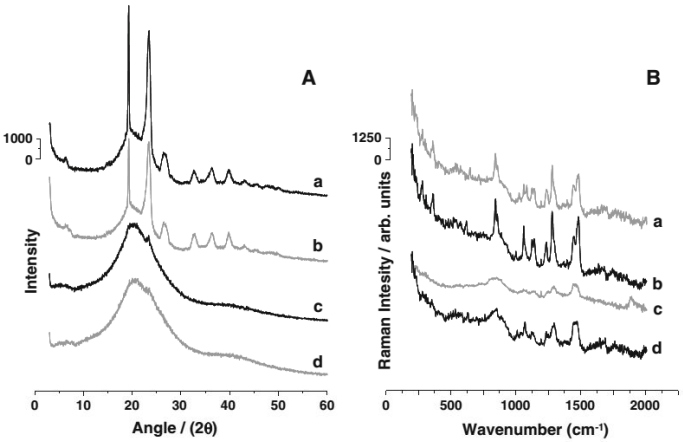

Fig. 10 X-ray patterns (**a**) and Raman spectroscopy (**b**) for Gelucire® 44/14 samples processed under two different humidity and temperature conditions. (*a*) = 37 °C, (*b*) = 25 °C, (*c*) = 37 °C, 25 wt% water, (*d*) = 25 °C, 25 wt% water

(12 weeks), unlike those prepared with a reduced proportion of this polymer.

Summarizing, this study allowed us to understand the different physicochemical processes involved in the SDs stability of SQVM, providing information for the design and improvement of the final formulation.

Acknowledgments We would like to acknowledge FAPESC/CNPq/MS/SES-SC (Grant Number 15.949/2009-2) for financial support. We also thank CAPES/MEC (Nanobiotechnology Network, Grant Number 759/2009) and CNPq/MCT for the authors fellowships. All the XRPD and SEM measurements were performed in multiuser facilities provided by the Federal University of Santa Catarina: Central Laboratory of Electron Microscopy (LCME) and X-ray Diffraction Laboratory (LDRX), respectively. Silvia L. Cuffini thanks to CAPES/PVE program.

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5. ARTIGO SUBMETIDO PARA AVALIAÇÃO

Effect of amphiphilic and hydrophilic carriers on dissolution, permeability and oral bioavailability of saquinavir mesylate

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 # In memoriam

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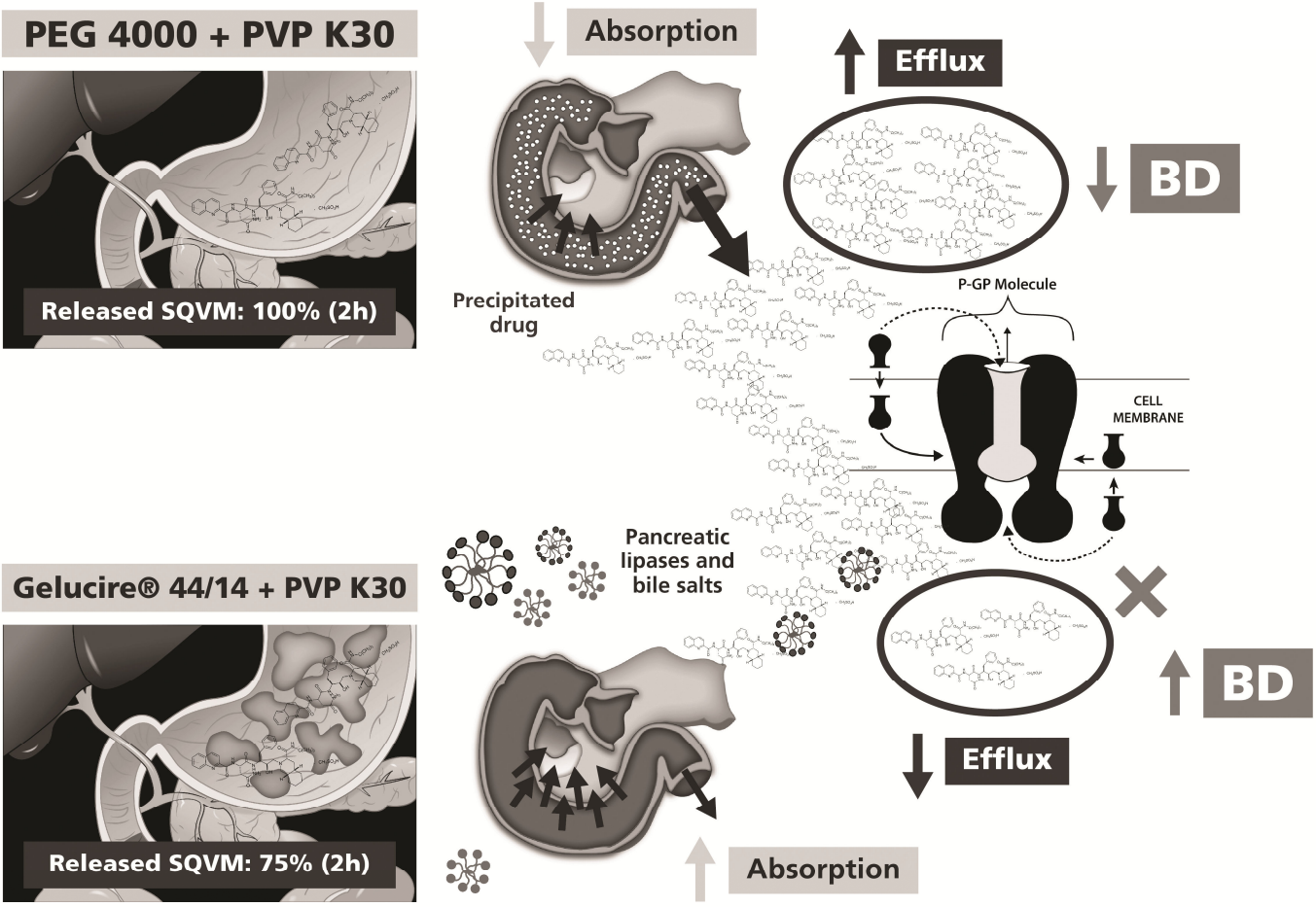
"Nós geralmente descobrimos o que fazer percebendo aquilo que não devemos fazer. E provavelmente aquele que nunca cometeu um erro nunca fez uma descoberta."
(Samuel Smiles)


PEG 4000 + PVP K30
Released SQVM: 100% (2h)
Absorption
Efflux
BD
Precipitated drug
P-GP Molecule
CELL MEMBRANE
Pancreatic lipases and bile salts
Gelucire® 44/14 + PVP K30
Released SQVM: 75% (2h)
BD
Efflux
Absorption

Abstract

The aim of this study was to investigate the effects of solid dispersions (SDs) containing a mixture of PVP K30 and different carriers (PEG 4000 or Gelucire® 44/14) on *in vitro* dissolution and intestinal permeability of saquinavir mesylate (SQVM), and to evaluate their impact on the oral bioavailability of SQVM in a dog model by comparing with Svir® (commercial formulation). Although hydrophilic carriers such as PEG may provide advantages in terms of drug release, their limited solubilization capacity led to precipitation of drug and therefore to a reduced and variable oral bioavailability, as observed for SQVM from this carrier. On the other hand, self-emulsifying systems composed of Gelucire® 44/14 or oleic acid/Castor oil mixture (Svir®) were more effective at improving oral bioavailability of SQVM (approximately 5-fold higher than PEG-based formulations), probably due to the improved solubility of SQVM in GI tract, reduced particle size and inhibitory effects on P-gp (a 2.3-fold reduction of efflux ratio). The selection of carriers and other excipients which display a solubilizing effect and inhibitory effect on P-gp seem to be key factors for increasing the oral bioavailability of SQVM. Gelucire might be considered as a promising carrier for the development of a SQVM commercial formulation.

Keywords: solid dispersion; bioavailability; precipitation; intestinal absorption; drug transport; amphiphilic and hydrophilic carriers; saquinavir mesylate.

1 Introduction

The successful structure-based design of HIV protease inhibitors (PI) introduced new and effective drugs to the HIV/AIDS therapy arsenal[1]. PI figure among first-line therapies since they prevent the early-stage assembly and maturation of infectious virions, and effectively inhibit the HIV replication by blocking the specific cleavage of viral polyproteins[2,3].

Saquinavir, the first representative of this class approved by the FDA, has initially been marketed as hard-gel capsule containing micronised saquinavir mesylate (Invirase® - Roche, USA) and, subsequently, the same manufacturer developed soft-gel capsules (Fortovase®). Although Fortovase® has enhanced oral bioavailability, it has caused gastrointestinal disturbances[4] and then was discontinued in the United States in February 2006[5]. In order to reduce treatment costs, a saquinavir mesylate-based formulation has been recently launched on the market, the soft-gel capsule Svir® (Cristália Pharmaceutical Industry, São Paulo, Brazil).

Given the low and variable oral bioavailability of these formulations in healthy volunteers (4-12%)[6] and the unfavorable pharmacokinetics properties of saquinavir (low absorptive permeability and rapid biotransformation into inactive metabolites)[7], the co-administration of ritonavir has been recommended[8]. Ritonavir competitively inhibits the P-glycoprotein (P-gp) efflux transporter and cytochrome P450 3A enzymes, enhancing the absorption and, consequently oral bioavailability of saquinavir[9].

Nevertheless, this approach is limited by both toxicity resultant from high serum concentrations, and high doses required to inhibit these enzymes/transporters[10]. Furthermore, HIV/AIDS patients often take concomitantly other drugs subjected to cytochrome P450 3A enzymes metabolization or P-gp efflux, potentially leading to drug-drug interactions and additional toxicity[5].

Therefore, novel pharmaceutical formulations that safely enhance the oral bioavailability of saquinavir are required. Different strategies involving processing or formulation modifications[11], development of prodrugs[7,10] or methods to improve the dissolution properties as reduction of particle size[12], and complexation with cyclodextrins[5] have been proposed. Although some of these techniques have been effective at enhancing oral bioavailability, success rates are usually marginal.

Alternatively, solid dispersion has been a promising method for improving the oral bioavailability of poorly water-soluble drugs and the selection of functional carriers has been a determining factor for the success of this technique[13]. Given that saquinavir is a P-gp substrate, this method might confer additional advantages by allowing the selection

of polymers/carriers and excipients that have effects on this transporter. In addition to that, advantages associated with production technology and stability compared to soft gel capsules may be observed and all these facts have led to research efforts in this direction. To the best of our knowledge, our research group was the first to report the development of SQVM solid dispersions[14]. Within this context, different carriers, an amphiphilic (Gelucire) and another hydrophilic (PEG), were used to prepare SDs and their effects on *in vitro* dissolution, intestinal permeability and oral bioavailability of saquinavir mesylate were compared in the present study. Mechanistic studies considering precipitation events as a function of pH were also considered. Comparative bioavaliability studies with the commercial formulation were performed to identify its potential benefits in clinical practice.

2 Experimental

2.1 Materials

Saquinavir mesylate (SQVM) was kindly provided by *Cristália Produtos Químicos Farmacêuticos Ltda* (São Paulo, Brazil), and the commercial formulation (Svir®) was a donation from the Brazilian Health Ministry (Batch 11042699). Gelucire® 44/14 was kindly donated by Gattefossé Corporation (Westwood, NJ). Polyethylene glycol (PEG) 4000, polyvinyl pyrrolidone (PVP) K30, potassium dihydrogen phosphate (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), Hank's balanced salt solution (HBSS), sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES), ethylenediaminetetraacetic acid (EDTA), trypsin, bovine serum albumin (BSA), verapamil hydrochloride, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) with high glucose, fetal bovine serum (FBS), nonessential amino acids (NEAAs), and antibiotics/antimycotic were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals and reagents used in this study were of the highest commercially available purity.

2.2 Preparation of solid dispersions

SDs were prepared by the fusion method. Briefly, SQVM (50 mg per capsule) was added to a mixture of molten carrier (PEG 4000 or Gelucire® 44/14) and PVP K30 (1:8:1 w/w/w). Mixtures were heated at 10°C above the melting point of each carrier for 10 min with continuous stirring (IKA Ultra Turrax® T25, Canada). Three agitation cycles of 1 min each at 13,500 rpm were employed. Hard gelatin capsules were filled (500 mg of formulation) and stored for 1 day at 25°C until the experiments were performed. The content uniformity was evaluated by

HPLC analysis (item 2.3) after extracting the drug from different matrices, and ranged from 99 to 102%. The SDs design and physicochemical characterization have been described previously[14].

2.3 *In vitro* dissolution and particle size measurement

Release profiles from SDs were determined in triplicate by the USP rotating paddle method. SDs or the commercial formulation (Svir®) were added to 900 ml of dissolution medium (0.01 M HCl - pH 1.2 or 34.0g KH_2PO_4 plus 35.3g Na_2HPO_4 in 10L of deionized water - pH 6.8)[15] at 37±0.5°C and stirred at 75 rpm on standard dissolution equipment (Nova Ética, Brazil). Acid and intestinal pH were selected based on recommendations from Guidance for Industry Dissolution Testing of Immediate Release Solid Oral Dosage Forms[16]. Samples of 5.0 ml were withdrawn at 10, 15, 20, 30, 45, 60, 90 and 120 min and filtered at 45µm, and the aliquots taken were immediately replaced by fresh dissolution medium. The amount of dissolved SQVM was assessed by HPLC in a Shimadzu LC-10A system (Kyoto, Japan), equipped with an ultraviolet detector (set at 240 nm), using a C18 column (Perkin Elmer, 5 µm particle size, 250×4.6 mm) as stationary phase. The mobile phase consisted of acetonitrile and 30 mM potassium dihydrogen phosphate (pH adjusted to 3.2 with orthophosphoric acid) in the ratio of 60:40 (v/v). Flow rate was 1 mL/min and retention time was 4.5 min. This method was previously developed and validated according to guidelines on analytical method validation [17] and it was linear in the range of 0.05 to 20 $\mu g.mL^{-1}$ (r>0.999), precise (intraday and interday relative standard deviations <2.15 and 3.07%, respectively), accurate (recoveries ranged from 99 to 101%) and specific. Data were reported as arithmetic mean values ± standard deviation (mean ± SD). Dissolution efficiency (DE) was calculated from the area under the dissolution curve at time t using the trapezoidal rule and expressed as a percentage of the area of the rectangle described by 100% dissolution in the same time[18]. Dissolution efficiency at pH 1.2 and 6.8 for each formulation were statistically compared by unpaired *t-test* and p<0.05 values were considered significant.

After this dissolution test of Gelucire and PEG-based formulations (2 h), samples (approximately 100 mL) were collected from the dissolution vessels for direct measurement of drug particle size in order to clarify the factors that limit drug absorption. The mean diameter of particles over the volume and number distribution ($d_{4.3}$) as well as particle size distribution were determined by laser diffractometry (Mastersizer 2000, Malvern Instruments, UK). The presence of particles was confirmed by optical microscopy (Olympus IX 71).

2.4 Transport of saquinavir mesylate across Caco-2 cell monolayers

Caco-2 cells were obtained from the American Type Culture Collection (ATCC # HTB-37, USA) and were maintained in a humidified 5 % CO_2 air atmosphere at 37°C. Cells from passage 28-37 were cultured in DMEM containing 4.5 g/L glucose with 20 % FBS, 1% NEAAs, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 25 µg/mL of amphotericin B, into Millicell® polycarbonate inserts (0.6 cm^2, 0.4 µm pore size – Millipore, USA) at a density of 10^5 cells/insert, for 21-25 days. Transepithelial electrical resistance (TEER) measurements were performed with a Millicell® ERS meter (Millipore, USA) connected to a WPI Endohm chamber (Sarasota, FL, USA) to control Caco-2 cell monolayers integrity. Only monolayers with TEER values >200 Ωcm^2, before and after transport studies, were considered.

In vitro transport experiments were carried out under sink conditions in both absorptive (apical to basolateral – AP/BL) and secretory (basolateral to apical – BL/AP) directions. HBSS pH 7.4 (10 mM HEPES) was used as transport buffer in both sides of the monolayer, as this allows direct comparison of the permeabilities in both directions without a need to consider pH-dependent effects on the charge of the ionizable compounds studied[19]. For experiments with P-gp inhibition, a solution of verapamil (100 µM) in HBSS pH 7.4 was pre-incubated with cell monolayers for 30 min and used as transport buffer during the experiments. To initiate the experiments, a solution containing 40 µM of SQVM (or its equivalent in SDs) was added to the donor compartment, while the receiver compartment was filled with buffer, and filters were incubated for 2 h at 37°C in an orbital shaker (100 rpm). At suitable time intervals, samples were collected from the receiver compartment and replaced with an equal volume of fresh HBSS.

SQVM was determined by HPLC as previously described, and the apparent permeability coefficients (P_{app}, cm/s) were calculated according to the equation $P_{app} = (\Delta Q/\Delta t) \times (1/AC_0)$, where $\Delta Q/\Delta t$ is the permeability rate (mol/s), A is the surface area of the filter (cm^2) and C_0 is the initial concentration in the donor compartment (mol/ml). The efflux ratio (ER) was determined as ER = ($P_{appBL-AP}/P_{appAP-BL}$). Data were reported as arithmetic mean values ± standard deviation (mean ± SD) of two experiments performed in triplicate (n = 6). Values obtained for each treatment were compared for statistical significance by unpaired Student's *t-test* in each one of the three groups (absorptive, secretory, efflux ratio) (Prism, GraphPad Software, Inc., San Diego, CA, USA). Statistically significant differences were considered at $p<0.05$.

2.5 Bioavailability studies in dogs

The University Institutional Animal Care and Use Committee approved this study (protocol number 23080.016346/2010-62), and appropriate guidelines for the use of animals were observed during all aspects of the study. The objective of this study was to characterize the bioavailability of SQVM from SDs in six fasted female Beagle dogs (±15 kg) as well as to compare the results with those obtained with the commercial formulation (Svir® capsules). Dogs were fasted for at least 16 h prior to formulations administration, and water was available *ad libitum*. Given that Beagle dogs were selected as the animal model for these experiments, a HCl pretreatment was desirable to ensure that gastric pH is consistent and similar to human levels[20]. Thus, the oral administration of 0.02 N HCl (10 mL) was carried out 10 min prior to the formulation administration. The commercial formulation (200 mg/dose) or one of the test formulations (200 mg/dose) were administered orally to the dogs in a randomized crossover manner with at least one week washout period. This SQVM dose was defined based on oral escalating dose studies in dogs[21]. Blood samples were collected from the jugular vein via direct venipuncture and placed into chilled heparin-containing polypropylene tubes at different periods of time: 0 (predose), 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8 h after dose. Plasma samples were obtained by centrifugation of whole blood samples and stored at -20°C prior to analysis.

Plasma samples (250 µL) were extracted by protein precipitation with 250 µL of acetonitrile (containing verapamil as internal standard at 10 µg L^{-1}). Following vortex mixing and centrifugation (12,000 rpm, 5 min), 250 µL of supernatant were removed and added to an equal volume of 0.1% formic acid in water. Following vortex mixing, 10 µL aliquots were injected in the LC–MS/MS system in order to quantify plasma concentrations of SQVM. The separation of this analyte from matrix components was achieved over 5-5.5 min using a 150×2.0 mm Phenomenex C18 (4 µm particle size) column and an isocratic elution (20 mM ammonium formate, pH 3, and acetonitrile in the ratio of 50:50 v/v) using an Agilent system (Waldbronn, Germany). The flow rate was set at 0.25 mL/min. The LC system was coupled to a mass spectrometry system consisting of a hybrid triplequadrupole/linear ion trap mass spectrometer Q Trap 3200 (Applied Biosystems/MDS Sciex, Concord, Canada). Analyst version 1.5.1 was used for the LC-MS/MS system control and data analysis. The analyses were performed using the TurboIon Spray source (electrospray-ESI) in positive ion mode. The capillary needle was maintained at 4,500 V. MS/MS parameters were: curtain gas (10 psi); temperature (450°C); gas 1 (45 psi); gas 2 (45 psi); CAD gas (medium). Optimization of the mass spectrometer was performed by the direct infusion of the analyte aqueous solution. This

method was previously developed and validated according guidelines on bioanalytical method validation[22] and it was linear in the range of 10 to 100 ng.mL^{-1} (r>0.999), precise (intraday and interday relative standard deviations <11.12 and 15.13%, respectively), accurate (recoveries ranged from 91.32 to 100.31%) and specific.

The maximum plasma concentration (C_{max}) and the time to reach peak concentration (T_{max}) were obtained directly from the concentration-time data of each dog. The area under the curve (AUC_{0-t}) was determined using the trapezoidal rule using the Excel vs. 2012 software (Microsoft Corporation, EUA). The relative bioavailability (F_{rel}) of the SDs was calculated using the equation F_{rel}=$AUC_{0-t(test)}$/$AUC_{0-t(reference)}$×100%. For each one of the PK parameters, data were reported as arithmetic mean values ± standard deviation (mean ± SD) and compared for statistical significance using analysis of variance (ANOVA), with post-hoc Tukey test (Prism, GraphPad Software, Inc., San Diego, CA, USA), at p<0.05.

3 Results

3.1 *In vitro* dissolution and particle size measurement

Svir® capsules released SQVM more rapidly at pH 1.2 than at pH 6.8 (100% in 10 and 45 min, respectively) and provided higher dissolved-drug concentrations than the SDs (Fig. 1). Interestingly, SQVM was released faster from the PEG 4000-based SDs than from those with Gelucire® 44/14 at pH 1.2 (about 100% vs. 75% of released SQVM from SDs after 2h, respectively) while an opposite effect was observed at pH 6.8.

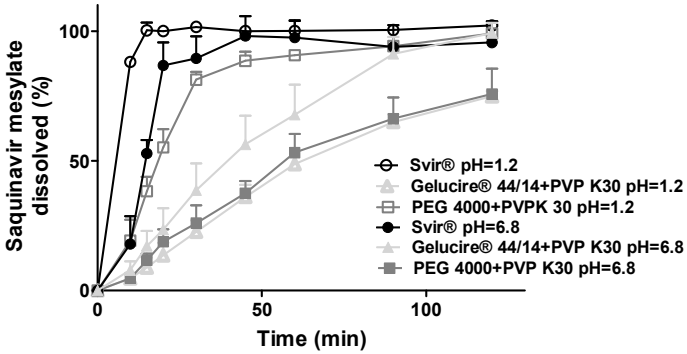


Fig. 1. Dissolution profiles from different preparations of saquinavir mesylate. Results are shown as mean ± S.D. (n=3). Testing was performed for 2 h in 900 mL of dissolution medium at pH 1.2 and 6.8.

Svir® showed a higher dissolution efficiency at pH 1.2 (96.69±0.41) compared to that at pH 6.8 (81.03±8.05%; p=0.0281), and the same pattern was observed for the PEG-based SDs (73.69±9.97 at pH 1.2 vs. 45.92±12.36% at pH 6.8; p=0.0389). Conversely, Gelucire-based SDs showed higher dissolution efficiency at pH 6.8 (61.67±10.83 vs. 43.07±2.765 at pH 1.2; p=0.0449).

In order to check the presence of undissolved drug or drug precipitation or even the formation of colloidal structures (in case of amphiphilic SDs) during *in vitro* dissolution studies, particle size assays were performed after dissolution at pH 1.2 and 6.8 (Fig. 2). Gelucire-based SDs displayed minor changes in mean particle size when pH varied from 1.2 to 6.8 ($d_{4.3}$ ranged from 10.4 to 27.6 µm, respectively), while more significant changes were noted for those SDs prepared with PEG ($d_{4.3}$ ranged from 7.8 to 68.6 µm, respectively). Furthermore, multimodal distributions with large variability were observed at pH 6.8, indicating the formation of agglomerates. To confirm that these findings on particle size could be associated with drug precipitation or aggregate formation, optical microscopy analysis were performed and PEG-based SDs clearly demonstrated drug precipitation events at pH 6.8 (Fig. 3).

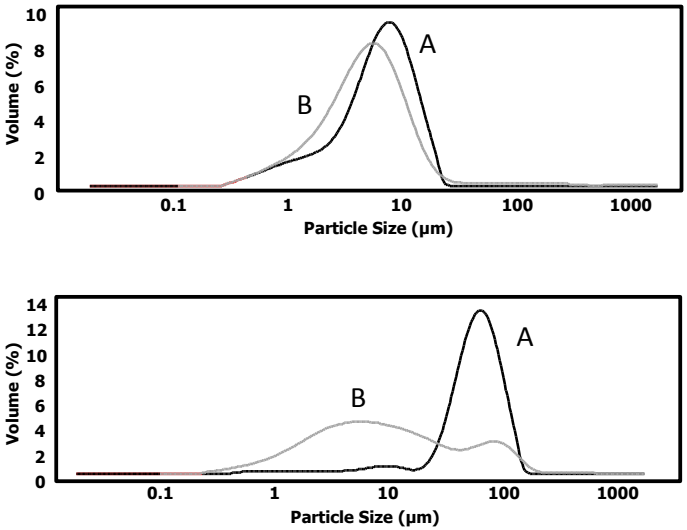


Fig. 2. Particle size distribution (PSD) after dissolution test of solid dispersions prepared with PEG 4000 and PVP K30 (A) or Gelucire® and PVP K30 44/14 (B) at pH 1.2 (upper) and 6.8 (bottom). Results are shown as mean ± S.D. (n=3).

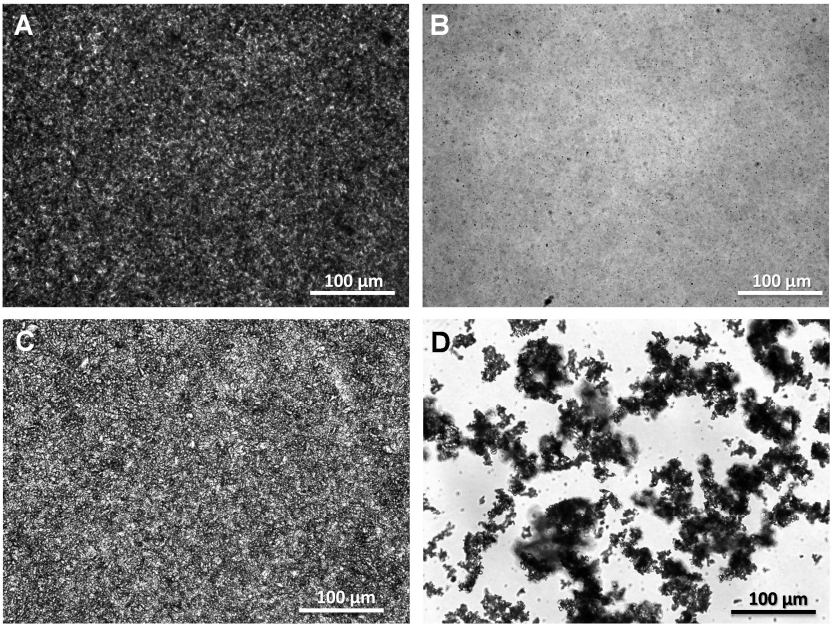


Fig. 3. Optical microscope images after dissolution test of solid dispersions prepared with PEG 4000 and PVP K30 (A,B) or Gelucire® and PVP K30 44/14 (C,D) at pH 1.2 (A,C) and 6.8 (B,D).

3.2 Transport of saquinavir mesylate in Caco-2 cell monolayers

Given that SQVM intestinal efflux mechanisms involving P-gp and MRP are already well-known[23], the effect of SDs excipients on the Caco-2 cells permeability of SQVM was investigated (Table 1). It is not feasible to add the entire content of SDs in cell culture and the partition into small fragments also presents some practical limitations (lack of uniformity in the fragment size particularly in Gelucire-based SDs since this material has a gel-like aspect; the drug release profile would be changed since the surface area is increased). Due to these limitations, solutions containing each carrier (Gelucire® 44/14 and PEG 4000) and PVP K30 were prepared in the same ratios to those of SDs with the help of a high speed homogenizer (to allow micellar solubilization of drug). This approach assumes that drug molecules are both dissolved and free to be absorbed in intestinal mucosa, similarly to that observed in vivo.

Table 1. In vitro intestinal permeability values of different SQVM preparations across Caco-2 cell monolayers.

	Transport direction		
	Absorptive P_{app}AP-BL (cm s^{-1}, x10^{-6})	Secretory P_{app}BL-AP (cm s^{-1}, x10^{-6})	Efflux ratio (ER)
SQVM	6.34 ± 0.91	31.23 ± 11.50	4.85 ± 1.12
SQVM/verapamil	13.46 ± 2.31**(p=0.0077)	17.47 ± 1.01*(p=0.01079)	1.31 ± 0.22**(p=0.0058)
PEG 4000/PVP K30/SQVM	4.82 ± 0.84	41.09 ± 22.73	8.23 ± 3.28
Gelucire® 44/14/PVP K30/SQVM	10.29 ± 3.31	21.92 ± 11.01	2.07 ± 0.41*(p=0.0156)

Data are shown as mean ± SD (n=6).
Each SQVM preparation was compared to drug-containing solution by unpaired Student's *t-test* in each one of the three groups (absorptive, secretory, efflux ratio).

The secretory permeability ($P_{aapBl-AP}$) of SQVM (40 μM) through Caco-2 cell monolayers was approximately 5-fold greater than that in the absorptive direction (A-to-B), which is consistent with efflux via Pg-p. Additionally, co-incubation with the P-gp inhibitor verapamil reduced secretory SQVM transport (*p*=0.1079, *t-test*) and efflux index (*p*=0.0058, *t-test*), as expected, demonstrating that this model is physiologically representative to assay samples that impair P-gp mediated efflux.
The presence of PEG 4000 and PVP K30 did not affect SQVM transport significantly (*p*>0.05, *t-test*). On the other hand, Gelucire® 44/14 and PVP K30 reduced the efflux ratio to a level similar to that of verapamil (*p*>0.05, *t-test*).

3.3 Bioavailability studies in dogs

The plasma concentration–time profiles and pharmacokinetic parameters obtained for the tested SDs are summarized in Fig. 4 and Table 2, respectively. No treatment-related clinical signs or mortality were observed. SQVM was rapidly absorbed by dogs from Gelucire-based SDs, displaying T_{max} values similar to those of the commercial formulation Svir® (p>0.05).

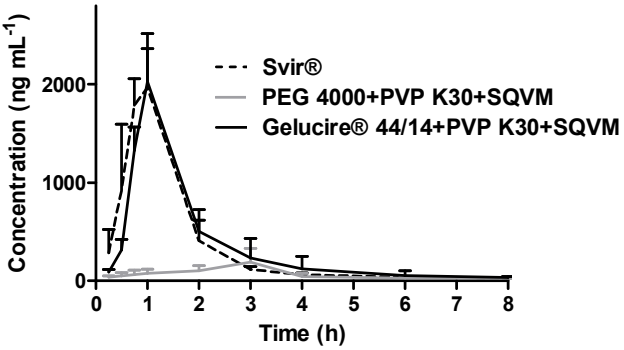


Fig. 4. Mean (±S.D.) concentration vs. time profile after oral administration of different saquinavir mesylate preparations (200 mg) in Beagle dogs under fasting conditions (*n*=6).

Table 2. Summary of pharmacokinetic parameters obtained from female Beagle dogs after oral administration (dose mg kg^{-1}) of SQVM-containing SDs and Svir® (commercial) capsules (*n*=6).

Parameters	SDs (PEG 4000+PVP K30+SQVM)	SDs (Gelucire® 44/14+PVP K30+SQVM)	Svir®
$AUC_{0\text{-}480}$ (ng h mL^{-1})[a]	536.2 ± 245.3***	2763.8 ± 515.3	2388.8 ± 744.5
C_{max} (ng mL^{-1})[b]	210 ± 130***	2120 ± 330	2130 ± 210
T_{max} (h)[c]	2.6 ± 0.8***	1 ± 0.00	0.9 ± 0.1
F_{rel} (%)	22.44	115.70	n/a

Differences among SQVM formulations administered orally were determined using one-way analysis of variance (ANOVA) followed by the Turkey's pos hoc test for each pharmacokinetic parameter individually.
[a]F=29.10, $p<0.0001$; [b]F=129.5, $p<0.0001$; [c]F=25.9, $p<0.0001$.
(***) $p<0.0001$
n/a = not applicable.

$AUC_{0\text{-}8h}$ values for the Gelucire-based SDs and Svir® (both formulations administered orally) were similar (p>0.05), whereas for PEG 4000-based SDs were approximately 4.5-fold lower (F=29.10, p<0.0001) than that of Svir®.

C_{max} value for the Gelucire-based SDs were at least 10-fold greater than that of the PEG-based SDs (F=129.5, p<0.0001), while C_{max} of the Svir® was similar to that of the Gelucire-based SDs (p>0.05).

The oral bioavailability of SQVM after administration of Gelucire-containing SDs was found similar to that of Svir®. For PEG-based SDs, the oral bioavailability of SQVM was approximately 5-fold lower than that of the commercial formulation. Overall, it is important to note that PEG-based solid dispersions of SQVM were characterized by significant variability in their PK parameters.

4 Discussion

The development of oral formulations able to delivery class IV drugs as SQVM[24], promptly to be absorved in intestine is a great challenge. In such scenario, SD is a valuable approach. In this process, the investigation of the role of different functional carriers or excipients able to avoid drug precipitation upon release from the dosage form in the gastrointestinal tract is extremely relevant[25,26]. It becomes even a greater problem if the salt form of the drug is considered, since it may precipitate in the GI fluid after its oral administration into their free acid and base forms and, in these situations, it might not be able to enhance bioavailability of drugs adequately[27].

Because dissolution time about 30-60 min is representative of gastric residence time[28] and PEG and Gelucire-based SDs showed an incomplete dissolution at pH 1.2 in this period (90 and 50%, respectively), one can expect a complete dissolution in the intestinal fluid. Gelucire-based SDs had its dissolution rate augmented as the pH varied from 1.2 to 6.8. This may be associated with a relatively higher erosion at pH 6.8, a phenomenon already reported involving a partial hydrolysis of Gelucire matrices in their ester bonds[25]. Even though PEG-based SDs have not provided significant SQVM release at pH 6.8 compared to those with Gelucire, the dissolution at pH 1.2 was enough to ensure high levels of released drug and, in this condition, a solubilizing effect for this carrier would be expected, especially in the intestinal lumen.

Although PEG-based SDs have shown rapid dissolution, low oral bioavailability was observed (Fig. 4), which may be associated with drug precipitation in intestinal medium, a common phenomenon in formulations with a high content of hydrophilic materials[25]. It is well described a lower solubility of the drug at intestinal pH compared to simulated gastric fluid[12]. Precipitation events were observed

microscopically for PEG-based SDs at pH 6.8 and an increased particle size was observed when the pH was changed from pH 1.2 to 6.8. In fact, a significant role of PEG in protein crystallization has also been reported, mainly when its molecular weight varies from 2000 to 6000[29]. PEG was selected since it is widely used for SDs preparation by the fusion method, offers advantages during the large-scale production and it can also be used to avoid decomposition of drugs after their melting[30]. In addition to that, several SDs using this polymer are commercially available[31]. Despite of the advantages of using PEG in SD formulations, its solubilization capacity seems to be lower than that of Gelucire® 44/14 upon contact with intestinal fluids. The acylglycerol fraction of Gelucire® 44/14 is responsible for the solubilization of lipophilic drugs, whereas the mono- and diesters of PEG present surfactant properties and free PEG acts as a cosolvent[32]. Based on these observations, Gelucire® 44/14 provides different solubilization mechanisms since it is a complex mixture of various compounds. Its chemical composition can be changed with the lypolysis in GIT, which could explain the higher solubilization capacity upon release from the dosage form compared to PEG. In solubility experiments carried out at 37°C at pH 1.2 and pH 6.8 in a period of 24 h, the apparent solubility of SQVM dispersed into Gelucire® 44/14 and PVP K30 was enhanced in approximately 15 times as the pH varied from 1.2 to 6.8 (296.11±6.26 vs. 4,299.01±10.30 μg mL^{-1}, respectively).When associated to PEG 4000 and PVP K30, SQVM had an apparent solubility approximately 3 to 20-fold lower than that of Gelucire® 44/14 and PVP K30 at pH 1.2 and pH 6.8, respectively. It is well known that water soluble co-solvents, such as PEG, loose rapidly their solvent capacity after the dispersion of the formulation in an aqueous phase[33], which may contribute to drug precipitation in intestinal medium (as observed in microscopic analysis), thereby further decreasing the amount of drug available for absorption.

The SQVM absorption through Caco-2 cell monolayer was also dependent on carrier type and the data obtained from this model showed an association with the pharmacokinetic profiles *in vivo*. Although the concentration/volume ratio from *in vitro* permeability studies has been lower than from *in vivo* experiments, concentrations in which the efflux is not saturated were selected in order to evaluate the effect of different excipients on this parameter. Regarding the improvement of solubility and dissolution provided by some formulations, it is expected that the passive permeability will have a greater influence in the absorptive process (the role of transporters would be less noticeable) in these situations since there is a higher amount of free drug available to be absorbed. Passive and carrier-mediated processes would coexist in drug transport[34]. The combination of PEG 4000 and PVP K30, in fact, increased significantly the efflux ratio values, unlike those with Gelucire and PVP (it reduced this parameter similarly to verapamil, a well-known

P-gp inhibitor). Interestingly, it was observed that the ratio between AUC_{0-8h} from Gelucire and PEG-based SDs was equivalent to the efflux ratio values of these same formulations. These observations lead us to hypothesize that not only the dissolution process but also the permeability (efflux) were the rate-limiting steps in absorption process of SQVM, which is expected in view of its biopharmaceutical classification[24]. Because Beagle dogs present low gut-wall CYP3A4 metabolism compared to humans[21] and Caco-2 cells display low expression of these enzymes[35], P-gp has played a primary role on intestinal transport of SQVM regarding its considerable expression in both systems (*in vitro* and *in vivo*).

Intestinal membrane damages were not the reason for an enhanced absorptive transport after treatment with Gelucire, since this carrier was used at non-cytotoxic concentrations (0.02% w/v)[36], and TEER values obtained after the experiments were consistent with the resistance values obtained prior to experiments (>200 Ωcm^2). However, reduction on P-gp expression to 65 and 52% has been reported after 24 h treatment with Gelucire® at 0.01 and 0.02% (w/v), respectively[36]. Although PEG has been shown to interact with P-gp[37], it did not reduce the efflux index values at 0.02% w/v. A concentration-dependent inhibitory effect of PEG on P-gp, which vary from 0.1 to 20% (w/v)[38], may justify these findings.

It is well known that the oral bioavailability of SQVM is higher at fed conditions compared to fasted conditions[39], but the experiments were carried out under fasting conditions in order to avoid the carrier or drug-nutrient interactions, changes in residence times in the GI tract, in gastric emptying rates or other interferences that could lead to misleading interpretation of the results.

The oral bioavailability of SQVM from Gelucire-based SDs was approximately 5-fold higher than that from the formulation with PEG. Similarly, oral bioavailability of DMP 323 in Beagle dogs treated with glycol-based vehicles was approximately 8-fold lower than that formulated with Gelucire® 44/14[40]. One of the reasons to explain these different behaviors of carriers may be the lipolysis process that occurs with Gelucire® 44/14 in the GI tract. This carrier is hydrolyzed by digestive lipases and their lypolitic products might play a relevant role in transport of drugs from formulation to the mixed micelles and/or the unstirred water layer next to the enterocytes[32]. Additionally, formulation components and endogenous lipids (bile salts, phospholipids) may act synergistically to further enhance the solubilization of SQVM in colloidal structures[41,42]. The formation and stabilization of a metastable supersaturated state of the drug before precipitation may also contribute to explain these findings (the enhancement of bioavailability for Gelucire-based SDs). This phenomena increase the thermodynamic activity of the drug beyond its solubility limit and, therefore, result in an increased

driving force for transit into and across the biological barrier[43]. Since the PEG-based SDs display low solubilizing effect, an uncontrolled precipitation of drug upon dosing would be observed instead of creating a supersaturated state, which would explain the low bioavailability provided for this formulation. On the other hand, Gelucire-based SDs would be able to increase free drug concentration *in vivo* due to the stabilization of the supersaturated state, increasing the oral bioavailability.

Given the similar bioavailability of Svir® and Gelucire-based SDs, a comparable absorption and solubilization mechanism might be suggested. Svir® is also able to form fine oil-in-water emulsions or micro-emulsions upon mild agitation *in vivo* since it is composed of oleic acid, polyoxyl 35 castor oil, ethanol and butylhydroxytoluene, which present self-emulsifying properties[44]. Furthermore, mono- and diglycerides of oleic acid as well as polyoxyl 35 castor oil have also presented effects on P-gp[36].

According to the results, the development of Gelucire-containing SDs is a promising carrier for SQVM, providing high oral bioavailability. Additionally, it does not require refrigeration during transport and storage, reducing logistic costs. Although comparative stability tests between Svir® and Gelucire-containing SDs have not been carried out, solid systems have been reported to be more physically stable due to their lower molecular mobility[45].

5. Conclusions

Given that SQVM is a P-gp substrate, dissolution and solubility alone may not be used to predict rank order of its oral bioavailability during SDs development phase. The increased dissolution rate observed for the PEG-based SDs at pH 1.2 did not yield a substantial enhancement of oral bioavailability probably due to the limited solubilization capacity of PEG added to precipitation of SQVM at elevated pH conditions of intestine. On the other hand, self-emulsifying Gelucire-based SDs increased amount of solubilized SQVM in GI tract, which would reduce drug precipitation in intestinal pH. Furthermore, this formulation reduced the P-gp mediate transport of SQVM, resulting in an increased oral bioavailability when compared with the PEG-based SDs.

Acknowledgments

We would like to acknowledge FAPESC/CNPq/MS/SES-SC (grant number 15.949/2009-2) for financial support. We also thank CAPES/MEC (Nanobiotechnology Network, grant number 759/2009) and CNPq/MCT for the authors fellowships. The authors would also like to thank Romina Carrera for her help in preparing the graphical abstract.

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6. ARTIGO SUBMETIDO PARA AVALIAÇÃO

Pharmacokinetic evaluation of oral self-emulsifying lipid based delivery systems containing a poorly soluble antiviral drug

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"Porque cada um, independente das habilitações que tenha, ao menos uma vez na vida fez ou disse coisas muito acima da sua natureza e condição, e se a essas pessoas pudéssemos retirar do quotidiano pardo em que vão perdendo os contornos, ou elas a si próprias se retirassem de malhas e prisões, quantas mais maravilhas seriam capazes de obrar, que pedaços de conhecimento profundo poderiam comunicar, porque cada um de nós sabe infinitamente mais do que julga e cada um dos outros infinitamente mais do que neles aceitamos reconhecer."
(José Saramago)

Abstract

The pharmacokinetics of a commercial formulation and a lipid based delivery system containing saquinavir mesylate (SQVM) orally administered to Beagle dogs were compared with that following na I.V. bolus infection of the drug (1 mg/Kg). Plasma concentration of the drug was determined by LC/tandem mass spectrometry. The curve disposition curve of SQVM when given intravenously was better described by a three-compartment model. On the other hand, plasma profiles obtained following the oral dose were fitted by a two-compartment model with lag time. Similar absorption rate constants (k_a) and peripheral to central compartment transfer rate constants (k_{21}) were also found for both oral lipid delivery systems.

Keywords: pharmacokinetics; lipid based carrier systems; saquinavir mesylate; non-compartmental analysis; compartmental analysis.

Typically, poorly soluble compounds have poor and erratic absorption as well as limited bioavailability, which require the development of new drug delivery systems[1]. Lipid-based systems have gained much interest as carriers for the delivery of these drugs in recent years, particularly due to their ability to improve the solubility/dissolution, to prolong the gastric residence time, stimulation of lymphatic transport, affeting intestinal permeability and reducing metabolism and efflux activity[2]. Lipid formulations include simple solutions, self-emulsifying drug delivery systems (SEDDS), and micellar solutions[3]. This approach has led over the past years to successfully enhance the bioavailability of some antiretroviral drugs such as efavirenz (Sustiva®), saquinavir (Fortovase® or Svir®) and ritonavir (Norvir®). Although various studies have evaluated the oral bioavailability and basic pharmacokinetics parameters of lipid systems containing poorly water-soluble drugs, compartmental pharmacokinetic analyses using different kinetic models has been poorly explored. Thus, the purpose of this study was to assess the compartmental plasma pharmacokinetics of self-emulsifying lipid based carrier systems containing saquinavir mesylate (SQVM). This drug was selected because of its low and variable oral bioavailability[4]. Our group has been investigating the development and characterization of another SQVM-based semi-solid system, which is composed mainly by Gelucire® and filled into hard gelatin capsules[5].

Semi-solid and liquid self-emulsifying drug delivery systems were then compared in order to understand if a similar absorption-disposition kinetics may be obtained for both systems.

Six female beagle dogs (±15 kg) were fasted overnight and water was provided *ad libitum*. Before experiments, a HCl pretreatment (10 mL of 0.02 M HCl administered 10 min prior to formulation) was carried out to obtain an gastric pH close to human levels[6]. Experiments were according to a randomized cross-over design (protocol 23080.016346/2010-62, approved by the Animal Ethics Committee, Universidade Federal de Santa Catarina). Each dog received an intravenous (i.v.) single dose of SQVM (1 mg/Kg in 10% DMSO administered into cephalic vein over a 120 s period) or oral formulation (Svir® or semi-solid formulation based on Gelucire®, 200 mg/dose, which was defined based on a study of escalating doses[7]) with a whashout period of 1 week. Blood samples were collected from the jugular vein via direct venipuncture and placed into chilled heparin-containing polypropylene tubes at different periods of time: 0 (predose), 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8 h or 0.17, 0.33, 0.5, 0.67, 0.83, 1, 2, 4, 5, 6, 7, and 8 h after oral and i.v. dose, respectively. Plasma samples were obtained by centrifugation of whole blood samples and stored at -20°C prior to analysis.

Plasma samples (250 µL) were extracted by protein precipitation with 250 µL of acetonitrile (containing verapamil as internal

standard at 10 μg L^{-1}). Following vortex mixing and centrifugation (12,000 rpm, 5 min), 250 μL of supernatant were removed, added to an equal volume of 0.1% formic acid and injected into the LC–MS/MS system. This method was previously validated and analytical parameters were optimized. Briefly, the separation of this analyte from matrix components was achieved over 5-5.5 min using a 150×2.0 mm Phenomenex C18 (4 μm particle size) column and an isocratic elution (20 mM ammonium formate, pH 3, and acetonitrile in the ratio of 50:50 v/v). The flow rate was set at 0.25 mL/min. The LC system (Agilent system, Waldbronn, Germany) was coupled to a mass spectrometry system consisting of a hybrid triplequadrupole/linear ion trap mass spectrometer Q Trap 3200 (Applied Biosystems/MDS Sciex, Concord, Canada). The Analyst version 1.5.1 software was used to the LC-MS/MS system control and data analysis. The analyses were performed using the TurboIon Spray source (electrospray-ESI) in positive ion mode. The capillary needle was maintained at 4,500 V. MS/MS parameters were: curtain gas (10 psi); temperature (450°C); gas 1 (45 psi); gas 2 (45 psi); CAD gas (medium). Optimization of the mass spectrometer was performed by the direct infusion of the analyte aqueous solution.

Pharmacokinetic profiles were analyzed using both non-compartmental and compartmental approaches. In the non-compartmental analysis, the area under the plasma concentration–time curve (Fig. 1) from time zero to time infinity ($AUC_{0-\infty}$) was determined using a combination of trapezoidal and log-trapezoidal methods plus the extrapolated area. The extrapolated area was determined by dividing the measured concentration at the time of last non-zero plasma concentration by the slope of the terminal log-linear phase. Apparent elimination rate constant (k_e) was estimated by linear regression analysis of the terminal portion of the log concentration-time data and apparent elimination half-life ($t_{1/2\ \gamma}$) as ln $2/k_e$. The mean residence time (MRT) was calculated using $AUMC/AUC_{0-\infty}$. Total blood clearance (CL_{tot}) was calculated using $D/AUC_{0-\infty}$, in which D represents the SQVM dose administered. The steady-state volume of distribution (Vd_{ss}) was calculated by multiplying CL_{tot} by MRT.

Compartmental analysis of individual concentration–time curves was performed by Scientist® software. The plasma profiles obtained after the intravenous and oral administration of the compound were best described by a three-compartment (equation 1) and two-compartment with a lag time between the time of administration and the onset of absorption (equation 2) open model, respectively.

$$C = A.e^{-\alpha.t} + B.e^{-\beta.t} + C.e^{-\gamma.t} \text{ (eq. 1)}$$

Where C is the total plasma concentration over time t; A, B and C are the intercepts of the distribution to the shallow peripheral

compartment, distribution to the deep peripheral compartment and elimination phase, respectively; and α, β and γ are the distribution rate constants for the shallow peripheral compartment, deep peripheral compartment and the elimination rate constant, respectively.

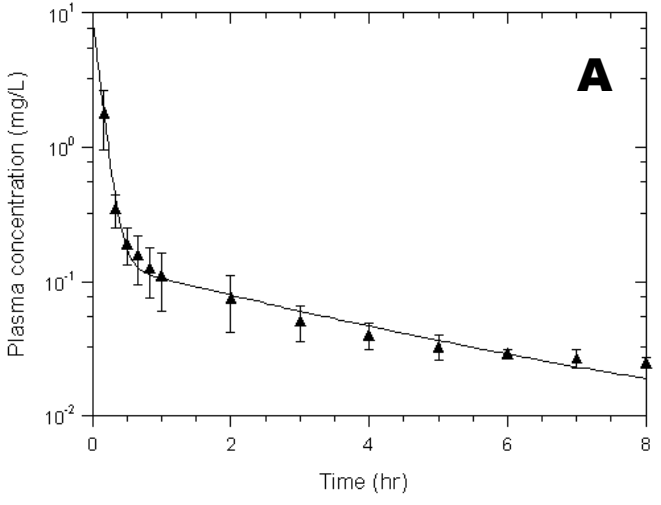
$$C = \frac{F.D.k_a}{V_c}\left[\frac{k_{21} - \alpha}{(\alpha - k_a).(\alpha - \beta)}.e^{-\alpha.(t-t_0)} + \frac{k_{21} - \beta}{(\beta - k_a).(\beta - \alpha)}.e^{-\beta.(t-t_0)} + \frac{k_{21} - k_a}{(k_a - \alpha).(k_a - \beta)}.e^{-k_a.(t-t_0)}\right] \quad \text{(eq. 2)}$$

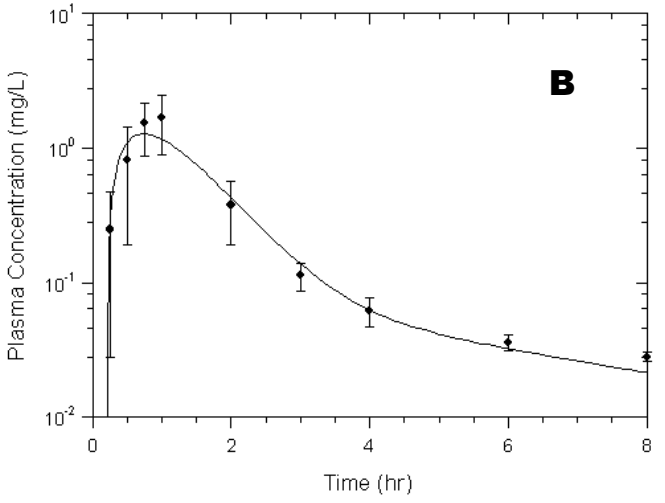
Where F is the bioavailability; D, dose; C is the total plasma concentration over time t; k_a is absorption rate constant; V_c, central volume of distribution; k_{21}, distribution rate constant from compartment 2 to compartment 1; α and β are the distribution and elimination rate constants, respectively; $t\text{-}t_0$ is the lag time between the time of administration and the onset of absorption.

In order to obtain the best fitting of the plasma profiles, non-weighted data were used for modelling. Model selection was guided using visual inspection of the observed versus estimated concentrations. Furthermore, model selection criteria (MSC) and the correlation coefficient (*r*) provided by the software were used.

The pharmacokinetic parameters determined by the non-compartmental and compartmental analysis after intravenous administration and those obtained after oral administration of different formulations were statistically compared by unpaired *t*-Student test (GraphPad Prism®, version 5).

Comparing the non-compartmental and compartmental analyses can be useful in determining consistency between stimated pharmacokinetics methods. Both methods provided similar V_c, V_{dss}, $AUC_{0\text{-}\infty}$, $t_{1/2}$ γ, CL_{tot}, MRT values (p>0.05) (Table 1), and therefore the selected compartmental model was found to be suitable for describing plasma concentration-versus-time data (Fig. 1A) after intravenous SQVM administration.





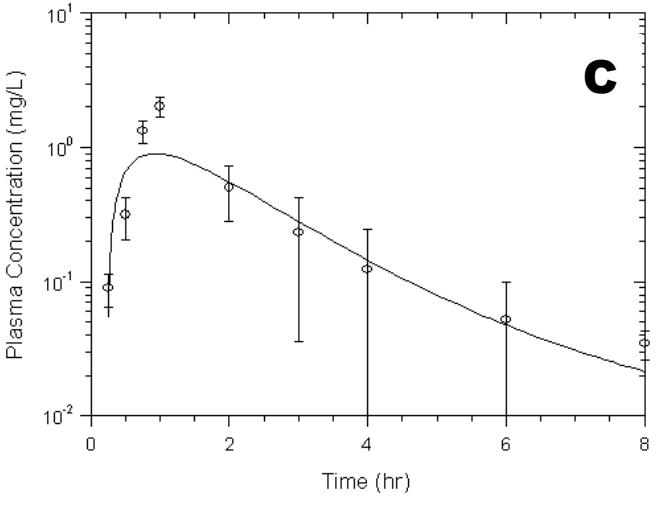


Fig. 1. Plasma-time concentration curves of saquinavir mesylate following intravenous administration of 1 mg/kg dose (A) or oral administration of Svir® (B) or Gelucire®-based dispersion solid (C) to Beagle dogs.

Table 1. Pharmacokinetic parameters obtained by compartmental and non-compartmental analysis following intravenous single dose (1 mg/Kg) of saquinavir mesylate (mean ± SD) (*n*= 6).

	Compartmental	Non-compartmental
V_c (mL.Kg^{-1})	8.67 ± 45.1	ND
Vd_{ss} (mL.Kg^{-1})	2,510.48 ± 1,742.33	1,648.49 ± 1,067.91
$AUC_{0-\infty}$ (ng.h.mL^{-1})	1,933.04 ± 578.61	1,605.83 ± 519.3
$t_{1/2}\gamma$ (h)	8.53 ± 4.00	9.45 ± 2.8
CL_{tot} (mL.h^{-1})	562.06 ± 194.06	670.8 ± 191.74
MRT (h)	4.36 ± 2.98	2.09 ± 1.19
MSC	1.09 - 6.38	ND
r	0.94 - 0.99	ND

ND, not determined. V_c, distribution volume of the central compartment; Vd_{ss}, volume of distribution at steady state; $AUC_{0-\infty}$, area under the plasma concentration; t½ γ, terminal half-life; CL_{tot}, total clearance; MRT, mean residence time; MSC, model selection criteria and *r*, correlation coefficient.

SQVM disposition was best described by a three-compartment open model after IV administration, which has not yet been reported for this drug concerning this administration route.

No statistical difference was found when each non-compartmental pharmacokinetic parameter of the oral lipid formulations (Svir® or Gelucire®-based semi-solid formulation) was compared to each other (Table 2).

Table 2. Non-compartmental pharmacokinetic parameters of saquinavir mesylate following oral administration of Svir® and Gelucire®-based formulation in beagle dogs (mean ± SD) (*n*= 6).

Pharmacokinetic parameter	Svir®	Gelucire®-based SD
k_e (h^{-1})	0.20 ± 0.06	0.25 ± 0.17
$t_{1/2}$ (h)	3.86 ± 1.33	5.12 ± 5.14
$AUC_{0-\infty}$ (ng.h.mL^{-1})	2,516.39 ± 752.59	3,006.02 ± 406.57
CL (mL.h^{-1})	1,0693.57 ± 4,909.05	9,434.39 ± 1,109.52
V_{dss} (mL.Kg^{-1})	2,6840.40 ± 1,7738.93	3,2289.56 ± 2,2968.93
MRT (h)	2.38 ± 0.43	3.43 ± 2.40

k_e, apparent elimination rate constant; t½, half-life; $AUC_{0-\infty}$, area under the plasma concentration; CL, clearance; Vd_{ss}, volume of distribution at steady state; MRT, mean residence time. No statistical difference was found when each pharmacokinetic parameter of the different formulations (Svir® or Gelucire®-based) was compared to each other (*t*-Student).

The oral plasma SQVM kinetics after oral administration of these formulations (Fig. 1B and 1C) was best described by a two-compartment model, as the distribution phase was easily masked by the absorption phase. Staats et al. (1991)[8] have reported that this kinetic model appears to be more adequate for oily vehicles, unlike the aqueous vehicles. In the latter case, gastrointestinal (GI) absorption in physiologically based pharmacokinetic models is typically described as first-order transfer from one compartment directly into the liver. Lunn and Aarons (1998)[9] have also suggested the suitability of a two-compartment model with biphasic zero-order absorption when single-dose oral formulations of saquinavir (P-8016) were administered to healthy human volunteers from various phase I studies. The authors stated that the absorption phase of orally administered saquinavir is somewhat erratic and difficult to accurately describe, however, they believe that this model is appropriate to estimate the disposition characteristics of these saquinavir oral formulations. Similarly, a linear two-compartment model, but with first-order absorption and lag time was also the most appropriate to describe the oral pharmacokinetic of saquinavir after its coadministration with amprenavir and efavirenz (both administered orally) to healthy HIV seronegative subjects.[10]

Estimation of absorption rate constant (k_a) and peripheral to central compartment transfer rate constant (k_{21}) following oral administration were found to be similar for both Svir® and Gelucire®-based formulation (p>0.05, Table 3), suggesting a similar oral absorption mechanism between these systems.

Table 3. Compartmental pharmacokinetic parameters of saquinavir mesylate following oral administration of Svir® and Gelucire®-based formulation in beagle dogs (mean ± SD) (n= 6).

Pharmacokinetic parameter	Svir®	Gelucire®-based SD
α (h^{-1})	2.45 ± 0.00	2.01 ± 0.00
β (h^{-1})	0.21 ± 0.00	0.19 ± 0.00
k_a (h)	0.82 ± 0.37	1.95 ± 1.78
t_{lag} (h)	0.23 ± 0.01	0.18 ± 0.04*
k_{21} (h^{-1})	0.22 ± 0.08	0.19 ± 0.09
V_c ($mL.Kg^{-1}$)	7,458.91 ± 4,036.97	6,569.26 ± 4284.59
MSC	0.98 - 1.39	0.9 – 4.27
r	0.91 - 0.97	0.91 – 0.99

α and β are the distribution and elimination rate constants; k_a, the oral absorption rate constant; t_{lag}, lag time between the time of administration and the onset of absorption; k_{21}, the disposition rate constant between the central and peripheral compartment; V_c, distribution volume of the central compartment; MSC, model selection criteria and r, correlation coefficient. (*) Significant difference, t-Student, p=0.0140. No statistical diferences were found for the other pharmacokinetics parameters obtained from compartmental analysis when Svir® and Gelucire®-based SD were compared each other (t-Student).

Even though one formulation is liquid (Svir®) and the developed one is semi-solid, SQVM dissolution does not seem to be a limiting factor for its absorption, since both preparations presented a similar k_a. The most probable hypothesis may be a similar inhibitory effect on P-glycoprotein (an enzyme involved in the efflux of saquinavir) provided by excipients from both formulations, which has already been reported by our research group.

In summary, the proposed system (semi-solid lipid formulation) is very promising for commercial applications given the similarity of SQVM absorption rate to that from the commercial liquid formulation. In addition, both pharmacokinetic profiles were best described by a two-compartment model with lag time, whereas the pharmacokinetics of SQVM after intravenous administration was best described by a three-compartment model. More targeted studies regarding a limphatic disposition of the drug should be carried out given that higher HIV-1 viral load may be found in this system.

Acknowledgments
This work was supported by FAPESC/CNPq/MS/SES-SC (grant number 15.949/2009-2) and CAPES/MEC (Nanobiotechnology Network, grant number 759/2009).

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7. DISCUSSÃO

Embora o desenvolvimento/aprimoramento de novas formulações a partir de outras existentes no mercado (com o mesmo princípio ativo) possa ser facilitado pela disponibilidade de informações biofarmacêuticas, dados físico-químicos, e outros aspectos do fármaco em questão, a necessidade de se obter um desempenho superior àquele da formulação comercial é muito desafiante pois, por exemplo, há necessidade de se alcançar uma absorção oral superior da formulação comercial.

Este desafio é ainda maior em se tratando de fármacos de classe IV, de acordo com o Sistema de Classificação Biofarmacêutica, uma vez que há limitações quanto à solubilidade e permeabilidade intestinal.

Além disto, é preciso formular considerando as vantagens e as desvantagens de processos e dos componentes a serem envolvidos em uma formulação, bem como os aspectos relativos aos seus custos. O objetivo deve ser a obtenção de um produto com custo mínimo, oferecendo maior acesso da população a medicamentos de qualidade, com segurança e eficácia (tríade básica para a aprovação de novos medicamentos pelas agências reguladoras).

Neste contexto e considerando toda a problemática do fornecimento de medicamentos antirretrovirais no Brasil, além da resistência antiviral que exige a utilização de fármacos que integram os esquemas de resgate, e das limitações farmacocinéticas dos mesmos (baixa absorção oral, efluxo intestinal, flutuações plasmáticas), o mesilato de saquinavir foi selecionado como fármaco de estudo.

Na última década, o número de publicações que tratam da utilização de DS para melhorar aspectos biofarmacêuticos de fármacos poucos solúveis tem crescido exponencialmente e, neste sentido, decidiu-se avaliar o potencial da incorporação do MS nestes sistemas. Existem vários métodos para a obtenção desses sistemas, porém, selecionou-se o método de fusão tendo em vista seu reduzido custo de produção comparativamente aos demais, bem como sua simplicidade operacional. Em resumo, todos os compenentes da formulação (MS e excipientes) foram inicialmente pesados, a mistura foi aquecida a 10^{o}C acima do ponto de fusão do carreador, procedeu-se para a homogeinização a alta pressão no Ultra-turrax® e, finalmente, realizou-se o envase individual em cápsulas de gelatina dura.

Quanto à seleção dos carreadores (poliméricos e não poliméricos), considerou-se o número de estudos realizados com os mesmos, custo de aquisição e natureza físico-química. Optou-se por selecionar um carreador de natureza anfifílica e outro hidrofílico, para

testar a possibilidade de se obter diferentes interações com os sistemas orgânicos (membranas, ligação a proteínas plasmáticas, por exemplo).

O polietilenoglicol (PEG) é um carreador hidrofílico e foi selecionado tendo em vista sua ampla aplicação no preparo de DS por diferentes técnicas, particularmente aqui selecionada, e seu baixo custo. Além disso, já estão disponíveis comercialmente várias dispersões sólidas utilizando PEG como carreador.

O Gelucire^{TM} 44/14, caracterizado como uma mistura de componentes lipídicos, alguns anfifílicos, foi selecionado uma vez que estudos recentes têm demonstrado um aumento da biodisponibilidade do fármacos por ele carreados (HUSSEIN et al., 2012[1]; FAISAL et al., 2013[2]). Também demonstrou um desempenho superior a sistemas hidrofílicos quando propriedades físico-químicas (DAMIAN et al., 2000)[3] ou biodisponibilidade oral (AUNGST et al., 1997)[4] de ambos sistemas foram comparadas. Além disto, o seu efeito inibitório sobre a glicoproteína-P (gpP) já tem sido descrito (SACHS-BARRABLE et al., 2007)[5], o que seria extremamente relevante para o fármaco selecionado, uma vez que ele é efluxado via gpP, explicando a sua baixa permeabilidade intestinal.

Para aumentar a interação química entre fármaco e carreador, particularmente quando da utilização do PEG (fármaco: lipofílico vs. carreador: hidrofílico), diferentes surfactantes foram testados. Optou-se pela utilização do Tween 80, particularmente por seu baixo custo e efetividade em diferentes sistemas bifásicos, bem como do colato de sódio e do deoxicolato de sódio, tendo em vista a ocorrência natural dos mesmos no trato gastrointestinal (o que reduziria problemas de incompatibilidade biológica).

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Outro adjuvante testado foi o ácido cítrico, geralmente incluído em formulações pelo seu efeito modulador do pH (ele está presente na formulação comercial do ritonavir que apresenta solubilidade pH-dependente). Uma vez que o mesilato de saquinavir apresenta uma solubilidade que descresce com o aumento do pH, a inclusão deste adjuvante poderia conferir vantagens neste sentido.

A polivinilpirrolidona (PVP), um polímero hidrofílico, também é utilizada no preparo de DS por evitar a cristalização de fármacos, basicamente por estabelecer interações químicas específicas com os mesmos, e foi incluída neste estudo.

Com o objetivo de selecionar os adjuvantes mais adequados para o preparo das DS, foram inicialmente realizados testes de solubilidade e, quando foram visualmente observadas formação de precipitado, alteração de cor ou quaisquer alterações físico-químicas indesejáveis, o adjuvante em questão foi descartado. Assim sendo, colato de sódio, deoxicolato de sódio e ácido cítrico foram descartados, uma vez que houve formação de precipitado amarelo em cada um dos casos.

Após esta triagem inicial para a seleção dos adjuvantes, preparou-se o primeiro lote de DS pelo método de fusão. Para tal, preparações isoladas de cada um dos carreadores (PEG ou Gelucire) e preparações de cada um destes carreadores com Tween 80 e PVP K30 foram produzidas. Resumidamente, os componentes foram inicialmente pesados, o carreador foi fundido a uma temperatura de 20^{o}C acima de seu ponto de fusão, a mistura foi agitada em homogeneizador de alta pressão (para garantir uma distribuição homogênea do fármaco através da matriz) e, finalmente, procedeu-se ao envase individual das cápsulas, as quais foram submetidas a diferentes taxas/temperaturas de resfriamento. Ainda que diferentes variáveis possam interferir no processo de produção das DS, neste estudo considerou-se apenas o efeito da temperatura de solidificação dada a sua relação com alterações da cristalinidade do fármaco ou do carreador (fato este já bem descrito para outros trabalhos, com outros fármacos).

Decidiu-se realizar ensaios de dissolução *in vitro* previamente à caracterização físico-química destas formulações, diferentemente da abordagem convencional utilizada no processo de desenvolvimento de novas formulações, dado que melhorias deste parâmetro poderiam indicar que esta técnica de preparo poderia ser efetiva e haveria tempo hábil para se avaliar outras estratégias, caso resultados negativos fossem observados.

Os métodos clássicos utilizados para a caracterização físico-química de sistemas sólidos, tais como difração de raios X, análises termoanalíticas, microscopia eletrônica de varredura, espectroscopia na região do infravermelho e espectroscopia Raman, foram utilizados para selecionar as formulações mais promissoras para a realização dos

testes subsequentes. Uma vez que a espectroscopia na região do infravermelho não foi uma técnica discriminatória, ou seja, não foi capaz de detectar diferenças entre as condições avaliadas, priorizou-se os resultados obtidos com a espectroscopia Raman. As formulações analisadas apresentaram certa complexidade, particularmente aquelas com Gelucire e, nesses casos, a sobreposição de picos nos espectros de infravermelho (IR) referentes aos modos vibracionais com frequências muito próximas pôde ser separada nos espectros Raman com modificação da geometria de espalhamento. Em análises subsequentes, também foi observado que a absorção de umidade pelas formulações, com posterior degradação química, pôde ser monitorada por espectroscopia Raman. Outra vantagem deste método é que as amostras são mantidas íntegras até o momento da análise, uma vez que o feixe de laser incide diretamente nas formulações, sem necessidade de preparar a amostra mediante estress físico, como acontece com a espectroscopia na região do infravermelho, onde amostras devem ser misturadas com KBR, sob agitação, o que poderia alterar relações amorfo/cristalino do fármaco devido a este processo físico ou, até mesmo, aumentar ou reduzir as interações entre os componentes da formulação.

Embora técnicas termoanalíticas, tal como a Calorimetria Exploratória Diferencial (DSC), proporcionem informações termodinâmicas relevantes para que se possa monitorar transições vítreas de sistemas sólidos, esta técnica não foi utilizada para a caracterização das formulações em questão devido à sua baixa sensibilidade para a detecção de eventos térmicos específicos do fármaco estudado. A baixa quantidade de fármaco utilizada, comparativamente aquela de polímero (1/9), explicaria a sua ineficiência.

A difração de raios X revelou alterações da relação amorfo/cristalino, tanto para o fármaco quanto para o polímero quando diferentes tempos de armazenagem foram considerados, particularmente para as DS preparadas sem PVP. Embora o processo de amorfização de fármacos tenha sido priorizado pela indústria farmacêutica, nos últimos anos, como estratégia para aumentar a solubilidade dos mesmos e, consequentemente, aumentar sua dissolução; a estabilização destes sistemas é bastante desafiadora. Há vários estudos na literatura que mostram incrementos significativos de solubilidade/dissolução, obtidos em função de amorfização dos polímeros ou fármacos em DS (ZHANG et al., 2012[6]; SHI et al., 2013[7]),

[6]ZHANG, M.; LI, H.; LANG, B.; O'DONNELL, K.; ZHANG, H.; WANG, Z.; DONG, Y.; WU, C.; WILLIAMS, R.O. Formulation and delivery of improved amorphous fenofibrate solid dispersions prepared by thin film freezing. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 82, p. 534-44, 2012.

contudo, todos os testes foram conduzidos imediatamente após a formulação e não foram relatados ensaios para avaliar a estabilidade físico-química ao longo do tempo (estudos consultados durante a preparação do projeto de doutorado). A absorção de umidade facilita não só a conversão do estado amorfo para o estado cristalino, como também a transição polimórfica; exigindo a incorporação de agentes que evitem esta conversão, tal como a PVP. Ainda que o mecanismo de estabilização proporcionado pela PVP não tenha sido investigado neste trabalho, estudos sugerem que este polímero é capaz de formar ligações de hidrogênio com grupamentos dos fármacos uma vez que a PVP é constituída por grupos doadores de elétrons como nitrogênio e oxigênio (KARAVAS et al., 2006)[8].

Neste estudo, foram detectados, por espectroscopia Raman, problemas de instabilidade química nas DS contendo Gelucire e armazenadas por um período superior a uma semana. Por este motivo e considerando-se outro trabalho já publicado (SVENSSON et al., 2004)[9], foram investigados os efeitos da umidade e temperatura sobre a estabilidade deste carreador, onde constatou-se maior influência da umidade.

As informações fornecidas pela microscopia eletrônica de varredura (MEV) facilitaram a compreensão do fato de que as DS contendo PEG e PVP apresentaram maiores taxas de dissolução. A formação de poros, devido a inclusão da PVP na formulação, já tinha sido descrita (TUNTIKULWATTANA et al., 2010)[10] e os resultados aqui obtidos apenas confirmaram este achado. Isto facilita o contato do meio de dissolução com a matriz sólida e, então, o fármaco seria liberado mais rapidamente.

A análise cruzada dos resultados da caracterização físico-química revelou que a PVP tem um papel importante na estabilização físico-química das DS, mantendo constante o perfil de dissolução do fármaco ao longo do tempo (após 7 dias). Desta forma, as formulações contendo este polímero e cada um dos carreadores (Gelucire ou PEG)

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[7]SHI, N.-Q.; LEI, Y.-S.; SONG, L.-M.; YAO, J.; ZHANG, X.-B.; WANG, X.-L. Impact of amorphous and semicrystalline polymers on the dissolution and crystallization inhibition of pioglitazone solid dispersions. **Powder Technology**, v. 247, p. 211-21, 2013.

[8]KARAVAS, E.; GEORGARAKIS, E.; SIGALAS, M.P.; AVGOUSTAKIS, K.; BIKIARIS, D. Investigation of the release mechanism of a sparingly water-soluble drug from solid dispersions in hydrophilic carriers based on physical state of drug, particle size distribution and drug-polymer interactions. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 66, p. 334-47, 2006.

[9]SVENSSON, A.; NEVES, C.; CABANE, B. Hydration of an amphiphilic excipient, Gelucire® 44/14. **International Journal of Pharmaceutics**, v. 281, p. 107-18, 2004.

[10]TUNTIKULWATTANA, S.; MITREVEJ, A.; KERDCHAROEN, T.; WILLIAMS, D.B.; SINCHAIPANID, N. Development and Optimization of Micro/Nanoporous Osmotic Pump Tablets. **AAPS PharmSciTech**, v. 11, p. 924–35, 2010.

foram selecionadas para os testes subsequentes (dissolução em pH gástrico e intestinal, avaliação do efeito dos excipientes sobre o efluxo do fármaco através do modelo celular Caco-2 e, finalmente, avaliação da biodisponibilidade em cães Bealge).

Uma vez que algumas formulações, particularmente aquelas preparadas com Gelucire, não apresentaram dissolução completa em pH gástrico, e com base nos fatos de que o MS tem solubilidade pH-dependente e uma absorção intestinal, foram realizados ensaios de dissolução em pH intestinal de modo a permitir maior correlação *in vitro*/*in vivo* e uma maior compreensão dos fatores que limitam a absorção oral deste fármaco. Embora formulações constituídas por PEG e PVP tenham apresentado melhores perfis de dissolução em pH gástrico (próximo a 100%), um comportamento contrário foi observado em pH 6,8. Contrariamente, formulações constituídas por Gelucire e PVP apresentaram maior dissolução em pH intestinal do que em pH gástrico, o que poderia ser explicado em função de hidrólise de alguns lipídeos presentes na sua composição, que facilitariam a liberação do fármaco no meio intestinal (uma hidrólise pH-dependente), particularmente nas ligações ésteres (MOHSIN et al., 2009)[11].

A próxima etapa incluiu uma avaliação *in vitro* do efeito dos excipientes sobre o efluxo intestinal do MS, que é intermediado, principalmente, pela gpP. Há outros transportadores de menor importância envolvidos no efluxo deste fármaco e, por este motivo, foram desconsiderados neste estudo. Nesta etapa, a permeabilidade do fármaco, em presença dos excipientes, foi avaliada em ambas as direções (sentido apical-basolateral e sentido basolateral-apical), utilizando-se verapamil como controle (ele é um inibidor de gpP bem conhecido, previamente validado no Laboratório). Preparações com PEG e PVP aumentaram o efluxo do fármaco em aproximadamente 2X, o que não é desejável, enquanto que, aquelas com Gelucire e PVP, o reduziram praticamente à metade. O efeito isolado do Gelucire sobre a expressão da gpP é conhecido (como anteriormente mecionado), porém, em presença da PVP, tal efeito poderia teoricamente ser alterado, mas isto não foi observado, nas condições experimentais testadas.

Para demonstrar que os efeitos sobre a gpP, e a maior ou menor dissolução em meio intestinal poderiam ter relação com o aumento ou com a redução da biodisponibilidade oral do MS, as mesmas formulações foram administradas oralmente em cães da raça Beagle. Em intervalos pré-definidos, amostras de sangue foram coletadas com o objetivo de se conhecer a concentração plasmática

[11]MOHSIN, K, LONG, M.A., POUTON, C.W. Design of lipid-based formulations for oral administration of poorly water-soluble drugs: precipitation of drug after dispersion of formulations in aqueous solution. **Journal of Pharmaceutical Sciences**, v. 98, p. 3582-95, 2009.

para, finalmente, calcular os parâmetros farmacocinéticos. Testes comparativos com a formulação comercial Svir™ também foram considerados a fim de demonstrar uma possível aplicabilidade prática para estes sistemas caso alta absorção oral fosse observada. Ainda que as DS preparadas com PEG tenham apresentado maior estabilidade físico-química, uma baixa biodisponibilidade oral foi observada (cerca de 4x menor do que a do Svir™). Por outro lado, as formulações com Gelucire apresentaram uma menor estabilidade físico-química, porém, biodisponibilidade oral similar à do Svir™, tornando-a promissora para fins comerciais. Além do efeito inibitório sobre o efluxo, sistemas baseados em Gelucire apresentaram maior capacidade de solubilização de fármacos com baixa solubilidade aquosa que aqueles constituídos por PEG. Após a dissolução, sistemas com Gelucire podem ser capazes de formar sistemas coloidais ou, então, evitar a formação de agregados/precipitados de fármaco, diferentemente daqueles a partir do PEG, o que facilitaria a absorção do MS. Tendo em vista a menor solubilidade do fármaco em pH intestinal e a limitada capacidade de solubilização do PEG, hipotetizou-se que o fármaco poderia precipitar no lúmen intestinal após sua liberação das DS contendo este carreador. Análises microscópicas das DS após a dissolução, em diferentes pHs, confirmaram esta hipótese uma vez que foi observada a formação de precipitado de MS, nas DS contendo PEG disperso em meio de dissolução com pH 6,8.

Uma vez que a formulação com Gelucire™ 44/14 e Svir™ apresentaram similar biodisponibilidade, a farmacocinética desses sistemas foi avaliada através de modelos compartimentais. Em um primeiro momento, verificou-se a adequabilidade do modelo compartimental para descrever os dados de concentração plasmática *versus* tempo, obtidos após administração intravenosa do MS. Um modelo de três compartimentos proporcionou um melhor ajuste dos dados após administração intravenosa de MS (1 mg/Kg) e, tendo em vista que os parâmetros farmacocinéticos obtidos por esta análise compartimental e por meio de uma análise não-compartimental foram estatisticamente similares (p>0.05), confirmou-se a adequabilidade do sistema. Na sequência, partiu-se para a análise compartimental das formulações orais (aquela com Gelucire™ 44/14 e o Svir™) e observou-se que um modelo de dois compartimentos com tempo de latência melhor descreveu os perfis plasmáticos para ambos os sistemas. Além disto, similar taxa de absorção (k_a) e de transferência do compartimento central para o periférico (k_{21}) foram obtidas, sugerindo mecanismos de absorção semelhantes.

8. CONSIDERAÇÕES FINAIS

- O método de preparo das DS com MS foi efetivo no sentido de aumentar a taxa de dissolução do fármaco, a qual variou em função do pH e do tipo de carreador utilizado.

- Adjuvantes, tais como ácido cítrico (modulador de pH), colato de sódio e deoxicolato de sódio (surfactantes naturais), foram descartados na triagem inicial de solubilidade, uma vez que levaram a formação de precipitado.

- Foi observada instabilidade química para as formulações com Gelucire através da espectroscopia Raman, o que pode ser atribuído à absorção de umidade.

- Foi detectada instabilidade física para as formulações contendo PEG, com aumento de cristalinidade tanto para o fármaco quanto para o carreador com variações no tempo de armazenagem (particularmente aquelas sem PVP K30).

- De forma geral, formulações solidificadas a -20^{o}C apresentaram menor variação nos perfis de dissolução em relação àquelas solidificadas a 25^{o}C.

- As formulações contendo PVP foram mais estáveis físico-quimicamente, após um armazenamento de curto prazo (7 dias), provavelmente devido a menor conversão da forma amorfo-cristalino, tanto para o fármaco quanto para o carreador, particularmente aquelas preparadas com PEG. Além disto, este grupo de formulações manteve estável o perfil de dissolução, após armazenamento à curto prazo. A formação de poros, observada por MEV, é característica da presença de PVP e contribuiu para explicar a maior dissolução dos sistemas contendo este polímero.

- Os testes de estabilidade acelerada indicaram instabilidade físico-química da formulação com PEG 4000 e PVP K30 (selecionada para o teste devido ao seu perfil de dissolução estável após 7 dias), após 60 dias de armazenagem, particularmente para a condição com maior umidade.

- As DS com PEG e PVP aumentaram ainda mais o efluxo do MS, mediado pela gpP nos ensaios de permeabilidade intestinal, e aquelas com Gelucire e PVP reduziram este parâmetro à aproximadamente a

metade, o que é relevante quando se considera a possibilidade de aumentar a absorção oral;

- A formulação com Gelucire e PVP mostrou biodisponibilidade oral similar à da formulação comercial Svir^{TM}, que foi aproximadamente 5X maior do que a da formulação preparada com PEG e PVP.

- A maior absorção oral de formulações preparadas com Gelucire pode ser explicada em função da maior solubilização do fármaco, da não precipitação do mesmo, e de seus efeitos sobre o efluxo mediado pela gpP. Através destes achados, é possível inferir que a incorporação de adjuvantes funcionais, tal como o Gelucire, poderia proporcionar vantagens adicionais aos fármacos cuja biodisponibilidade é limitada, não apenas pela dissolução como também pela permeabilidade (fármacos de classe IV).

- Uma taxa similar de absorção (k_a) do MS foi obtida para as formulações orais com Gelucire e Svir^{TM}. Além disto, uma disposição cinética similar do MS foi obtida após administração oral de sistemas lipídicos (dispersões sólidas com Geluire e Svir^{TM}), os quais foram melhores descritos por um modelo de dois compartimentos com tempo de latência. A farmacocinética do MS, após administração intravenosa, foi melhor descrita por um modelo de três compartimentos.

CAPÍTULO II

PERMEABILIDADE BUCAL DE DIFERENTES FÁRMACOS

Os experimentos desta etapa foram realizados na *Monash University* (Melbourne, Austrália) sob orientação do professor Dr. Joseph Nicolazzo.

Fontes de financiamento:
Projeto: financiado pela *Monash University* (materiais de consumo)
Recursos humanos: CAPES/MEC “Programa de Doutorado Sanduíche” (BEX 12349/12-7)

"Os resultados provêm do aproveitamento das oportunidades e não da solução dos problemas. A solução de problemas só restaura a normalidade. As oportunidades significam explorar novos caminhos."
(Peter Drucker)

1. APRESENTAÇÃO

Todos os resultados envolvendo permeabilidade bucal constam deste capítulo II, tanto os obtidos com o mesilato de saquinavir quanto aqueles do cloridrato de donepezila. A investigação de vias alternativas para a administração de fármacos, tal como a bucal, é particularmente relevante nos casos em que certas inconveniências relativas à administração oral possam são observadas (problemas farmacocinéticos, fármacos efluxados via glicoproteína-P, efeitos adversos, etc.). A mucosa bucal ainda apresenta vantagens particulares, tal como a ampla irrigação sanguínea pela veia jugular interna, e também por evitar o metabolismo hepático de primeira passagem. Em alguns casos, quando se observa alta permeabilidade bucal, é possível reduzir a dose administrada e, desta forma, minimizar efeitos adversos dose-dependentes, bem como flutuações plasmáticas, com inúmeras vantagens aos pacientes.

Uma vez que o MS apresentou baixa permeabilidade bucal, investigaram-se as possíveis causas deste achado, as quais parecem estar associadas particularmente com o alto peso molecular do fármaco. Neste sentido, preparou-se um manuscrito de revisão considerando os principais fatores que interferem na permeabilidade bucal de macromoléculas, bem como as principais estratégias que poderiam ser utilizadas para reforçá-la. Ao mesmo tempo em que o peso molecular pareça ser o fator limitante da absorção bucal do MS, macromoléculas peptídicas, tais como insulina e calcitonina, têm sido preferencialmente administradas por esta rota, incluindo até mesmo alguns medicamentos já sendo comercializados. Este contraposto parece estar relacionado com a estratégia de reforço utilizada, que inclui a combinação de mais de um reforçador químico ou, então, a combinação de estratégias químicas e físicas de reforço. No entanto, o uso de estratégias individuais para o MS não foram efetivas em reforçar sua absorção e, isto pode ser melhor compreendido com a leitura do artigo de revisão deste capítulo.

Considerando que este manuscrito de revisão aborda, de forma abrangente, aspectos estruturais e fisiológicos da mucosa bucal, vantagens da administração bucal frente a outras vias, e estratégias físicas e químicas de reforço da absorção bucal, acredita-se que este tipo de discussão seja suficiente para a compreensão da etapa experimental conduzida posteriormente com o cloridrato de donepezila (DPZ).

Segundo extensiva busca na literatura, a permeabilidade bucal do DPZ ainda não tinha sido testada e, neste trabalho apenas estratégias químicas de reforço da absorção bucal foram testadas para esse fármaco em vista da sua alta permeabilidade intestinal (já bem

estudada). Este avaliação é relevante dada à importância clínica do DPZ, o qual é utilizado no tratamento da Doença de Alzheimer, e suas limitações farmacocinéticas quando administrado oralmente (ele atinge concentrações plasmáticas muito rapidamente e podem ser observadas flutuações plasmáticas). Embora já tenham sido realizados estudos da absorção transdérmica deste fármaco, os coeficientes de permeação obtidos foram significativamente menores que aqueles obtidos em ensaios de permeabilidade intestinal (absorção oral), o que torna necessária a pesquisa de outras alternativas farmacotécnicas ou de novas rotas de administração, tal como a bucal.

2. OBJETIVOS

2.1. OBJETIVO GERAL

- Avaliar o potencial da mucosa bucal como rota de administração do MS e do cloridrato de donepezila (DPZ), bem como o impacto do uso de reforçadores químicos de penetração sobre a absorção bucal destes dois fármacos.

2.2. OBJETIVOS ESPECÍFICOS

- Compreender os fatores que limitam a absorção bucal de macromoléculas com potencial aplicação biotecnológica ou farmacêutica e as estratégias que podem ser utilizadas para aumentar tal absorção;
- Avaliar a permeabilidade bucal de soluções aquosas do MS e do DPZ;
- Avaliar o impacto de reforçadores de penetração conhecidos, tais como dodecil sulfato de sódio, taurodeoxicolato de sódio, Azone^{TM} e do ácido deoxicólico, sobre a permeabilidade bucal do MS;
- Avaliar a influência da combinação de ácido oleico e polietilenoglicol sobre a permeabilidade bucal do MS;
- Comparar os coeficientes de permeabilidade bucal, intestinal e transdérmica do DPZ, a fim de determinar a relevância da sua administração bucal;
- Avaliar o impacto do pré-tratamento com Azone^{TM} na permeabilidade bucal do DPZ;
- Avaliar a influência de diferentes concentrações do ácido deoxicólico (acima e abaixo da concentração micelar crítica), bem como de variações nos protocolos de tratamento (pré- e co-tratamento) sobre a permeabilidade bucal do DPZ;
- Avaliar o efeito do co-tratamento de diferentes concentrações de PEG 400 (5 e 50% m/m) sobre a absorção bucal do DPZ;
- Avaliar o impacto do pré- e co-tratamento com a combinação de ácido oleico e polietilenoglicol sobre a absorção bucal do DPZ.

3. ARTIGO DE REVISÃO SUBMETIDO PARA AVALIAÇÃO

Novel approaches for enhancing the buccal permeation of macromolecular therapeutics

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"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê."

(Arthur Schopenhauer)

Abstract

With the rapid development of the biotechnology and genetic engineering in recent years, there has been a dramatic increase in the availability of new therapeutic agents, particularly macromolecules, for the treatment of various diseases. Although most of these macromolecular therapeutics exhibit high potency, characteristics such as their large molecular weight, susceptibility to enzymatic degradation, immunogenicity and the tendency to undergo aggregation, adsorption, and denaturation have limited their application through the traditional oral route. In particular, peptides and proteins are extensively degraded by the proteases in the gastrointestinal tract, which represents a major impediment to oral drug delivery. So far, different noninvasive alternative routes have been investigated for systemic delivery of these macromolecules, one of which is the buccal mucosa. The buccal mucosa offers a number of advantages over oral route making it a very suitable route for delivery. While buccal mucosal permeability of small drug molecules has been well assessed, there is a growing use of the buccal mucosa as a administering site to rescue the clinical utility of macromolecule therapeutics. However, the buccal mucosa still exhibits some permeability-limiting properties, and as a result, various methods are employed to enhance the delivery of macromolecules via this route including the use of chemical penetration enhancers, physical methods (iontophoresis) and mucoadhesive formulations. The incorporation of anti-aggregating agents in buccal formulations as well as the use of proteins as carrier molecules also appear to show promise and have not yet been exploited. The intent of this review is to provide an update on recent pharmaceutical approaches which have been investigated to improve the buccal mucosal transport of macromolecules, mainly focusing on the proteins and peptides.

Keywords: buccal permeability, macromolecules, chemical penetration enhancers, particulate systems, mucoadhesion.

1. INTRODUCTION

For many years, the lack of industrial manufacturing processes for peptides and proteins had limited their use as therapeutic agents (1). However, the biotechnology and genetic engineering as well as alternative protein delivery technologies have advanced dramatically recently, leading to the availability of numerous macromolecular therapeutic agents for clinical use (2-4). The most frequently marketed macromolecules include monoclonal-antibody-based products for the treatment of different types of cancer and autoimmune diseases, therapeutic vaccines for immunization against hepatitis A and/or B, insulin for diabetes treatment, human growth hormone for supplementation in hormone deficiency, and interferon α for treatment of hepatitis B and/or C (5). Although the posttranscriptional gene silencing approach by RNAi have been tested for various diseases including Parkinson's disease, Lou Gehrig's disease, HIV infection, wet age-related macular degeneration, type 2 diabetes, obesity, hypercholesterolaemia, rheumatoid arthritis, respiratory diseases and cancers, new drugs regarding this strategy has not yet been clinically approved (6).

Intestinal absorption of macromolecules such as peptides and proteins following conventional oral administration is generally limited due to their hydrophilic characteristics and large molecular size. Moreover, some other common properties of these macromolecules such as susceptibility to enzymatic degradation, short plasma half-life, pH-dependent permeability, immunogenicity, the tendency to undergo aggregation, adsorption, and denaturation have also been limiting their oral use for treatment of various diseases (7, 8).

The parenteral administration route (e.g. intravenous or subcutaneous injection) has been the traditional routine for macromolecules to date (5, 9). However, disvantages associated with the parenteral route such as local site discomfort, inconvenience, and poor patients compliance have always limited the clinical utility of administering such agents via these routes (10). As a result, alternative routes of delivery such as nasal, ophthalmic, buccal, vaginal, transdermal and pulmonary routes have been extensively investigated (11). The nasal route is attractive because of the ease of administration; however, side effects including rhinitis, rhinorrhea, and allergic rhinitis induced by excipients (such as absorption enhancers and surfactants) have been reported (12). Moreover, long term treatments can compromise the integrity of the mucosa and there are various reports regarding the influence of penetration enhancers on the mucociliary activity (13, 14). Intravaginal delivery of peptides is characterized by poor and variable bioavailability, which have been associated with

formulation factors, vaginal physiology, age of the patient and menstrual cycle (15). Moreover, formulations to be administered via intravaginal may provide low retention to the epithelium and local irritation thereby causing inconvenience to the patient *(16)*. The colorectal delivery route presents low enzymatic activity and provides longer residence times; however, it has a very limited bioavailability (17). While the transdermal route has the advantages of easy access and the availability of a large surface area, irritation at the site of application and visibility issues of the formulation (such as patches) can limit the clinical utility of this route (18). Therefore, while each of these routes can be exploited for the delivery of macromolecular therapeutics, each is associated with various disadvantages that can limit the usefulness of the drug delivery route in patients.

Over the last two decades, much attention has been given to the buccal mucosa as an alternative route for drug administration because of the excellent accessibility, ~~its~~ physical robustness, and the avoidance of intestinal and hepatic metabolism, which is particularly favorable for peptide and protein delivery (19, 20). Unlike the skin, the human buccal mucosa consists of 40 to 50 layers of non-keratinized cell layer, which is more suitable for the systemic delivery of hydrophilic macromolecular compounds such as peptides, proteins and oligonucleotides (21). The easy accessibility of the buccal mucosa favors the administration of drugs at this site, and in the event of the appearance of adverse reactions, formulations can be easily removed. In addition, buccal and sublingual regions have lower enzymatic activity with enzymes such as trypsin, chymotrypsin and pepsin (which are present in the gastric and intestinal fluids) are largely absent in the oral mucosa (22). This specific property of the buccal mucosa is particularly favorable for protein and peptide delivery given their susceptibility to enzymatic degradation, which is often the most common reason for poor oral absorption (23). Furthermore, the buccal mucosa is a well vascularized tissue, from where blood vessels drain directly into the jugular vein. Therefore, molecules which are able to penetrate the buccal mucosa epithelium are likely to be rapidly delivered into the systemic circulation, avoiding the hepatic first pass effect and hydrolysis in the gastrointestinal tract (24). It is should also be noted that, particularly for sustained-release mucoadhesive devices, cellular turnover time in the buccal mucosa is slower (4-14 days) than in the gastrointestinal tract, allowing formulations to adhere to the buccal mucosa for relatively longer periods of time without being affected by cellular turnover (25). It should be noted that buccal mucosa has an relatively small absorption area, and a constantly flowing salivary film which affect the transport of macromolecules across the buccal mucosa. However, various enhancing strategies may be used to minimize this effect and maximize the buccal absorption. In addition, molecules which exhibit a strong taste

are also not desirable for buccal delivery and therefore taste issues must be considered prior to application (21). Although some of these limitations may not be controlled, the benefits associated with buccal mucosal administration outweigh the limitations, representing a promising route for the administration of macromolecular therapeutics. By utilizing such a favorable route of delivery, the total amount of macromolecule required for dosing would also be decreased, thus reducing expensive manufacturing or recombinant expression processes and limits the potential for undesirable side effects associated with higher doses.

Given that peptides and proteins have a very large variation in their molecular weight compared to most conventional drugs (600 to 100,000 Da) and their buccal absorption is reduced exponentially with molecular weights above 300 Da (26), the buccal delivery of macromolecules is extremely challenging and it usually requires long-term researches. The first attempts to use the buccal mucosa for insulin delivery, for example, have been made as early as 1925. A number of attempts have been made over time to improve its buccal absorption by adding chemical enhancers or by modifying its lipophilicity. A buccal system for this drug, the Oral-lyn® (Generex), was approved by the FDA only in 2009, which is used in treatment of Type 1 or Type 2 diabetes mellitus (27). It is a liquid formulation combining the formation of microfine, thin membrane, as well as mixed micelles made from the combination of insulin and specific chemical enhancers, which encapsulate and protect this peptide (28). This successful example further highlights the importance of knowing what strategies may be used to enhance the buccal absorption of macromolecules so that therapeutic plasma concentrations may be achieved and also to reduce the time required for approval of new buccal delivery systems. In general, individualized approaches have been considered.

The application of chemical enhancers such as surfactants, fatty acids and chitosan in the buccal delivery of peptides is the most common approach, however, minimal increases on absorption and severe histological damage are often observed in those studies (27, 29). Subsequently, new approaches to improve the buccal mucosal delivery of macromolecules have been exploited including chemical modification of therapeutics, physical methods (such as iontophoresis), and the incorporation of particulates systems and anti-aggregating agents. The purpose of this review, therefore, is to provide an update on recent pharmaceutical approaches that have been successfully employed to improve the buccal delivery of macromolecules, with novel directions that may be exploited based on technologies that are being investigated for other alternative routes of drug delivery.

2. STRUCTURE AND PHYSIOLOGY OF THE BUCCAL MUCOSA

The understanding of buccal mucosa structure allows optimizing strategies to enhance the permeability of drugs more rationally and understand the route by which drugs/dosage form cross the buccal mucosa for penetration into blood capillaries.

The oral cavity comprises the lips, cheek, tongue, hard palate, soft palate and floor of the mouth (23). While each of the regions have its physiological roles, sublingual (under the tongue and on the floor of the mouth) and buccal (located on the soft palate) are the most important in drug delivery since they are more permeable (30). Both mucosae are nonkeratinized (Fig. 1-I), providing flexibility and elasticity to mastication and speech processes (31). On the other hand, mastigatory mucosa, which include hard palate and gingiva, is an example of non-keratinized tissue (Fig. 1-II), which is developed in regions subjected to physical or chemical stress or abrasion resistant (32). Nonkeratinized and keratinized tissues also differ in their composition. The keratinized epithelia contain neutral lipids like ceramides and acylceramides which have been associated with the barrier function. In contrast, non-keratinized epithelia do not contain acylceramides and only have small amounts of ceramides (33). They also contain small amounts of neutral but polar lipids, mainly cholesterol sulfate and glucosyl ceramides (34).
Basically, the buccal mucosa is composed of a stratified squamous epithelium, a basement membrane, and an underlying connective tissue (Fig. 1a). The epithelium consists of 40-50 cell layers (500-600 μm thickness), which migrate from the basal layer to the superficial, and protects the underlying tissue against fluid loss and entry of potentially harmful environmental agents. It represents one of the major barriers limiting the development of buccal delivery systems (25). Once saturation of membrane transport is observed, this layer is unable to absorb more compound, regardless of the duration of exposure, and it diffuses into the deeper layers at a constant rate (35). Membrane coating granules (MCGs) also display barrier properties, particularly for drugs transported paracellularly, since they produce ‘‘lipid contents’’ during differentiation and discharge them into the intercellular space, a typical process in viable epithelial cells with metabolically active organelles (31). The connective tissue supports the epithelium and consists of lamina propria, which contains a network of capillaries, and submucosa. Once a drug has permeated the epithelium and the basement membrane, it can easily penetrate the capillaries and enter the blood circulation (25). The details of the structure were discussed in other reviews (23, 24, 36, 37).

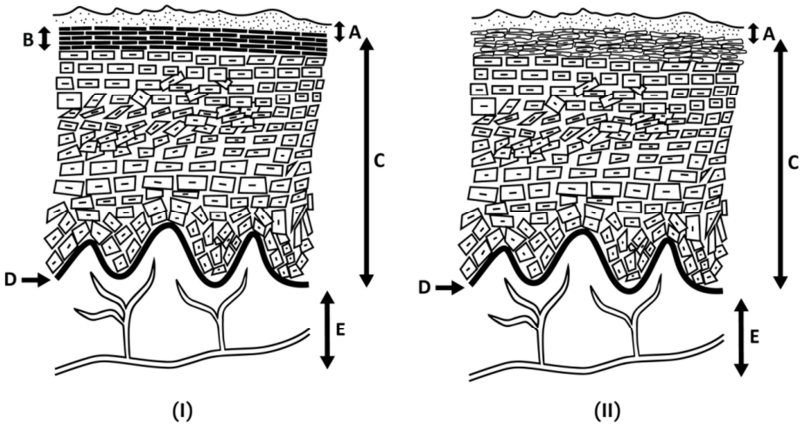

Fig. 1. Structure of keratinized (I) and nonkeratinized (II) stratified squamous epithelium of oral mucosa. This structure includes mucus layer (A), stratum corneum (B), stratified squamous epithelium (C), basement membrane (D) and submucosa (E).

2.1 Saliva and mucus

Saliva is secreted by three of major salivary glands (parotid, submaxillary and sublingual) and minor salivary or buccal glands (38). The parotid and submaxillary glands produce watery secretion, whereas the sublingual glands produce mainly viscous saliva with limited enzymatic activity (23). The pH of the saliva is slightly acidic (6.6) in rest and goes up to 7.4 when stimulated (39). It mainly consists of water (95–99% per weight), enzymes, inorganic salts, lipids, and glycoproteins, so-called mucins. MG1, a high molecular weight mucin composed of disulfide-linked subunits, is able to adhere to the surface of the oral epithelium, representing other penetration barrier, the mucus layer (40) (Fig. 1). Saliva not only lubricates the oral cavity, making possible functions such as swallowing and speaking, but it also helps to maintain integrity of the hard tissues of the teeth. It also allows carbohydrate digestion and regulates oral microbial flora by maintaining the oral pH and enzyme activity (41). Due to movement of the tongue and the jaw during the salivation, it may facilitate removal of the drugs from the site of absorption thereby limiting the administration unless the formulation can be retained (42, 43). This limitation may be overcome by using bioadhesive formulations since they are able to maximize the

drug concentration gradient and prolong adhesion, which would lead to increased bioavailability (44). On the other hand, saliva provides a water rich environment which can be favourable for drug release from delivery systems based on hydrophilic polymers (it facilitates polymer dissolution) (23).

2.2 Enzymatic Activity

Buccal and sublingual regions have less enzyme activity compared with GI tract, which is especially favorable to protein and peptide delivery, however, the metabolic activity of enzymes in the oral cavity should not be ignored as a number of peptides have been degraded in presence of buccal tissue homogenates including insulin, proinsulin (45), enkephalin analogues (46), thyrotropin releasing hormone (47), calcitonin (48) and substance P (49). Enzymes such as aminopeptidases, carboxypeptidases, phosphatases, carbohydrases, cytochrome P450 enzymes, cyclo-oxygenases, lipoxygenases, esterases and endopeptidases of oral cavity have been showed to limit the buccal bioavaliability of macromolecules (46, 50-52) and may require strategies to overcome this barrier.

Given that the oral cavity is composed of various regions, which present different types and levels of drug-metabolizing enzymes (51), studies on buccal delivery of macromolecules should prioritize enzymes from the buccal tissues but also consider other enzymes of the oral cavity. The catalytic activity of enzymes from other oral cavity regions should be studied concurrently in order to ensure the drug stability and its action on target tissues. While phosphatases and carbohydrases are present in the saliva, most of the enzymes originate from buccal epitelial cells. Cyclo-oxygenases and lipoxygenases, are possibly the products of inflammatory cells (51). Cytochrome P450 (CYP) enzymes, which are the most important initial phase drug-metabolizing enzymes, also has been found in the oral cavity (52). CYP1-3 enzyme subfamilies are expressed in human salivary gland parenchyma, leading to activation or inactivation of a series of drugs (52).

The activities of aminopeptidase and esterase in the buccal mucosa tissues have also been evaluated in different animal models (rat, rabbit, guinea pig, and dog), using L-leucine-β-naphthylamide as the substrate (53). For the aminopeptidase activity, the relative ranking was dog < rat < guinea-pig < rabbit, whereas for the esterase activity, the ranking was guinea-pig < rat < rabbit. In view of these differences, models should be carefully selected to predict the buccal transport of compounds.

Since enzymes are selective for their substrates and can be differently distributed in the regions of buccal mucosa (mucus, epithelium, connective tissue), targeted studies should be performed to

better understand where each peptide is preferentially degraded so that targeted approaches can be efficiently applied in developing bioadhesive drug delivery systems, avoiding its degradation. For the insulin, an intracellular degradation by cytosolic-bound proteases is suggested considering the lack of effect of aprotinin (a serine protease inhibitor) on its transport and no hydrolysis when it is applied to the surface of porcine buccal mucosa (54). In another study, the location of dipeptidyl peptidase IV activity in buccal tissues was determined by assessing endomorphin-1 (ENI) stability in partial thickness buccal epithelium. In the presence of full thickness buccal mucosal epithelium, ENI was unstable with only 23% intact drug detected, which suggest the location of the peptide metabolizing enzyme in the epithelial layer. In the presence of diprotin-A, a enzyme inhibitor, buccal stability of ENI was significantly enhanced, where 71% of the drug remained intact (55). Although the incubation of peptides in mucosal tissue homogenates have been used to characterize the proteolytic action (56), this test is not able to discriminate cytosolic, membrane-bound and intercellular proteolytic activities, leading to inconclusive results (57).

It should be noted that though some peptides are substrates for enzymes in the oral cavity, metabolism may not occur if there is no contact between them. For example, intracellular enzymes may be inaccessible for peptides which are paracellularly transported. It is know that a small percentage of oligopeptides escape from the hydrolysis process and enter the blood intact because they do not reach the internal cellular compartment (58). Insulin and enkephalin, which are predominantly transported via paracellular pathway, may escape an extensive metabolism because the proteolityc activity against these substrates is primarily cytosolic (26).

2.3 Drug transport mechanisms

Passive diffusion has been the primary mechanism for the transport of drugs across the buccal mucosa, which involves two main routes - the intracellular or transcellular route (drug passes through the cells), and the intercellular or paracellular route (drug passes through the spaces between the epithelial cells) (Fig. 2) (59). Both routes may coexist for all drugs, but the route with the least penetration resistance is usually preferred (60). It is suggested that hydrophilic compounds such as peptides/proteins permeate the buccal mucosa via the paracellular route as they can dissolve more readily in the aqueous fluids filling the intercellular spaces (36). Although the transcellular route is the shortest pathway to transport drugs, the paracellular route offers a low physical resistance, which makes it to be preferentially used, resulting in diferences in lag time. When drugs are transported via the paracellular pathway, it diffuses laterally over a wider area of the mucosa,

demonstrating higher lag time (61). Rate of penetration depend on the physicochemical properties of the drugs such as molecular geometry, lipophilicity, and charge (42). In addition, studies have demonstrated the presence of carrier-mediated transport, particularly for sugars (62) and drugs with a monocarboxylic acid residue (63).

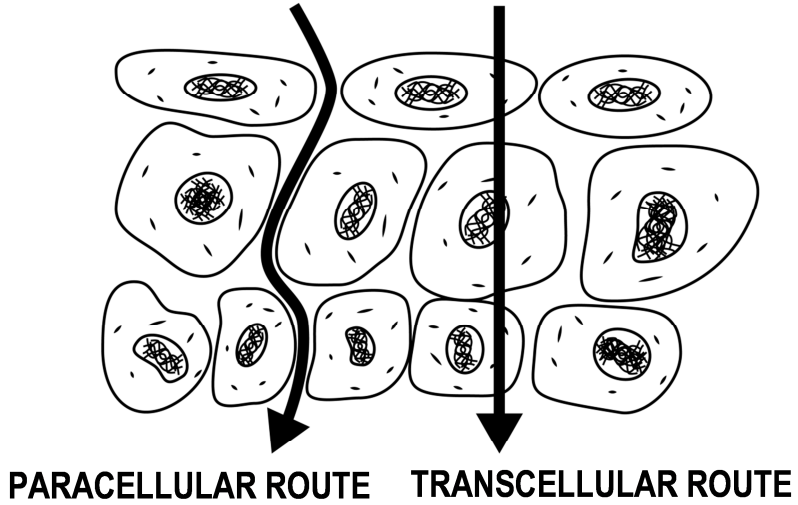

Fig. 2. Transepithelial transport pathways though buccal mucosa.

3. NOVEL APPROACHES FOR ENHANCING MACROMOLECULAR BUCCAL TRANSPORT

3.1. Chemical modifications

Because of their polarity and size, peptides are very poorly transported across biological membranes. As a result, chemical modifications have been undertaken to improve the biopharmaceutical properties of peptides and proteins, to assist in transport across biological membranes, including the buccal mucosa; basically increasing their resistance towards enzymatic degradation and/or buccal permeability (64). Overall, the chemically modified derivatives are suggested to be either cleaved within the cytoplasm of the epithelial cells, in the systemic circulation and/or targeted tissue, resulting in delivery of the free active peptide to the site of action following absorption (65).

The commonly used approach of chemical modification is to introduce the acyl chains to the amino-acid side of the terminal positions of peptides and proteins has been performed in order to increase lipophilicity and improve stability of peptides and protein-like substances (66, 67). For example, a lipophilic derivative (Trp-Leu) has been modified by covalent attachment of myristic acid to the terminal amino group (Myr-Trp-Leu), which can be found at the N-terminal end of many natural proteins (64). The acylated peptides displayed greater affinity to the buccal epithelium components and accumulation in the tissue (achieving levels close to 80% at 2 μg/mL), however, the amount which reached the receptor chamber was reduced significanlty (close to zero at 2, 4 and 6 μg/mL). On the other hand, the non-acylated peptide (control) had lower retention in the buccal mucosa (between 3 and 4%) and 55% of the dipeptide was found in the receptor compartment at lower tested concentration. These findings suggest that the N-terminal acylation is not effective to increase the buccal absorption of this peptide, however, it made the peptide resistant towards degradation by aminopeptidases. Despite its improved lipophilic properties, the sensitivity to carboxypeptidase have not been modified.

Thyrotropin-releasing hormone (TRH), a peptide considered in different buccal delivery systems (68, 69), also has been modified by N-acylation of the imidazole group of its histidine residue with chloroformates to improve its lipophilicity and thereby protect it against rapid enzymatic inactivation in the systemic circulation. Whereas TRH is rapidly hydrolyzed in human plasma by a TRH-specific pyroglutamyl aminopeptidase, N-alkoxycarbonyl derivatives have shown more resistant to cleavage, suggesting that the reduction of plasma metabolism would contribute to increase the buccal bioavailability (70). Thus, it is important to consider not only the enzymatic activity of the buccal mucosa but also that of regions where macromolecules are transported to exert their effect. Plasma stability and in the oral cavity should be observed for drugs where is expected a systemic and local effect, respectively.

3.2. Chemical penetration enhancers

Whenever the absorption of the drug is important for its action (systemic delivery), the use of absorption enhancers may be mandatory to overcome the barrier represented by the mucosal epitelium. The suggested possible mechanisms for these absorption enhancers include (i) increasing the partitioning of drugs into the tissue, (ii) extracting intercellular lipids, (iii) interacting with epithelial protein domains, and/or (iv) increasing the retention of drugs at the buccal mucosal surface (37). Hassan et al. (2010) (71) have also proposed that penetration enhancers are able to change mucus rheology, reducing its viscosity,

and to increase the thermodynamic activity of the permeants, however, limited research has been performed in the area to elucidate the exact mechanisms involved. The commonly used and evaluated penetration enhancers for increasing peptide and protein buccal delivery include surfactants and bile salts, fatty acids and polymers such as chitosan (72). Special attention has been given to polymers since they may be considered safer and generally are not absorbed bucally. Furthermore, they can not only improve permeability profile but also the mucoadhesive properties, inhibit the enzymatic activity of peptidases, or present antimicrobial action (73) and thus can be called “multifunctional polymers”. Among multifunctional polymers, chitosan is one of the more extensively studied materials (73).

One of the major concerns of applying chemical enhancers is their toxicity on the buccal membrane, though buccal mucosa is normally able to recover rapidly after the removal of the enhancers (74). However, it is still essential to select the appropriate type and concentration of enhancer to minimize irritation of the mucosal membranes (75). It is also recommended that the effects of chemical enhancers on the epithelial damage, local irritation, long term toxicity and enhanced permeability of pathogenic microorganisms should be carefully considered prior to their selection for buccal transport studies (76).

3.2.1. Surfactants and bile salts

There are a number of examples demonstrating the beneficial effects of surfactants and bile salts on the buccal mucosal absorption of peptide therapeutics (Table 1).

Table 1. Effects of surfactants on buccal permeability of macromolecules.

Macromolecule	Penetration enhancer	Method	Results	Ref.
Buserelin	Sodium glycodeoxycholate (SGDC)	Buccal delivery devices were administered to pigs *in vivo*	The coadministration of the SGDC at 0.45% increased the absolute buccal bioavailability to 5.3%	(77)
Calcitonin (CT)	Sodium deoxyglycocholate (SDGC)	*In vitro* permeation with excised pig buccal mucosa	Flux of CT increased 18-fold in presence of SDGC	(29)
Endomorphin-1 (ENI)	Sodium glycocholate (GC) and sodium taurocholate (TC)	*In vitro* permeation with porcine buccal epithelium	GC and TC were not effective in enhancing the permeation of ENI	(55)
Fluorescein isothiocyanate (FITC)-labeled dextrans (4, 10 nad 20 kDa)	Sodium glycodeoxycholate (SGDC), sodium taurodeoxycholate (STDC), sodium glycocholate (SGC) and sodium taurocholate (STC)	*In vitro* permeation with excised pig buccal mucosa	A maximal enhancement of approximately 2,000 times was obtained to lower molecular weight dextran (4 kDa) after treatment with SGDC. No significant differences in the degree of permeation enhancement were observed between these four bile salts	(77)
Fluorescein isothiocyanate (FITC)-labeled dextran (4 kDa)	Sodium glycodeoxycholate (SGDC)	Buccal delivery devices were administered to pigs *in vivo*	Co-administration of 10 mM SGDC increased the absolute bioavailability of 4 kDa dextran from 1.8 to 12.7%	(78)
Insulin	Laureth-9	Insulin solution was administered to rats *in vivo* and its absorption was monitored on the basis of cumulative hypoglycemic response	Hypoglycemic response of insulin increased from 3.6 to 27.2% in presence of laureth-9	(79)
Insulin	Laureth-9, polysorbate 20, PEG laurate, propylene glycol laurate, sorbitan laurate, glyceryl monolaurate, octoxynol- 9, sodium dodecyl sulfate, sodium glycocholate, sodium deoxycholate, sodium laurate, sodium lauryl sulfate	Administration of solutions to rats *in vivo*	In the absence of an absorption enhancer, buccal insulin was less than 4% as effective as i.m. insulin. Bile salts, sodium laurate, sodium lauryl sulfate and Laureth-9 were the most effective (efficacy relative to i.m. insulin > 20%)	(80)
Insulin	Sodium cholate (SC), sodium taurocholate (STC), lysophosphatidylcholine (LPC)	Buccal administration of insulin in anesthetized dogs *in vivo*	Glucose back permeation flux remained unchanged for SC and it has been decreased by 80% in 5-8 h after exposure to STC and LPC (> hypoglycemic effect)	(81)

Macromolecule	Penetration enhancer (cont.)	Method (cont.)	Results (cont.)	Ref.
Insulin	Brij-35, sodium taurocholate (STC), sodium lauryl sulfate (SLS), sodium deoxycholate (SDC)	Application of solution formulation to rabbits *in vivo*	Brij 35 provided the greatest hypoglycemic effect, followed by STC, SLC, SDC	(82)
Insulin	Octylglucoside and dodecylmaltoside	Buccal administration of solution formulation to rats *in vivo*	Hypoglycemic effect have enhanced from 1% (control) to 20 and 30% in the presence of octylglucoside and dodecylmaltoside, respectively.	(83)
Insulin	Soybean lecithin	Application to rabbits and rats in vivo	Blood glucose levels decreased significantly in diabetic rabbits (54%) and rats (60%) after buccal administration	(84)
Insulin	Lysalbinic acid	*In vitro* permeation in hamster cheek pouch	Lysalbinic acid increased buccal permeability of FITC–insulin for above five times in 10 min	(85)
α-interferon	Sodium taurocholate (STC), Tween 80 and sodium dodecyl sulfate (SDS)	Application of buccal preparation to rats *in vivo*	Buccal bioavaliability increased 18-, 6- and 8-fold with the addition of STC, Tween 80 and SDS, respectively.	(86)
α-interferon	Lysalbinic acid	*In vitro* permeation in hamster cheek pouch	Lysalbinic acid increased α-interferon transport for 6 and 9 times in 2 and 10 min, respectively.	(85)
Luteinizing hormone releasing hormone (LHRH)	Sodium taurodeoxycholate (STDC), sodium deoxycholate (SDC) and sodium cholate (SC) – tested separatelly	Bilayer mucoadhesive devices were administered to beagle dogs *in vivo*	The following rank order was observed for relative buccal bioavaliability: SDC > SC > STDC (237, 151 and 84%, respectively)	(87)
Pituitary adenylate cyclase-activating polypeptide (PACAP)	Sodium deoxycholate (SDC), cetrimide	*In vitro* permeation studies were performed on freshly excised buccal mucosa and Ussing chambers	Buccal permeation enhancement of PACAP was 18.6-and 46.5-fold in the presence of SDC and cetrimide, respectively.	(88)
Recombinant human basic fibroblast growth factor (rhbFGF)	Sodium glycocholate (SGC)	*In vitro* permeation using excised rabbit buccal mucosa and side-by-side diffusion systems	Flux of rhbFGF increased 2.3-fold with the addition of SGC	(89)

*Studies in which drug has not been tested alone (no enhancer) were not considered in this analysis.

Although some authors have suggested that surfactants cause removal of the superficial cell layers (90-92), affecting the barrier properties, a greater body of evidence suggests that intercellular lipid extraction is the main mechanism by which these agents enhance buccal permeability. This lipid-solubilizing effect generally modifies paracellular transport of agents, however, transcellular transport has been suggested to also be increased if high concentrations of surfactant are present (i.e. when lipids from the cell membranes are also extracted) (93, 94). For example, Hoogstraate (1996) (95) observed in *in vitro* experiments using porcine buccal epithelium that 10 mM sodium glycodeoxycholate (SDGC) is able to enhance the flux of fluorescein isothiocyanate–dextran (a hydrophyllic macromolecule having a MW comparable with that of peptides extensively used in therapeutics) only through the paracellular route while higher concentrations of 100 mM SDGC enhanced permeability of this macromolecule through both the paracellular and transcellular pathways. Jasti et al. (2000) (96) have also enhanced the *in vitro* buccal permeability of a phosphorothioate antisense oligonucleotide (ISIS 3082) in approximately 17 times by co-application of sodium glycocholate (SGC) at 100 mM. Phosphorothioate antisense oligonucleotides are malfunctioning gene modulators with application in treating diseases such as cancer (97), AIDS and genetic disorders (98). It has been suggested that SGC contribute to solubilize intercellular lipids, facilitating larger diffusivity of hydrophilic compounds since it decreased the resistance of paracellular pathway. Moreover, low interaction of ISIS 3082 with intracellular lipids is expected since molecule presents multiple charged species (96).

High concentrations of surfactant may not necessarily result in improved buccal absorption. Physico-chemical features such as the log P and charge of the macromolecule should also be considered when considering the use of surfactants or bile salts for improving buccal absorption. Different concentrations of sodium deoxyglycocholate at 1%, 2%, and 5% (comparable with 21.2, 42.4, and 105.9 mM, respectively) have been tested in experiments regarding the buccal absorption of calcitonin and 1% has been defined as the optimal concentration. Calcitonin is transported via the paracellular pathway due to effect of its strongly positive charge and transcellular transport seems not to occur even at high concentrations of sodium deoxyglycocholate (99). Although Nicolazzo et al. (2003) (90) have considered small molecules in their studies, they also suggest that the effect of surfactant is not only governed by its concentration, but also the physicochemical properties of the permeant. A reduced buccal absorption was observed for the estradiol at higher concentrations of sodium dodecyl sulfate (0.1 and 1%) after a pre-treatment protocol. This result was associated with a micellar solubilization of drug, which would decrease thermodynamic activity and, consequently, the driving force for drug absorption. This

hypothesis was not considered by Oh et al. (2011) (29) to explain the effect of the surfactant in buccal delivery of calcitonin.

In addition to the micellar solubilization and lipid-solubilizing effects of bile salts and surfactants on the buccal epithelium, bile salts may also present inhibitory effects on buccal membrane peptidases, which would contribute indirectly to improve the buccal absorption. Dyhydroxy bile salts (sodium deoxychoolate, sodium glycodeoxycholate, sodium taurodeoxycholate, sodium chenodeoxycholate, sodium glycochenodeoxycholate, sodium taurochenodeoxycholate) have been effective to reduce the calcitonin degradation rates in rat oral mucosa homogenate (it was reduced from 14 to 2 h^{-1}/mg protein) (48) as well as sodium glycocholate has inhibited insulin metabolism (a 5-fold maximum reduction) in homogenates of rabbit buccal mucosa (45). The selection of new absorption enhancer candidates for macromolecules should consider this aspect as enhancing the buccal delivery and preventing degradation are equally crucial, particularly for peptides and proteins.

As mentioned above, it is often times a dilemma that high concentration of synthetic surfactant or bile salts is required to enhance the macromolecules buccal absorption when irritation of the buccal mucosa will be induced. Therefore, natural surfactants were exploited to overcome the limitation. Lysalbinic acid, a non-ionic surfactant obtained from alkaline hydrolysis of albumin, have successfully enhanced the buccal absorption of α-interferon and insulin in Hamster cheek mucosa. Co-administration of 1 and 5% lysalbinic acid resulted in 5 and 9-fold increase of the buccal permeability of α-interferon. For insulin, 0.3% surfactant increased buccal permeability for above 5 times. Although these results may not be directly be extrapolated to human due to the fact that the hamster mucosa is keratinized, the experimental data suggested that lysalbinic acid is effective in improving the buccal delivery of macromolecules. More importantly, irritating effects of lysalbinic acid on the buccal mucosa were not identified in histological investigations. The molecular mechanism by which lysalbinic acid increases transport is not completely clear yet, but it seems be similar to that of other surfactants, i.e., providing an intercellular lipid solubilization (85).

3.2.2 Fatty acids

Despite its mechanism of action as the penetration enhancer has not been completely elucidated, it is suggested that fatty acids decrease the lipid packing in buccal epithelial cells (100). Factors such as fatty acid chain length of triglyceride, the saturation degree and the volume of lipid administered could potentially affect buccal delivery and should be considered during the development of buccal formulations (101).

Oleic acid, one of the most widely investigated fatty acids, has been incorporated into the cubic phase of glyceryl monooleate (GMO) to improve the *ex vivo* buccal permeability of [D-Ala^2, D-Leu^5]enkephalin, an opioid peptide with analgesic properties (102). When incorportaed in the cubic phase of GMO alone, oleic acid exhibited a poor permeability enhancing effect (ER=1.09), whereas a significant enhancing effect was observed with the addition of PEG 200 (enhancement ratio ranged from 1.15 to 3.99 depending on PEG concentration). The addition of PEG 200 also increased the *in vitro* release of oleic acid from this system up to 7-fold. In another study, the effect of oleic acid on buccal permeability of ergotamine tartrate (ET / MW 1,313 g/mol), also known as peptide-type ergot alkaloid, was investigated and compared with that of cod-liver oil extract (CLOE) using a keratinized epithelial-free membrane of hamster cheek pouch mucosa (103). Pretreatment with CLOE provided a 8 and 2-fold higher permeability flux and solubility, respectively. Although pretreatment with oleic acid has increased the permeation flux of ET by approximately 2-fold compared with the control group (no enhancer), this enhancing effect was approximately 3-fold lower than that of CLOE. It was suggested that CLOE mainly exhibit a direct action on buccal mucosa and solubilizing effect in the donor chamber.

While fluorescence polarization studies have suggested that oleic acid strongly reduces the lipid packing in both the hydrophobic and the polar head-group region of the non-keratinized buccal epithelial cell bilayer (100), Coutel-Egros et al. (1992) (104) have suggested that it may improve the permeability of compounds by an increase in partitioning of drugs into the tissue, however, little evidence to support this hypothesis. A number of experiments have been performed to explain the mechanism regarding the effect of oleic acid on the transdermal permeation (105), however, the results from these studies can not be extrapolated to the buccal mucosa in view of that the transport mechanisms of drugs are different in these membrane models (skin – transcellular transport vs. buccal mucosa – mainly paracellular) as well as their the lipid composition.

Other fatty acids have also been employed to enhance the buccal delivery of peptides. Unsaturated fatty acids (oleic, eicosapentaenoic or docosahexaenoic acid) were added individually into a Pluronic F-127 (PF-127) gel in order to maximize the buccal absorption of insulin (106). The presence of unsaturated fatty acid has decreased the insulin release from the gel, however, higher bioavaliability was observed. Oleic acid was the most effective since it increased the bioavaliability to 15.9% (compared to formulation containing only Pluronic F-127).

3.2.3 Chitosan

Chitosan is a biodegradable and biocompatible polysaccharide which has been widely used in the pharmaceutical formulations (107). Recently, it has also been employed in the transmucosal drug transport due to its bioadhesive property, which is resulting from the cationic charge of the primary amino groups (108). Ionic interactions between the cationic amino groups of chitosan and negative functional groups of the mucus (109) or epithelial cells (110) may be responsible for mucoadhesion, which is mainly responsible for enhancing effect. To futher enhance the mucoadhesive properties of this polymer, additional chemical modifications are carried out at its two hydroxyl groups (primary or secondary) and one primary amine group (107).

Several types of chitosan are commercially available differing in the molecular mass, deacetylation degree and presence of free or substituted by acid/amino groups (chitosan base or chitosan salt). Chitosan hydrochloride and chitosan glutamate are the two most widely used salts (73). The effect of different concetrations of chitosan glutamate ranged from 1 to 100 μg mL^{-1}) on the buccal permeability of fluorescein isothiocyanate labeled dextrans (FD) of different MW (4,10 and 20 KDa) have been evaluated in TR146 cell culture model, an *in vitro* model of the buccal epithelium (111). The most effective chitosan concentration for absorption enhancement was 20 μg mL^{-1}, which increased the permeability coefficient values to 2.9, 1.8, 1.7 times for FD4, FD10 and FD20, respectively (compared to the control in the absence of chitosan). The permeability of FDs was decreased as the MW increased. For chitosan concentrations higher than 20 μg mL^{-1}, the solutions become more viscous, and the diffusion of the macromolecules is hindered, especially those with high MW.

Since chitosan is only soluble in acidic solutions of pH below 6.5, which is required to insure the protonation of the primary amine, an elegant way to improve or to impart new properties to chitosan is the chemical modification of the chain, generally by grafting of functional groups, without modification of the initial skeleton (107). Sandri et al. (2005) (112) subjected two diferent chitosans (MW 1,460 and 580 kDa) to a methylation reactions obtaining two series of three trimethyl chitosans, using fluorescein isothiocyanate dextran (FD4, MW 4,400 Da) as drug model. The mucoadhesive performance increased on increasing the quaternization degree. Trimethyl chitosans (TMC) derived from the lower MWchitosan and characterized by the highest degree of quaternization shows the best mucoadhesive and penetration enhancement properties (permeated FD4 amount was approximately 8-fold higher compared to the control after 6 h at pH 6.4) and is the most promising TMC to improve the bioavailability of hydrophilic and large MW molecules (like peptides and proteins). In another study, this same

research team investigated the penetration enhancement mechanism of trimethyl chitosan hydrochloride and they have suggested that it involve a repackaging of the epithelial cells up to the basal membrane and a partial disarrangement of desmosomes (113).

Another modified form of chitosan, the thiolation of chitosan, has been also developed to improve the buccal permeability (114). These thiolated chitosans have numerous advantageous features in comparison to unmodified chitosan, such as significantly improved mucoadhesive and permeation-enhancing properties (115). For instance, the mucoadhesive properties of a chitosan-4-thio-butylamidine conjugate are greater than unmodified chitosan (adhesion time on freshly excised porcine mucosa was extended more than 140-fold by using the thiolated version) (116). Moreover, because of the capability of thiomers to bind divalent metal ions such as zinc ions, these polymers may inhibit zinc dependent proteases such as carboxypeptidases A and B as well as most membrane-bound peptidases (117). The strong cohesive properties of thiolated chitosans render them highly appropriate excipients in controlled drug release dosage forms (116). Langoth et al. (2006) (118) have selected thiolated chitosan aiming to enhance the bioavailability of adenylate cyclase-activating polypeptide (PACAP), a peptide designed to behave superior to insulin. A bioavailability of about 1% was reached by using drug delivery systems consisting of thiolated chitosan in pigs, whereas no PACAP was detected in plasma with use of unmodified chitosan. In addition to these results, the authors also suggest that cationic therapeutic peptides should be embedded in a cationic or nonionic mucoadhesive polymer such as thiolated chitosans because the incorportation in anionic polymers may lead a strong reduction in the mucoadhesive properties, and the drug release might be hindered by too strong ionic interactions between the therapeutic agent and the polymeric network (118).

Overall, the safety of this polymer and its derivates, its ability to prolong residence time in mucosal membranes, and its ability to enhance absorption by increasing cellular permeability have been major factors contributing to its widespread application (119, 120). Moreover, it offers remarkable biological properties, especially haemostatic (121) and antibacterial activity (122), making it a multifunctional matrix.

3.3 pH modulation

pH modulation can affect the buccal delivery in two aways (i.e. stability and ionized form). The control of pH is critical for successful buccal delivery of ionizable drugs (123, 124) as maximal permeability usually occurs at the pH at which macromolecules are predominantly in the unionized form (125). pH modifiers can be included in formulations in

order to temporarily modulate the microenvironment at the application site improving not only the buccal absorption (42) but also the stability against enzymatic degradation given that various enzyme-catalyzed degradation processes are pH-dependent (126). Acid and basic pH modifiers have been added into luteinizing hormone releasing hormone (LHRH)-loaded mucoadhesive formulations to evaluate the effect of pH on the buccal absorption in Beagle dogs given that this hormone has three ionization sites (87). It was found that the protonation of histidine at the acid pH resulted in higher plasma profile and greater bioavailability (C_{max} and bioavailability were, respectively, 3.1 and 2.6 times those of formulation without pH modifier), though higher mucosal irritation response was also observed.

Polymers are often used to prepare the mucoadhesive formulations for buccal delivery and they can be differentially ionized depending on the pH, which may affect the strength of mucoadhesion and subsequently the buccal permeability of the formulations. Polycarbophil has been used to prepare mucoadhesive formulation to improve buccal transport. Studies have showed that at a pH higher than the pKa of polyacrylic acid, polycarbophil tends to absorb water up to 100-800 times of its weight, which compromise the adhesive bonding between the buccal mucosa and the mucoadhesive formulations prepared by polyacrylic acid (127). Therefore, pH control strategies should be considered when this polymer is used in buccal delivery systems.

Absorption or effectiveness of some peptides may also be reduced if physico-chemical properties such as solubility are affected by pH. Insulin is less soluble at pH 3.4 and 5.4 than at pH 7.4 (pI=5.4), however, relative buccal efficacy to i.m. insulin is similar at different pHs (1, 1.2 and 0.7 at pH 3.4, 5.4 and 7.4, respectively). In the presence of 5% laureth-9, clear solutions were obtained at pH 3.4 and 7.4, but insulin was not completely soluble at pH 5.4. The efficacy of buccal insulin was the lowest at pH 5.4 (approximately 2-fold lower compared to the other pHs). In the presence of 5% sodium fusidate, another absorption enhancer, the lowest efficacy was also observed at pH 5.4 (2-fold lower than for other pHs) and insulin was incompletely soluble at each pH at room temperature (80). Although the authors have not argued on the probable mechanism responsible for the reduced effectiveness (blood glucose levels), a relationship between solubility and absorption should be considered.

Another aspect to be considered during buccal permeability prediction regarding pH changes is the fact that the ionization condition of epithelial or mucus structures also play an important role in the buccal transport of peptides and proteins. At physiological pH or at a pH above the isoelectric point (pI), of which epithelial structures (due to the sialic acid and sulfate residues) are negatively charged and are selective to

attract positively charged solutes (26). Taken together, pH needs to be carefully selected to optimize buccal permeation and the development of mucoadhesive formulation.

3.4 Iontophoresis

Recently, iontophoresis has been increasingly used to improve buccal delivery. It is a non-invasive and patient-friendly method that enables hydrophilic charged molecules penetrate through biological barriers to achieve both local and systemic effects (128). Iontophoresis enhances the rate of movement of ionic compounds across membranes by an externally applied electric potential (129). The iontophoretic system consists of a donor solution containing the drug in its ionic form and a receptor solution separated by a limiting membrane, in this case buccal mucosa, where is generated a voltage gradient by connecting an anode and cathode to a voltage source that supplies direct constant electric current. The circuit is completed as the ions carry the current through the tissue barrier. Similarly the skin, the buccal membrane has a total negative charge facilitating the delivery of positively charged compounds (130). This method has potential to be applied in buccal delivery systems of drugs with poor penetration properties, especially for high molecular weight electrolytes such as proteins, peptides and oligonucleotides (131).

Molecular transport during iontophoresis can be attributed to three component mechanisms: (enhanced) passive diffusion, electromigration and convective solvent flow, also called electroosmosis (128). Epithelia proteins from buccal mucosa are negatively charged at physiological pH and, acting as a cation-selective ion-exchange membrane (132). As a consequence, under the influence of an electric field, a convective solvent flow is generated in the anode-to-cathode direction. Assuming that each phenomenon is independent, the total flux of a molecule during iontophoresis can be described as the sum of the fluxes resulting from those three processes described previously (128).
Results from *in vitro* transbuccal experiments using iontophoresis suggest that the total drug flux is proportional to current density applied and is an exponential of the initial donor concentration under influence of competitive ions (21). Previous transdermal studies have considered that a level of electrical current of approximately 0.5 mA is physiologically acceptable, however, Guy (1996) (133), suggests a reduction in these levels for buccal mucosa given its lower barrier properties compared to skin. In addition to that, iontophoresis can negatively affect physico-chemical stability of macromolecules (134). For example, 65% degradation of thyrotropin-releasing hormone have been reported at a current value of 0.5 mA (135). Therefore, studies monitoring the tissue viability should be carried out until optimal cut-off levels are defined and

experimental parameters such as electrode orientation should also be carefully defined. Depending on the charge of drug molecule, the electrode orientation for successful iontophoretic delivery may be cathode-to-anode (anodal) or anode-to-cathode (cathodal) (136). The selection of appropriate electrodes is also highly relevant in order to avoid tissue irritation, reduction in drug stability and variations in its release. Ag/AgCl active electrodes are commonly selected because inactive electrodes such as carbon or platinum induce proton prodution, which may lead to those problems described above (137).

The role of iontophoresis has been investigated to enhance the buccal delivery of four model macromolecular compounds (dextrans – 3 and 10 kDa; bovine serum albumin – 64 kDa and parvalbumin – 12 kDa). The effect of parameters such as electrode polarity, pH of donor solution and different levels of electric current on buccal absorption of large molecules was also evaluated. Dextrans and parvalbumin were successfully delivered across porcine buccal mucosa after an anodal pulsed electrical stimulation (cathode to anode orientation). Flux enhancement ratios ranged from 32 to 38 for dextrans and 36 for parvalbumin. Iontophoretic delivery were approximately 37 times faster than passive diffusion. These results clearly indicated that the use of a physical technique is efficient to deliver peptides though the buccal mucosa. Parvalbumin (pI 4.1) had its buccal absorption influenced strongly by pH, which it was not successful at pH 3 and 10. The iontophoretic delivery of peptides with a pI between 4 and 7 has been challenging. For example, if a peptide becomes uncharged, the iontophoretic force will no longer apply; and precipitation phenomena have been observed when protein cations start to permeate the tissue. The bovine serum albumin did not present any buccal absorption and that result was associated with its molecular size (138).

In addition, the combination of chemical absportion enhancer and iontophores has also been studied recently (99). ER of calcitonin from the groups subjected to iontophoresis alone was 66-fold higher than that of the control group. The combination of iontophoresis and chemical enhancers further enhanced the transbuccal delivery of calcitonin to an approximately 165-fold increase in the ER value, demonstrating that it could be a potential strategy for the enhancement of peptide transbuccal delivery. The synergistic effect has been attributed to the increasing intercellular spacing resulting from chemical enhancers, which then improve the effectiveness of iontophoresis. More importantly, the combination of chemical enhancer and iontophoresis is clinically practical as the device and the processes required to combine both approaches is not likely to be significantly more complicated compared to that required for iontophoresis alone (139).

In future studies regarding the use of this technique, it is suggested that different parameters should be evaluated simultaneously

in order to optimize the buccal absortption, such as the composition of fomulation (drug concentration, pH of donor solution, presence of co-ionic, ionic strength), physicochemical properties of permeant (molecular weight and size, charge) and experimental conditions (current density and profile, duration of treatment, electrode material and polarity of electrodes) (140).

3.5 Formulation design

3.5.1 Particulates systems/delivery vectors

Particulate systems, such as emulsions, liposomes, micro- and nanoparticles have been widely used and remain as a promising approach for buccal delivery of macromolecules. The design and development of colloidal systems, which are very fine solid particles (typically ranging from 10 nm to 10 μm) suspended in a fluid phase, can help to overcome limitations of macromolecules buccal transport such as poor stability, low bioavailability and short half-lives (141). Additionally, these systems can provide a sustained and targeted release (142).

Although more hydrophobic polymers have been preferentially selected to increase the affinity by lipid bilayers, this could potentially lead to precipitation of proteins during storage or physiological conditions due to differences of the polarity (143). Thus, the addition of surfactants in order to increase the affinity between protein and polymer or the incorporation in hydrophilic polymers such as polyethylene glycol, chitosan, alginates, poloxamers may be used alternatively (144).

Among the hydrophilic polymers studied, chitosan seems to be a more suitable polymer given that it is able to retain the peptide in buccal mucosa upon its release. Insulin-loaded chitosan-ethylenediaminetetraacetic acid hydrogel films showed that the mucoadhesive force of the hydrogel remained over 17,000 N/m^2 during 4 h in the simulated oral cavity. A pronounced hypoglycemic effect following buccal administration to healthy rats was obtained, achieving a 17% pharmacological availability compared with subcutaneous insulin injection (145).

Alternatively, the use of alginates and poloxamers (both hydrophilic) as encapsulation materials has been studied and also represented a promising alternative to overcome those restrictions as they are able to swell large amounts and retain significant fractions of water in their structure without dissolving (144).

Despite the polymers mentioned above have provided promising results, instability problems have already been reported in insulin-loaded poly (lactic-coglycolic acid) (PLGA) microspheres. The erosion of PLGA reduces the microenvironmetal pH, leading the deamidation reactions and insulin instability. The relative precentages of

desamido insulin extracted from PLGA microsphere samples were 25 and 45% after 6 and 18 days release (146). Thus, PLGA may not provide a microenvironment that is unequivocally suitable for all protein and peptide drugs.

With the recognition of the role of surfactant in increasing the affinity between protein and polymer, lecithin and propanediol encapsulated in nanostructured system has been tested in rabbits aiming to improve the buccal delivery of insulin (147). The average bioavailability of insulin-loaded nanoparticle system was 18.3% (buccal delivery versus subcutaneous injection), indicating that this system may be promising for the buccal delivery of insulin. It is worth mentioning that the authors used phenol for previous drug solubilization, however, toxicity data were not presented.

Diblock copolymer composed of a poly(D,L-lactide) (PLA) core with a hydrophilic chain of poly(ethylene glycol) (PEG) was also considered. Nanoparticle system coated with PEG has been designed to increase the stability of this peptide when in contact with physiological fluids and provide a controlled release. Moreover, it is well established that PEG has a mucoadhesion promoting effect. Although the authors have suggested that the proposed system can provide a controlled buccal delivery of proteins based on *in vitro* release experiments, buccal transport studies from optimized systems were not carried out (141). In another study, biodegradable and redox-reponsive complex systems have also been prepared for the transmucosal delivery of proteins and peptides. Insulin-loaded chitosan/poly (L-aspartic acid) submicron capsules prepared by layer-by-layer technique are able to release the peptide from these systems when exposed to different levels of glutathione (GSH) (148). This system has shown acceptable biocompatibility (cell viability was above 90% at 40-500 μM) and mucoadhesive properties (adsorbed mucin amount achieved up to 48.1 μg/mg submicron capsules) able to extend the residence time after mucosa administration. Moreover, they observed that the release of insulin may be controlled by the content of thiol groups in the particles since submicron capsules with the more disulfide linkages had the lowest release rate (69% vs. 91%). The buccal permeability experiment with this capsule system has not been performed yet, but it is certainly worth investigating.

The combination between particulate system and bile salts has been employed to improve the buccal delivery of macromolecules. Phospholipid deformable vesicles (transfersomes) with and without sodium deoxycholate have been prepared by reverse phase evaporation methods in order to enhance the buccal insulin delivery (149). Differences in buccal permeability were not observed between vesicles with and without surfactant. On the other hand, the relative pharmacological bioavailability and the relative bioavailability in the

insulin-deformable vesicles group were 15.59% and 19.78%, respectively (compared to subcutaneous administration of insulin solution). This result also was significantly higher than that of conventional insulin vesicles, blank deformable vesicles and insulin mixture groups. It has been suggested that transfersomes may respond to external stresses by rapid shape transformations requiring low energy, which allows them to deliver drugs across barriers. Given that these systems presented high interaction with the buccal mucosa, its use for buccal delivery of other protein drugs is also suggested (149).
In view of the fact that aqueous suspensions/solutions do not provide high retention of the dosage form in buccal mucosa due to continous dilution by salivary flow, particles have been immobilized in buccal films or other solid systems.

3.5.2 Immobilized drug delivery systems

The buccal mucosa is a very suitable region for bioadhesive systems because of its smooth and relatively immobile surface as well as it easy accessibility (60). Mucoadhesive systems are essential to maintain an intimate and prolonged contact of the formulation at the absorption site, allowing that therapeutic drug levels to be maintained for the desired period of time (23). Interactions between polymer and mucosal surfaces occur via physical entanglement (diffusion theory) and/or chemical interactions such as electrostatic, hydrophobic, hydrogen binding and van der Waals interactions (adsorption and electronic theories) (42). In general, bioadhesive strength increases with the molecular weight, polymer mucoadhesive concentration, presence of hydrogen bond-forming groups (hydroxyl, carboxyl, amines and amides), chain flexibility, positively or negatively charged groups, reduced cross-linking density and a critical degree of hydration (150). Factors such as saliva secretion, food absorption, local pH, turnover of mucus layer also strongly affect bioadhesion (60). For those mucoadhesive formulations designed to deliver peptides and protein is also desirable that the used polymers are able to inhibit certain proteolytic enzymes since these macromolecules present various catalytic sites susceptible to enzymatic attack.

Solid bioadhesive dosage forms are more convenient for the patient than gel and ointments (151); and solid systems typically offer greater drug stability, improved residence time in buccal mucosa and hence may provide longer periods of therapeutic drug levels at diseased sites (152). The design of buccal dosage form, particularly films, patches and tablets may include (i) single-layer devices in that the drug is released multidirectionally, (ii) devices presenting an upper impermeable layer which minimize the loss of drug into the oral cavity or (iii) unidirectional release devices, from which drug loss is minimal since the

drug is released only from side adjacent to the buccal mucosa (60). Mucoadhesive polymers used in these formulations have been classified as first and second generation (152). The older generation includes charged hydrophilic polymers, which adhere to the mucus non-specifically, and present short retention due to the turnover rate of mucus. On the other hand, second-generation systems based on lectin, thiol and various other adhesive functional groups are able to interact more strongly with the cell surface than mucus (42).

Buccal tablets have been the most commercially available dosage form, however, the lack of physical flexibility has led to poor patient compliance for long-term and repeated dose and other alternatives have been studied. Mucoadhesive buccal films share some of these advantages and more. Due to their small size and thickness, and its flexibility, they have improved patient compliance (145).

In addition to traditional solid mucoadhesive forms, solid systems such as resorbable polymeric wafers and sponges have been proposed recently to deliver macromolecules through buccal mucosa. A low number of *in vivo* studies can be found in literature even if these systems seem promising. Unlike semi solid polymer gels, wafers can maintain their swollen gel structure for a longer period and therefore longer residence time (153). Due to their porous nature and higher surface area, they are able to load higher amount of drug compared to the thin and continuous solvent cast equivalent (154). Polymeric wafers are usually prepared by freeze-drying technique, offering more stable products, extend shelf life, as well as it enables storage of products at room temperature (155). On the other hand, drug or excipient crystallization may be observed during freezing or storage, requiring a rigorous product physico-chemical characterization during its development.

Portero et al. (2007) (156) have also suggested the use of chitosan sponges as carrier of macromolecules in buccal systems. In this sense, insulin was loaded in a mucoadhesive chitosan layer, coated with another ethylcellulose-based impermeable protective layer to provide unidirectional drug release. Unfortunately, the buccal experiments have not been carried out and the applicability of the macromolecules-based formulations require further studies. Alternatively, the performance of these new systems (polymeric sponges and wafers) with those traditional (buccal tables and films) should be compared.

4. FUTURE DIRECTIONS

The purpose of this section is to discuss strategies that seem promising for improving the buccal absorption of macromolecules, however, their effectiveness has not yet been confirmed/tested. The

addition of anti-aggregating agents would be an indirect approach to reduce or avoid physico-chemical instability problems of macromolecules [self-aggregation phenomena, a common process for some peptides (157)], which could lead to precipitation and thus reducing the buccal absorption. On the other hand, the use of cell-penetrating peptides is an opportunity to improve the biodistribution of therapeutic molecules, delivering molecules by routes which are not otherwise achievable (e.g., skin penetration) or into compartments or tissues which minimize potential toxicities (158).

4.1 Addition of anti-aggregating agents

Unlike other small molecular compounds, peptides and proteins possess some specific characteristics such as self-aggregation. A considerable number of soluble proteins have been converted into insoluble fibrils due to self-aggregation phenomena occurring in an intermolecular level under particular solvents, temperature and pH conditions (157). Nonspecific forces, such as hydrogen bonding could also contribute to this protein self-assembly (159). Moreover, the lipid bilayer has been suggested to provide an environment in which the aggregated state of polypeptide chain appears to be more thermodynamically favorable than its monomeric form (160). In fact, protein aggregation has been recognized as a major manifestation of instability that can severely affect protein functions *in vivo* and *in vitro* and also induce toxicological reactions (161). Overlooking the experimental and physiological conditions which accelerate the self-aggregation of these macromolecules may lead to a reduction of their absorption in different biological membranes such as the buccal mucosa. Indeed, this self-aggregation behavior has been reported for the two peptides most commonly tested in buccal transport experiments – calcitonin and insulin. Calcitonin has presented a tendency to aggregate in aqueous solutions and to form long, thin fibrillar aggregates resulting in viscous and turbid dispersions easily detected by microscopic techniques (162). *In vitro* insulin fibrillation occurs very fast, particularly at low pH, high temperature, high ionic strength and on hydrophobic surfaces (161). Thus, additives or co-solvents that prevent protein aggregation could be employed in order to stabilize macromolecules such as insulin and calcitonin in buccal permeability studies. Compounds that can either prevent unfolding of the native protein or sequester partially folded aggregation-competent intermediates have been effective in increasing the native insulin stability (163). Carbohydrates and glycerols as well as low molecular weight compounds such as ectoine, trehalose, and citrulline has enhanced the stability of insulin though preferential exclusion of these co-solutes from the protein surface and subsequent enhancement of hydrophobic interactions within

the native structure. Lecithins, cyclodextrins, and polymeric surfactants reduce insulin aggregation by binding either to hydrophobic interfaces or to hydrophobic insulin domains (164). Therefore, applying these compounds has the potential to prevent protein aggregation, optimizing the macromolecular buccal delivery. Anti-aggregation agents presenting an additional enhancement effect on buccal permeability should be prioritized.

4.2 Cell-penetrating peptides as macromolecules carries

The use of cell-penetrating peptides (CPP) as carrier system as a novel approach for delivery of macromolecules across biological membranes and tissue barriers has been recently proposed (165). Complex systems in which nanostructured lipid carriers are coated with CPP have already been exploited for the oral delivery of peptide (166), which showed improved bioavailability. The relative oral bioavailability of the nanostructured-lipid carriers coated with CPP compared to that of tripterine suspension and T-NLCs were 484.75% and 149.91% respectively. Peptide transporters are perhaps the most versatile of all of the membrane carrier systems that have been discovered so far because of their capacity and broad substrate specificity (167). These systems act by transporting their cargoes into the cytoplasm via perturbation of the lipid bilayer of the cell membrane or by endocytosis (168). These peptides carrier are relatively short (up to 30 amino acids in length), water soluble, cationic and amphiphilic, being able to penetrate the cell membrane at low micromolar concentrations without using any receptors or causing any significant membrane damage (165). Although promising results have already been observed, the uptake mechanism of these conjugates by epithelial cells and its subsequent enzymatic degradation are unclear (169). However, another advantage of this system is that since buccal mucosa possesses minimal enzyme activity, it would present additional advantages compared to the intestinal mucosa as the stability of peptide delivery system could be affected by the various enzymes present at the intestine. Whether similar enhancement can be achieved when the cell-penetrating peptides system applied in the buccal delivery will require further investigation, however, this approach has showed promising delivery of peptides and is certainly worthing more exploitation.

5. CONCLUSION

Over the last two decades, buccal mucosa has drawn a lot of attention as a absorption site for poorly permeating macromolecules as buccal mucosa possesses some unique properties. Given the characteristics of peptides and proteins such as hydrophilicity and large

molecular weight, physical, chemical and formulation techniques have been applied to improve transport through the paracellular route, each with its advantages and limitations. Therefore, special attention should be paid so that the most efficient technique can be selected considering the particular physical and chemical characteristics of each macromolecule. To achieve better buccal penetration or bioavailability of peptides and proteins, two or more of these approaches are often employed simultaneously to present a synergistic effect. Furthermore, cell-penetrating peptides and co-application of anti-aggregation agents are two promising methods, which have showed their potentials to stabilize and enhance the buccal permeability of macromolecules.

Acknowledgements
T. Caon and C.M.O. Simões thank, respectively, to CAPES/MEC and CNPq/MCTI, for providing his PhD scholarship (BEX 12349/12-7/“Sandwich” PhD Program) and her research fellowship. The work is also supported by the National Health and Medical Research Council (number #1042481).

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4. ARTIGO PUBLICADO

RESEARCH ARTICLE – *Pharmaceutics, Drug Delivery and Pharmaceutical Technology*

Exploiting the Buccal Mucosa as an Alternative Route for the Delivery of Donepezil Hydrochloride

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Received 15 December 2013; revised 26 February 2014; accepted 27 February 2014

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23950

ABSTRACT: The potential of the buccal mucosa as an alternative route for the systemic delivery of donepezil (DPZ) hydrochloride, and the impact of various skin penetration enhancers on DPZ buccal permeability, was assessed using an *in vitro* model. DPZ was applied to porcine buccal mucosa in modified Ussing chambers either alone (20 μg/mL) or with different treatment protocols of various enhancers including Azone® (AZ), deoxycholic acid (DA), polyethylene glycol (PEG) 400, and oleic acid (OA)–PEG 400. DPZ permeated the buccal mucosa very rapidly with a permeability coefficient of $35.6 \pm 4.9 \times 10^{-6}$ cm/s, which was not significantly affected by AZ pretreatment. Coapplication of DA 0.6% (w/w), but not DA 0.01% (w/w), reduced the buccal permeation of DPZ (3.5-fold), and PEG 400 reduced the absorption of DPZ in a concentration-dependent manner (1.6- and 18.0-fold reduction at 5% and 50%, w/w, PEG 400, respectively). Coapplication of a combination of OA 1% (v/w) and PEG 400 5% (w/w) further reduced DPZ permeability (5.5-fold), which was demonstrated to result from excipient-induced DPZ precipitation as assessed by light microscopy analysis. These results confirm the feasibility of a novel buccal delivery system for Alzheimer's disease, and suggest various approaches that may be exploited for controlled buccal delivery of DPZ.

Keywords: Permeability; buccal; transmucosal drug delivery; drug transport; absorption enhancers; donepezil hydrochloride; Azone®; deoxycholic acid; polyethylene glycol; oleic acid

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that impairs memory and cognitive function mainly in the elderly.[1] Given that AD is a multifactorial disease, various approaches have been proposed to either slow down the progression or prevent the onset of AD; however, the cholinergic hypothesis is still the only hypothesis on which currently approved treatments are based.[2,3] According to this hypothesis, the degradation of cholinergic neurons in the basal forebrain and the loss of cholinergic neurotransmission in the cerebral cortex and other brain regions contribute significantly to cognitive decline.[4] Consequently, therapeutic agents that inhibit acetylcholinesterase have shown beneficial effects in improving cognitive function, albeit they do not prevent the progression of the disease.[5] Different acetylcholinesterase inhibitors are currently approved for use in AD treatment such as donepezil (DPZ), galantamine, rivastigmine, and tacrine. DPZ has shown significant advantages over the other inhibitors because it is approximately 10 times more potent than tacrine, 500–1000-fold more selective for acetylcholinesterase over butyrylcholinesterase,[6] and it also exhibits a longer plasma elimination half-life (70–80 h) relative to other acetylcholinesterase inhibitors, resulting in longer dosing intervals.[7]

The most commonly reported adverse effects of orally administered DPZ occur in the gastrointestinal tract, including nausea, vomiting, and diarrhea.[8,9] These effects are dose-dependent[9] and are more prominent in patients who exhibit poor metabolism (~50% of this population cluster), resulting in adverse events even at low doses.[3] Furthermore, there may be large fluctuations in plasma concentrations after oral administration[10,11] as a result of the rapid absorption characteristics of DPZ, and the interpatient variability in metabolism. For these reasons, and the fact that patients with memory deficits may be at risk of forgetting to self-medicate (further impacting on steady-state plasma concentrations of DPZ), alternative routes of DPZ delivery could significantly enhance therapeutic options available for AD patients. Although one report suggests the possibility of subcutaneous injection of poly lactic-*co*-glycolic acid microparticles for controlled DPZ delivery,[12] this administration route can be inconvenient for many patients, and thus, alternative routes for drug delivery should be investigated. To this end, a recent preclinical study has evaluated the transdermal absorption of DPZ across hairless mouse skin.[13] However, the barrier nature of the skin resulted in an extremely low permeability coefficient of DPZ (25.0×10^{-9} cm/s), requiring a large surface area to achieve the desired plasma levels.

The buccal mucosa represents another route for the delivery of therapeutic agents, and has been exploited clinically for the systemic delivery of fentanyl citrate, miconazole nitrate, and midazolam.[14] The buccal mucosa exhibits high permeability and appreciable bioavailability of therapeutics as it is highly vascularized, with direct access to the systemic circulation through the internal jugular vein and avoidance of hepatic first-pass metabolism.[15] Not only would this route allow for a

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reduction in the dose and, consequently, plasma fluctuations of DPZ, but would minimize the potential for the gastrointestinal side effect profile associated with DPZ. Furthermore, this route is a more convenient route (relative to the proposed subcutaneous injection paradigm), allowing for easy administration and removal of a DPZ dosage form in the case of emergency or overdosage.[14] The potential of the buccal mucosa as an alternative route of delivery for DPZ has not been investigated; however, given the higher permeability of the buccal mucosa relative to the skin,[16] such a delivery route may be of extreme benefit to AD patients. However, as the buccal mucosa still exhibits some barrier properties, chemical enhancers may be necessary to improve the buccal delivery of DPZ. This may be particularly important for DPZ, given the p*K*a of DPZ of 8.8, and with experimental studies being undertaken at pH 7.4 (where DPZ will be approximately 96% ionized),[17] the permeability of DPZ may be limited. Therefore, the purpose of this study was to investigate the potential of the buccal mucosa for the systemic delivery of DPZ and the impact of different skin chemical penetration enhancers [Azone®, AZ; deoxycholic acid, DA; polyethylene glycol (PEG) 400; and oleic acid (OA)–PEG 400 combination] on DPZ permeability using an *in vitro* porcine buccal mucosa model previously established in our laboratory.[18–20]

MATERIALS AND METHODS

Materials

DPZ was purchased from EMD Chemicals (San Diego, California). Krebs bicarbonate Ringer (KBR) buffer was prepared with 115.5 mM NaCl, 4.2 mM KCl, 21.9 mM $NaHCO_3$, 12.2 mM glucose, 4.0 mM HEPES, 1.2 mM $MgSO_4{\cdot}7H_2O$, 2.5 mM $CaCl_2{\cdot}2H_2O$, and 1.6 mM $NaH_2PO_4{\cdot}2H_2O$, and adjusted to pH 7.4 with carbogen (95% O_2 + 5% CO_2) bubbling. AZ was purchased from Yick-Vic Chemicals and Pharmaceuticals (HK) Ltd. (Hong Kong, China). Ammonium acetate, OA, PEG 400, and DA were obtained from Sigma–Aldrich (St. Louis, Missouri). Acetonitrile (Mallinckrodt, Paris, Kentucky) was of HPLC grade and all other chemicals were of analytical grade and were used as received. Water was obtained from a Milli-Q water purification system (Millipore, Milford, Massachusetts).

Methods

DPZ Transport Studies

Porcine buccal tissue was obtained from a local abattoir immediately after slaughter and was transported in ice-cold KBR (pH 7.4). Within 2 h of slaughter, the buccal epithelium was carefully separated from the underlying connective tissue using forceps and surgical scissors. The separated epithelial tissue (~500 μm in thickness) was kept in ice-cold KBR and supplied with carbogen bubbling until being placed in modified Ussing chambers (diffusional area 0.64 cm^2). To determine the impact of AZ, DA, PEG 400, and combinations of PEG 400 and OA on the transport of DPZ, different treatment protocols were considered (pretreatment and coapplication) and compared with that of control (DPZ in KBR). Different pretreatment approaches were considered depending upon the type of penetration enhancer used.

For pretreatment protocols, porcine buccal mucosa was placed in the modified Ussing chambers, which were clamped together, and the donor chamber was filled with 1.5 mL of either KBR alone or KBR containing DA 0.6% (w/w) or OA 1% (w/w)–PEG 400 5% (w/w) for 30 min. The receptor chambers were filled with 1.5 mL of KBR and the chambers were kept at 37°C for 30 min and supplied with carbogen bubbling (95% O_2 and 5% CO_2). For the AZ pretreatment, a 10-μL aliquot of AZ 50% (w/w) in ethanol (EtOH) 95% (v/v) or EtOH 95% (v/v) alone was applied to the exposed buccal mucosa area and the Ussing chambers were clamped together after 30 min. After all pretreatment approaches, solutions were removed and the donor and receptor chambers were filled with 1.5 mL of DPZ solution (20 μg/mL in KBR) and KBR, respectively, and the permeability study was commenced (described in the following paragraph). A donor chamber concentration of DPZ of 20 μg/mL was used for all experiments based on the sensitivity of the HPLC assay and aqueous solubility of DPZ.

For cotreatment approaches, the modified Ussing chambers were clamped together immediately after the porcine buccal mucosa was inserted, and both the donor and receptor chambers were incubated with 1.5 mL of KBR for 30 min at 37°C and supplied with carbogen bubbling. After this equilibration period, both donor and receptor solutions were removed and replaced with either 1.5 mL of DPZ (20 μg/mL in KBR) with or without enhancers [DA 0.01 or 0.6% (w/w), PEG 400 5% (w/w) or 50% (w/w), or OA 1% (w/w)–PEG 400 5% (w/w)] in the donor chamber and KBR in the receptor chamber. From this step forward, similar procedures were considered for both the pretreatment and coapplication protocols. Immediately after addition of the DPZ solution (alone or in the presence of penetration enhancer), a 20-μL aliquot of the donor solution was taken to determine the initial concentration of DPZ present in this chamber. At time intervals of 30 min over 4 h, samples were taken simultaneously from the donor chamber (20 μL) and receptor chamber (200 μL). Although the receptor chamber was replenished with 200 μL of fresh KBR after each receptor sample was taken, the donor chamber was not replenished to prevent continual dilution of the donor chamber solution. Donor and receptor chamber samples were diluted with an equal volume of acetonitrile to precipitate buffer salts and proteins (which may have been extracted from the buccal tissue over the 4-h period). Samples were vortexed and centrifuged for 5 min at 13,147*g* and the supernatant was then transferred into a vial and analyzed by HPLC (according to the method described below). All experiments were conducted using the buccal mucosa of at least two pigs with six replicates.

The steady-state permeation flux (J_{ss}) was determined from the linear slope of the cumulative amount of DPZ permeated versus time curve. The apparent permeability coefficient (P_{app}) of DPZ was then determined by dividing J_{ss} by the initial donor chamber concentration, and P_{app} values from different treatments were compared using a one-way analysis of variance followed by a Tukey's post-hoc test. All statistical analyses were performed using GraphPad Prism software (version 5; GraphPad, San Diego, California).

HPLC Analysis

Chromatographic conditions were evaluated and optimized to obtain good resolution, a narrow peak shape without tailing, adequate sensitivity, and a short retention time of DPZ in KBR (which had been previously exposed to buccal mucosal tissue for 4 h). The concentration of both samples and standard solutions (0.1–10 μg/mL) was assessed by HPLC using a Shimadzu

DOI 10.1002/jps.23950

Table 1. Permeability Coefficient of DPZ Across Different Biological Membranes

Route of Administration	Model Membrane/Diffusion System	Permeability Coefficient (Mean ± SD) (cm/s)	Reference
Buccal	Pig buccal epithelium/Ussing chambers	$35.6 \pm 4.9 \times 10^{-6}$	Current study
Transdermal	Hairless mouse skin/Keshary–Chien diffusion cells	$25.0 \pm 5.6 \times 10^{-9}$	Choi et al.[13]
Oral	MDR1–MDCK cell system/Transwell inserts	$14.7 \pm 0.7 \times 10^{-6}$	Summerfield et al.[23]

LC-10A system (Kyoto, Japan), equipped with an ultraviolet detector, pump (LC-20ADXR), autoinjector (SIL-20AC), online degasser (DGU-20A3), and column heater (CTO-20AC). Chromatographic separation was performed at 40°C using a C_{18} analytical column (250 × 4.6 mm^2, 5.0 μm internal diameter) (Phenomenex®, Torrance, California) preceded by a guard column of the same packing material. The mobile phase consisted of acetonitrile and 10 mM ammonium acetate in a ratio of 50:50 (v/v). The flow rate was set at 1 mL/min and the total sample acquisition time was 10 min. The detector and the injection volume were 270 nm and 100 μL, respectively. The quantification method was validated according to US FDA[21] and EMA[22] guidelines. Individual r^2 values for each replicate of the calibration curve were higher than 0.999, suggesting good linearity for the considered range of DPZ standard solutions in KBR (0.1–10 μg/mL). Instrumental, intraday and interday precision ($n = 6$) showed relative standard deviation values less than 3.3%, 11.7%, and 7.5%, respectively. Intraday and interday accuracy ranged from 95.7% to 100.2% and from 98.2% to 99.6%, respectively.

Isothermal Titration Calorimetry

Calorimetric measurements were carried out using a high-sensitivity MicroCal isothermal titration calorimeter (GE Healthcare, Piscataway, New Jersey) to identify any alterations in thermodynamic activity of DPZ with increasing concentrations of PEG 400. A 250-μL volume of DPZ (20 μg/mL in KBR pH 7.4) containing 5% or 50% (w/w) PEG 400 was placed in the sample cell, and 15 injections of DPZ (20 μg/mL in KBR pH 7.4) were titrated (with each injection being 2.6 μL, an injection duration of 5 s and an interval of 220 s between each injection). All aqueous solutions were degassed under vacuum prior to calorimetric studies to eliminate air bubbles. The measurements were carried out at 37°C and the heats of dilution were recorded with MicroCal Origin 5.0 software used to process the data. The heat of dilution profile was plotted as kcal per mol of injectant titrated against the number of injections.

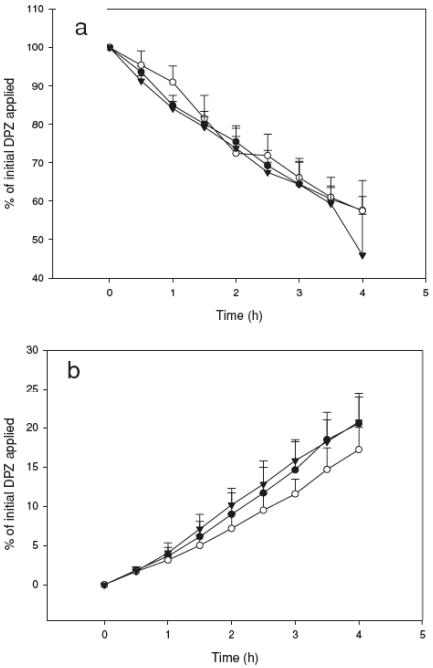

Figure 1. Percentage of initial DPZ applied disappearing from the donor chamber (a) and appearing in the receptor chamber (b) through porcine buccal epithelium pretreated with KBR (▼), EtOH 95% (○), or AZ 50% (●) for 30 min. Data are presented as mean ± SD ($n = 6$).

RESULTS

Once performance characteristics of the developed HPLC method were deemed adequate for the intended use, buccal permeability studies were undertaken. DPZ exhibited a very high buccal permeation with the final DPZ amount (relative to the amount applied) being 54% and 21% in the donor and receptor chamber, respectively. This resulted in a P_{app} of 35.6 ± 4.9 × 10^{-6} cm/s, which was higher than the P_{app} of DPZ across the skin and intestinal cells (Table 1). Pretreatment of porcine buccal mucosa with EtOH 95% or AZ 50% (w/w) in EtOH 95% exhibited similar disappearance and appearance profiles as DPZ (Fig. 1), with the P_{app} value in AZ-pretreated tissue being similar to that of EtOH-pretreated and KBR-pretreated tissue ($p > 0.05$) (Table 2).

Although coapplication with DA 0.01% (w/v) had no significant effect on neither the appearance nor disappearance of DPZ through porcine buccal mucosa, coapplication of DA 0.6% (w/v) significantly retarded the disappearance of DPZ from the donor chamber and the appearance of DPZ in the receptor chamber (Figs. 2a and 2b). This coapplication at the higher concentration significantly reduced the P_{app} of DPZ 3.5-fold ($p < 0.0001$), as summarized in Table 2. To confirm that the effect of DA was because of being at a concentration greater than its critical

Table 2. Impact of Different Treatment Approaches (Pretreatment and/or Coapplication) with AZ, DA, PEG 400, and Combination of PEG 400 and OA on the Buccal Permeability Coefficient (P_{app}) of DPZ

Sample (Treatment Protocol)	$P_{app} \pm$ SD (cm/s) $\times 10^{-6}$
DPZ alone	35.6 ± 4.9
AZ (pretreatment)	36.5 ± 6.9
EtOH (pretreatment)	29.6 ± 5.2
DA 0.6% (w/v, coapplication)	10.2 ± 1.9*
DA 0.01% (w/v, coapplication)	31.5 ± 4.3
DA 0.6% (w/v, pretreatment)	31.1 ± 1.5
PEG 400 5% (v/v, coapplication)	22.1 ± 3.1*
PEG 400 50% (v/v, coapplication)	2.0 ± 0.3*
PEG + OA (pretreatment)	25.9 ± 3.3*
PEG + OA (coapplication)	6.4 ± 2.2*

Data are shown as mean ± SD ($n = 6$).
Each treatment group (DA, PEG 400, or combination of PEG 400 and OA) was statistically compared with that of the control (DPZ alone) by a one-way ANOVA followed by Tukey's post-hoc test.
*$p < 0.001$.

micelle concentration (CMC) (which is 0.1245%, w/v),[24] a pretreatment approach was employed where DA 0.6% (w/v) was applied to the donor chamber for 30 min, rinsed with fresh KBR, and the permeability of DPZ assessed. In such studies (Figs. 2c and 2d), it is clearly observed that the retarding effect of DA on DPZ disappearance and appearance was abolished.

Coapplication of DPZ with PEG 400 significantly retarded the disappearance of DPZ from the receptor chamber and also the appearance of DPZ into the receptor chamber ($p < 0.0001$) in a concentration-dependent manner from 5% to 50% (Fig. 3, Table 2). A 1.6- and 18.1-fold reduction in P_{app} was observed after the simultaneous treatment with PEG 400 at concentrations of 5% and 50%, respectively. Isothermal calorimetric studies demonstrated that an interaction appeared between DPZ and increasing PEG 400 concentrations, with 5% PEG inducing an exothermic reaction and 50% PEG 400 inducing an endothermic reaction (Fig. 4).

Given that PEG 400 appeared to retard the permeability of DPZ across the buccal mucosa, the impact of coapplying PEG 400 with OA was assessed. Coapplication of OA 1% (v/v)–5% (v/v) PEG 400 significantly reduced DPZ permeability (5.6-fold) without dramatically affecting the disappearance of DPZ from the donor chamber (Fig. 5). This effect was unlikely because of PEG 400 as PEG 400 5% alone only induced a 1.4-fold reduction in DPZ permeability. Interestingly, this retarding effect of

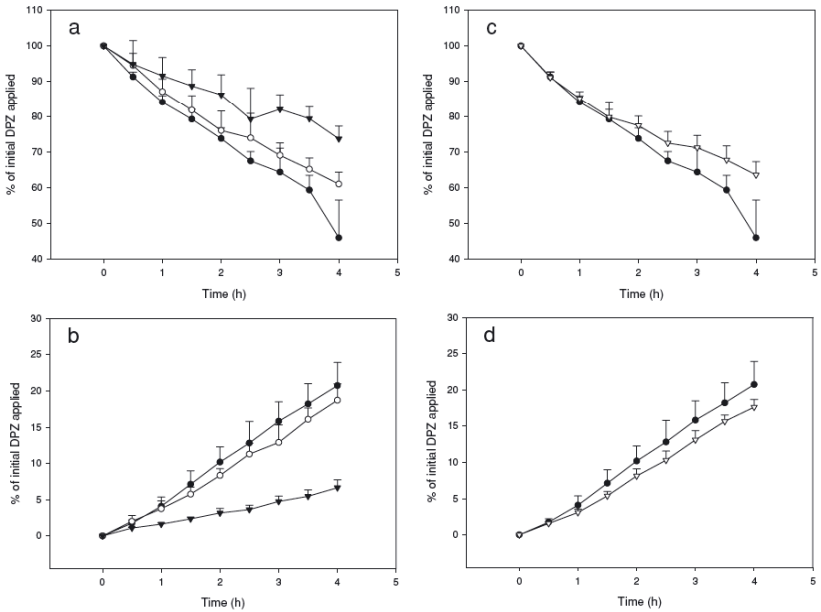

Figure 2. Percentage of initial DPZ applied disappearing from the donor chamber (a, c) and appearing in the receptor chamber (b, d) through porcine buccal epithelium after administration of DPZ (20 μg/mL) alone (●), DPZ (20 μg/mL) coapplied with DA 0.01% (w/v) (○), DPZ (20 μg/mL) coapplied with DA 0.6% (w/v) (▼), and DPZ (20 μg/mL) through tissue pretreated with DA 0.6% (w/v) for 30 min (▽). Data are presented as mean ± SD ($n = 6$).

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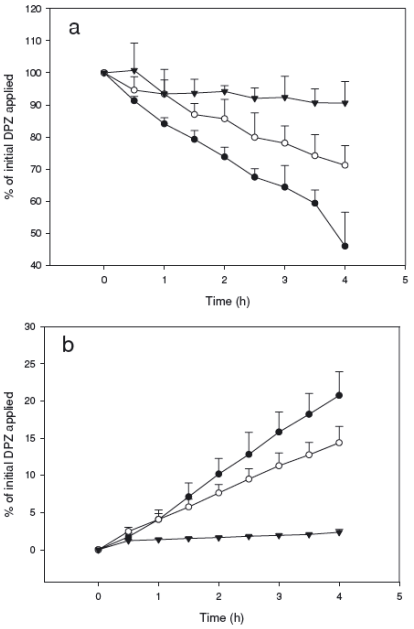

Figure 3. Percentage of initial DPZ applied disappearing from the donor chamber (a) and appearing in the receptor chamber (b) through porcine buccal epithelium after administration of DPZ alone (20 μg/mL) (●), DPZ (20 μg/mL) coapplied with PEG 400 5% (v/v) (○), and DPZ (20 μg/mL) coapplied with PEG 400 50% (v/v) (▼). Data are presented as mean ± SD ($n = 6$).

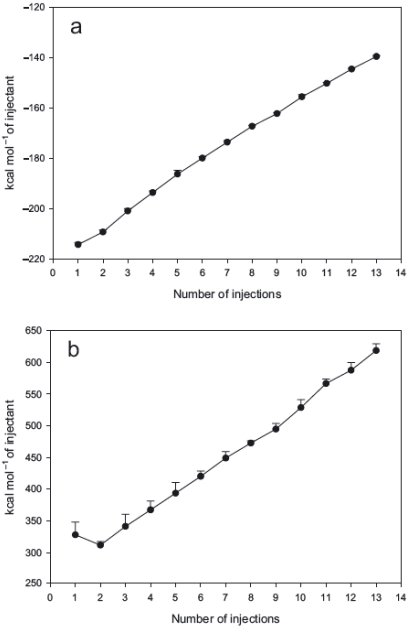

Figure 4. Isothermal titration calorimetric profiles obtained following injections of DPZ (20 μg/mL in KBR, pH 7.4) into solutions of DPZ (20 μg/mL in KBR pH 7.4) containing either (a) 5% (v/v) PEG 400 or (b) 50% (v/v) PEG 400 at 37°C. Data are presented as mean ± SEM ($n = 3$).

OA–PEG 400 combination was substantially dampened when DPZ was applied to porcine tissue that had been pretreated (and subsequently rinsed) with this combination for 30 min (Fig. 5). Light microscopic analyses of samples taken from the donor chambers indicated the presence of precipitated material when DPZ was coapplied with OA–PEG 400 (Fig. 6). Such an observation was not made when a donor chamber sample was taken from experiments where DPZ was added to the donor chambers after a 30-min pretreatment with OA–PEG 400 and a subsequent rinse (Fig. 6). As shown in Figure 6, there was notable precipitation when DPZ, PEG 400, and OA were present, and this appeared to be a result of the presence of drug and excipients, as DPZ alone or excipients alone did not exhibit such precipitation events.

DISCUSSION

Although DPZ has shown high intestinal permeability, and exhibits high oral bioavailability,[25] oral administration of DPZ is associated with peripheral adverse events[7] and large fluctuations in plasma concentration levels,[26] which may limit patient compliance. In addition to minimizing plasma concentration fluctuations associated with oral administration, alternative routes aiming to minimize dosage frequency of DPZ would be beneficial given the cognitive deficits associated with AD patients (and the possibility of missing doses). To this end, transdermal delivery of DPZ has been evaluated in *in vitro* hairless mouse skin and a permeation coefficient of 25.0 ± 5.6 × 10^{-9} cm/s was obtained.[13] Although the authors suggested that DPZ may achieve plasma levels necessary for action following this route (EC_{50} > 15.6 ng/mL), potential transdermal systems would still be limited by the barrier nature of the skin and the large surface area required for appropriate concentrations to be reached (20 cm^2). Because of these limitations, the buccal mucosal absorption of DPZ was investigated.

Although the permeability across a biological membrane is usually greater for the unionized species compared with the ionized species,[27] DPZ in its ionized form appeared to permeate the buccal mucosa very rapidly with a P_{app} value of 35.6 ± 4.9 × 10^{-6} cm/s, which is substantially higher than the P_{app} of DPZ through skin[13] and slightly higher than the P_{app} of DPZ

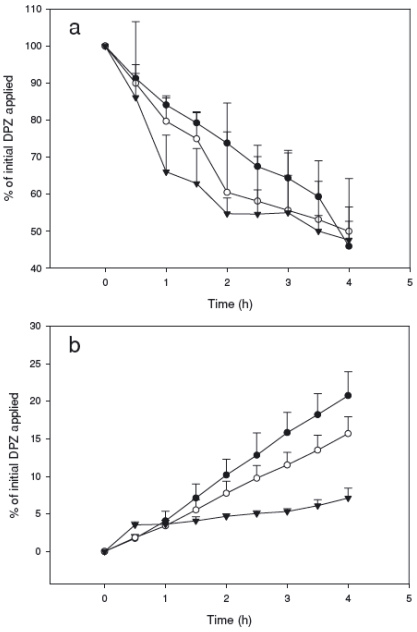

Figure 5. Percentage of initial DPZ applied disappearing from the donor chamber (a) and appearing in the receptor chamber (b) through porcine buccal epithelium after application with DPZ alone (20 μg/mL) (●), DPZ (20 μg/mL) coapplied with PEG 400 5% (v/v)/OA 1% (w/v) (▼), and DPZ (20 μg/mL) through tissue pretreated with PEG 400 5% (v/v)/OA 1% (v/v) for 30 min (○). Data are presented as mean ± SD ($n = 6$).

through intestinal cells.[23] Although the permeability of ionized species is expected to be lower than the unionized species (which we did not assess), the high permeability of DPZ across the buccal mucosa may be attributed to the likely paracellular route of transport utilized by this compound. The paracellular route of transport has been suggested to be the main route of transport for hydrophilic drugs, and given the intercellular lipids of the buccal mucosa exhibit some polarity,[28] this may assist in the absorption of the ionized DPZ species. On the basis of this high permeability (with a flux of 2.464 μg/cm^2 h), an exposed surface area of 5 cm^2, and a plasma volume of 2.58 L (~0.043 L/kg),[29] the amount of DPZ that would need to be applied to the buccal mucosa to result in therapeutic plasma concentrations of 50 ng/mL[30] is estimated to be 22 μg, which is considered clinically feasible.

Although the high buccal permeability of DPZ is sufficient to justify the development of a buccal formulation, the impact of different permeation modifiers on *in vitro* buccal permeability of DPZ was also evaluated. Given the varying effects of skin penetration enhancers on buccal permeability,[18,31] different pretreatment and cotreatment paradigms were assessed to assist in elucidating potential mechanisms contributing to the altered permeability of DPZ.

Although the skin penetration-enhancing effects of AZ have been extensively studied,[32–34] few mechanistic studies have been performed to assess the effects on AZ on the buccal mucosa. Because the barrier nature of these two tissues is quite different (intercellular spaces of the buccal epithelium contain lipids of a more polar nature and these lipids are in a less organized state in the buccal epithelium),[31] it is not surprising that AZ has been shown to either have no effect or a retarding effect on buccal mucosal delivery of drugs. For example, AZ has been shown to have no effect on caffeine[35] and risperidone[20] buccal permeability, but significantly reduces the permeability of estradiol by creating a reservoir in the buccal mucosa, resulting in a slow release of estradiol into the receptor chamber.[35] Our studies demonstrated that AZ had no effect on either the disappearance of DPZ from the donor chamber or appearance into the receptor chamber (both relative to KBR and to EtOH alone), which is surprising given the lipophilic nature of DPZ (log P value of 3.08–4.11).[27] However, given that DPZ is positively charged under physiological conditions because of its tertiary amine,[17] any reservoir effect of AZ may not assist in creating a depot of DPZ in the buccal mucosa, as it is likely that such a depot effect is more relevant for unionized lipophilic molecules (such as estradiol).

Bile salts have been shown to improve the permeability of the buccal mucosa to various compounds.[36–38] A large body of evidence suggests that intercellular lipid extraction is the main mechanism by which these agents improve buccal permeability, altering usually the paracellular or the polar route of transport, albeit at higher concentrations, cellular membrane lipids may be extracted, also modifying the transcellular transport of molecules.[31] Given bile salts can form micelles above their CMC, the impact of DA on the buccal permeability of DPZ was assessed at concentrations above and below the CMC, as it has been demonstrated that micellar solubilization can lead to reduced buccal permeability.[39] Indeed, in this study, DA 0.6% (w/v) resulted in a slower disappearance of DPZ from the donor compartment and a slower appearance of DPZ in the receptor chamber, an effect that was not observed with coapplication of DA 0.01% (w/v). This suggests that DPZ is rapidly internalized into the micelles when a concentration of DA 0.6% (w/v) was used, thus decreasing the thermodynamic activity of DPZ and subsequent permeability across the buccal mucosa. In line with this, the CMC of DA has been reported to be 3 mM (0.1245%, w/v) in saline.[24] To support the hypothesis that any permeability retarding effects of DA 0.6% were because of micellar solubilization (and not an effect that could be attributed to tissue integrity), the buccal mucosa was pretreated with DA 0.6% (w/v) for 30 min prior to the DPZ permeability study. After 30 min, the donor chamber was rinsed and DPZ was added to the donor chamber and permeability assessed. With this paradigm, permeability retarding effects of DA were abolished, likely because of the donor chamber concentration of DA being below the CMC. Although this is a proposed mechanism, such conclusions can only be made if the concentrations of DA in the donor chamber were indeed measured.

Coapplication of PEG 400 decreased the disappearance rate of DPZ from the donor chamber as well as reducing the rate of DPZ appearance in the receptor chamber. Furthermore, this effect was concentration dependent with a greater permeability

DOI 10.1002/jps.23950

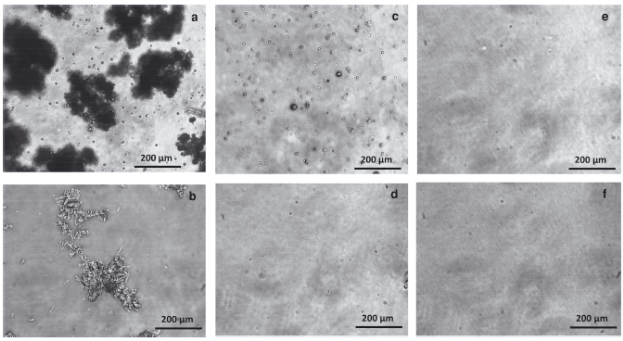

Figure 6. Optical microscope images (100× magnification) for OA–PEG 400 combination in the absence (a) and presence (b) of DPZ in KBR, OA in KBR (c), OA and DPZ in KBR (d), DPZ in KBR (e), and OA in KBR (f). Drug precipitation was only observed when DPZ was in contact with the OA–PEG 400 combination (b).

reducing effect observed with 50% (v/v) PEG 400, relative to 5% (v/v) PEG 400. The impact of PEG on buccal permeability has not been well studied, unlike its effects on intestinal[40–42] and transdermal permeability.[43,44] The effect of different concentrations of PEG 400 on the intestinal permeability of carbamazepine has been evaluated using an *in situ* intestinal perfusion technique, and intestinal permeability of carbamazepine varied inversely with the percentage of PEG 400 (0%, 10%, and 50%, v/v) applied to the perfusate solution.[45] This decreased permeability was reported to be associated with a reduction in the thermodynamic activity of carbamazepine at higher concentrations of PEG 400, and also with solvent drag considerations because of the osmolarity of the perfusing solutions. Similarly, progesterone permeability across rat jejunal segments was also significantly reduced by PEG 400 (0%, 5%, 10%, 20%, and 30%, v/v) as a suggested result of reduced thermodynamic activity of the permeant in the small intestinal lumen.[46] The effect of different concentrations of PEG 400 (10%, 20%, 30%, and 40%, v/v) on the solubility, partitioning and permeation coefficient of estradiol was also evaluated in hairless mouse skin tissues (whole and stripped skins).[43] The equilibrium solubility of estradiol at 37°C increased exponentially with increasing concentrations of PEG 400. Conversely, the partition and permeation coefficient values of estradiol were found to decrease as the PEG 400 concentration increased. The permeability coefficients of estradiol through the *stratum corneum* decreased 6-, 16-, 49-, and 98-fold at 10%, 20%, 30%, and 40% PEG 400, respectively, suggesting an effect of PEG 400 in reducing the partitioning of the drug through the skin. Given that similar conclusions for both membrane models (skin and intestinal cells) were obtained, this supports the hypothesis that an improved drug solubility and reduced thermodynamic activity of DPZ in the donor chamber could be contributing to the DPZ-retarding effects of PEG 400 in the buccal mucosa. Studies using isothermal titration calorimetry demonstrated that a direct interaction between DPZ and PEG 400 occurs in KBR, suggesting that the thermodynamic activity of DPZ was indeed influenced by the presence of PEG 400. It therefore appears that the buccal mucosa reacts similarly to PEG 400 as other biological membranes, an observation which has not been reported previously.

Similarly, there are a limited number of studies that have considered the impact of OA on the buccal permeability of compounds,[47–49] and it has been suggested that this fatty acid should be solubilized by a cosolvent so that it can effectively provide an enhancer effect.[50] As PEG 400 is able to solubilize lipophilic compounds,[40] OA and PEG 400 were blended in order to optimize the enhancing properties. The proportion of each component was defined according to studies of Lee and Kellaway,[50] where the buccal permeability of [D-Ala^2, D-Leu^5]enkephalin was enhanced 2.2- and 3.4-fold in the presence of 1% OA and 5% or 10% PEG 400, respectively. For this reason, a ratio of 1:5 was used for the combination of OA–PEG 400, respectively. Interestingly, the combination of PEG 400 and OA did not show any enhancing effect on the buccal permeability of DPZ, but rather further reduced the diffusivity of DPZ through the buccal mucosa, greater than that observed for PEG 400 alone. These findings could be related to the formation of precipitates in the donor chamber, which were first observed visually and then confirmed by microscopic analysis. Precipitation events were not observed for the DPZ or excipient alone, suggesting that the combination of PEG 400, OA, and DPZ contributes to the formation of a drug precipitate. This combination therefore likely decreases the free fraction of DPZ available for absorption after a coapplication protocol, potentially leading to the low appearance rate of DPZ in the receptor solution. In line with this, a similar reducing effect was not observed when OA–PEG 400 was applied as a pretreatment (and subsequently rinsed), where this combination is not in the donor chamber (or in very low concentrations) when DPZ was applied to the donor chamber. Given that DPZ is positively charged in physiological conditions, it may be capable of forming a complex with the carboxylic acid group of OA, which could contribute to a drug aggregation or precipitation. Ionic complexation between basic drugs such as DPZ and OA has already been reported,[51] and this interaction was consistently attributed to the presence of a tertiary amine on the drug and the carboxylic acid group of OA, supporting our hypothesis. However, this effect appears to

be more pronounced in the presence of PEG 400, likely because PEG 400 enhances the solubility of DPZ in the aqueous buffer potentially promoting this DPZ–OA interaction to occur. Given these results, the clinical utility of OA for controlling the absorption of DPZ should be avoided, as such an approach is not only pharmaceutically not desirable but is also likely to lead to extreme variability in the absorption characteristics of DPZ.

In summary, DPZ exhibited high buccal permeability and the application of various skin penetration enhancers substantially decreased the permeability of DPZ across the buccal mucosa. These studies suggest that the buccal mucosa may be exploited as an alternative route for the delivery of DPZ in AD. The slow-release properties of DPZ into the receptor chamber imparted by the investigated excipients could provide extreme benefit in this patient cohort, where the dosing interval of DPZ may be substantially prolonged, minimizing the requirement of multiple dosing. Further studies assessing the impact of these chemical penetration enhancers when they are incorporated in bioadhesive formulations should be carried out to confirm the permeability retarding effects of these excipients on DPZ buccal permeability.

CONCLUSIONS

DPZ exhibited high permeability across the buccal mucosa compared with values reported for transdermal and intestinal permeability. The buccal permeability of DPZ, however, can be significantly decreased to provide a controlled release of DPZ, through the use of various excipients, potentially of benefit in the clinical treatment of AD.

ACKNOWLEDGMENT

Thiago Caon especially acknowledges CAPES for providing PhD scholarship (BEX 12349/12-7/"Sandwich" PhD Program).

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5. DISCUSSÃO

Uma vez que o mesilato de saquinavir tem baixa solubilidade aquosa, limitada absorção oral, rápida metabolização, e efeito de efluxo intermediado principalmente pela gpP, faz-se necessária a administração de doses elevadas e frequentes deste fármaco. Além de modificações farmacotécnicas, como exposto no capítulo anterior, a investigação de rotas alternativas para administração de inibidores da protease do HIV, tal como o saquinavir, também pode ser interessante. A aplicação vaginal do MS tem já foi proposta, tendo em vista sua ação virucida, a fim de evitar a infecção por HIV, durante ou imediatamente após relações sexuais. No entanto, a absorção bucal ou transdérmica deste fármaco ainda não foi explorada.

A mucosa bucal é mais promissora para administração de grandes moléculas tais como peptídeos, comparativamente à pele e, tendo em vista a alta massa molecular do MS, investigou-se o potencial desta via para administração do mesmo. Adicionalmente, o fluxo sanguíneo a partir do epitélio bucal é capaz de acessar diretamente a veia jugular interna, o que facilita a absorção bucal. Outra vantagem é que os metabolismos hepático e intestinal de primeira passagem poderiam ser evitados. Isto é particularmente interessante para fármacos com natureza peptídica, dado que a mucosa intestinal apresenta maior atividade enzimática que a bucal.

Neste sentido, o potencial da mucosa bucal para a administração do MS foi investigado e o cenário parecia favorável, tendo em vista que essa mucosa não apresenta sistemas de efluxo intermediados por Pgp. Para tal, utilizou-se o modelo de câmaras de difusão de Ussing, tendo em vista suas vantagens no controle das trocas gasosas teciduais (níveis de CO_2 e O_2), o que contribuiria para maior viabilidade tecidual durante o período do experimento. No nosso conhecimento, e até o momento, não há relatos da administração bucal de inibidores da protease do HIV pela via bucal. Esta etapa foi realizada na *Monash University*, sob a supervisão do Professor Dr. Joseph Nicolazzo.

Após revalidação analítica de um método de quantificação do MS por CLAE, iniciaram-se os experimentos de permeabilidade bucal. O compartimento doador foi preenchido com solução aquosa do fármaco (33,33 µg/mL), diluída em tampão Krebs, e o experimento teve uma duração total de 4 h, com amostragens em intervalos fixos de 30 min. A quantidade do MS absorvida foi inferior ao limite de quantificação (0,01 µg/mL) e, desta forma, novos ensaios incluindo reforçadores químicos de permeação foram realizados. Inicialmente, efetuou-se um pré-tratamento com 1% (m/m) de dodecil sulfato de sódio (SDS) durante 2 h e, então, foi feito o tratamento com a solução de MS (33,33 µg/mL) na solução doadora durante 4 h. A quantidade do MS absorvida também foi

inferior ao limite de quantificação. Uma nova tentativa foi realizada considerando-se um pré-tratamento com 1% (m/m) de taurodeoxicolato de sódio ou 50% (m/m) de Azone^{TM}, durante este mesmo intervalo, mas o MS também não permeou a mucosa em níveis detectáveis.

A co-administração de 0,5% (m/m) de ácido deoxicólico ou da combinação de 1% (m/m) de ácido oleico e 5% (m/m) de polietilenoglicol também não foram efetivos no aumento da absorção bucal do MS. Nestes últimos experimentos, aumentou-se a concentração do MS para 200 µg/mL, de modo a verificar se incapacidade de observar algum efeito poderia estar relacionada com a baixa concentração do MS utilizada no compartimento doador. Pelo menos três experimentos com cada um dos reforçadores foram realizados e, muitos destes, já tinham sido previamente testados para peptídeos ou macromoléculas, porém, não foram efetivos para o MS. Acredita-se que não apenas o alto peso molecular do MS (766,95 g/mol) como também o uso de estratégias de reforço individuais (apenas um reforçador químico de penetração) possam explicar o fracasso desta tentativa, com uma permeabilidade bucal inferior ao limite de quantificação do método de CLAE.

Considerando os resultados negativos desta etapa experimental e a existência de poucos trabalhos revisão, na literatura, sobre absorção bucal de macromoléculas, optou-se pela redação de um artigo de revisão, onde foram apresentadas as estratégias clássicas e as mais atuais para o reforço da absorção bucal destas moléculas. Além disto, na última década, muitos produtos de base biotecnológica (geralmente grandes moléculas, tais como proteínas recombinantes, peptídeos e anticorpos monoclonais) foram investigados e lançados no mercado, porém, grande parte dos mesmos é administrada por via intravenosa, causando desconforto e dificuldade de adesão ao tratamento por parte dos pacientes. Neste sentido, vias alternativas de administração, como a bucal vêm sendo extensivamente investigadas e, dada a complexidade estrutural destas moléculas, grandes esforços são necessários para o aumento da sua absorção. Embora estratégias individuais tais como o uso de reforçadores químicos ou de métodos físicos (ultrassom, iontoforese, etc.) tenham sido consideradas, a literatura mostra que, ao longo do tempo, foi se estabelecendo a tendência de usar a combinação de diferentes técnicas, tendo em vista a complexidade destas moléculas, particularmente peptídeos. Neste último caso, não é necessário apenas aumentar a absorção bucal, como também fornecer maior estabilidade físico-química, uma vez que peptídeos são altamente suscetíveis à degradação enzimática. Para exemplificar, é possível utilizar inibidores enzimáticos e reforçadores químicos de permeação em uma mesma formulação. A abordagem de combinar reforçadores químicos e tratamento físico, como a iontoforese, já foi testada, com resultados de reforço da absorção bem superiores aos testes individuais (somente iontoforese ou uso isolado de

determinado reforçador químico) (OH et al., 2011)[12]. O uso combinado de diferentes reforçadores químicos de penetração (ex. ácido oleico e polietilenoglicol) também pode contribuir para um aumento da absorção, que pode estar associado a diferentes mecanismos (efeito sinérgico, interação diferenciada entre os reforçadores, interação diferenciada dos reforçadores com proteínas/lipídeos da mucosa bucal, etc). Ao mesmo tempo em que é possível aumentar a absorção bucal de macromoléculas, o comprometimento da viabilidade tecidual também pode ser observada, o que exige monitoramento contínuo, incluindo os marcadores químicos (inclusão de compostos químicos de alto peso molecular que não apresentam absorção bucal) ou físicos (medidas de resistência elétrica transepitelial) de controle de integridade, bem como o uso de métodos diretos que avaliam funções celulares específicas (ex. ensaio colorimétrico do sal de tetrazólio, que avalia a função mitocondrial). Outro aspecto que deve ser observado é a capacidade de recuperação da mucosa bucal após a indução de dano, por determinado agente químico, o que reestabelece a homeostase. Caso tenha ocorrido rápida recuperação, é possível incluir determinado reforçador químico de absorção na formulação, mesmo que danos reversíveis possam ser observados com sua utilização.

Com o conhecimento dos principais fatores que interferem na permeabilidade bucal de macromoléculas, decidiu-se selecionar um novo fármaco para continuar os ensaios de permeabilidade bucal. Nesta decisão, foi considerada a relevância clínica do mesmo, priorizando-se seu uso crônico, suas características físico-químicas (coeficiente de partição octanol/água bem como massa molecular) e ausência de ensaios de permeabilidade bucal com esta molécula.

A partir deste raciocínio, o cloridrato de donepezila mostrou ser uma opção interessante tendo em vista suas limitações farmacocinéticas quando administrado por via oral (ele atinge concentrações plasmáticas máximas muito rapidamente, observando-se flutuações plasmáticas), sua relevância clínica na Doença de Alzheimer, um problema de saúde pública bem conhecido e que tem crescido exponencialmente nos últimos anos, sua reduzida massa molecular e seu coeficiente de partição intermediário (se o fármaco é muito lipofílico, observa-se alta retenção na mucosa bucal e lenta taxa de transferência sistêmica, pois demonstra maior afinidade por lipídeos da membrana; se o fármaco é muito hidrofílico, a penetração na mucosa é baixa).

Os testes iniciais, realizados apenas com o fármaco, em várias concentrações (10, 20 e 50 µg/mL), demonstraram um efeito

[12]OH, D.H.; CHUN, K.H.; JEON, S.O.; KANG, J.W.; LEE, S. Enhanced transbuccal salmon calcitonin (sCT) delivery: effect of chemical enhancers and electrical assistance on in vitro sCT buccal permeation. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 79, p. 357-363. 2011.

concentração-dependente e sugeriram que a mucosa bucal é uma rota promissora para a administração do cloridrato de donepezila, uma vez que o coeficiente de permeabilidade bucal foi aproximadamente 2X maior que o coeficiente de permeabilidade intestinal, e em torno de 1000X maior que o coeficiente de permeação transdérmica. Estes achados, por si só, seriam suficientes para justificar o preparo de formulações bucais mucoadesivas com este fármaco para fins comerciais.

Visando aumentar ainda mais a absorção bucal do fármaco, o impacto de diferentes reforçadores químicos de absorção foi investigado e diferentes protocolos de tratamento foram considerados (pré- e tratamento simultâneo). Com isto, foi possível hipotetizar se estes reforçadores químicos teriam ação direta sobre o composto/fármaco (ex. formação de par iônico) ou, então, se teriam algum efeito sobre os componentes da mucosa bucal. Tendo como base outros experimentos já relatados na literatura, em alguns casos, é possível também definir se há certa seletividade de ação (por exemplo, interferência na via paracelular ou transcelular).

Reforçadores químicos de penetração com natureza físico-química diferenciada foram selecionados, sendo que o ácido oleico e o Azone^{TM} são lipofílicos, o polietilenoglicol é hidrofílico (com pequena porção hidrofóbica) e os sais biliares tal como o ácido deoxicólico tem natureza anfifílica. Surpreendentemente, estes reforçadores não aumentaram o coeficiente de permeabilidade bucal do DPZ e, em alguns casos, houve até redução significativa (adição de polietilenoglicol, por exemplo). Em algumas situações, estes achados podem ser explicados em função do fármaco apresentar uma via de absorção que não é compatível com aquela da ação do reforçador de permeação. Em outros, há um aumento da solubilização do fármaco no compartimento doador, reduzindo sua atividade termodinâmica e, consequentemente, sua absorção bucal. O polietilenoglicol é bem descrito como co-solvente de fármacos com solubilidade limitada e alguns estudos tem demonstrado a possibilidade dele reduzir a absorção oral de alguns fármacos (RIAD & SAWCHUK, 1991[13]; MILLER et al., 2012[14]), porém, este efeito ainda não tinha sido descrito para a absorção bucal. Ensaios com tensoativos, tal como o ácido deoxicólico, também sugeriram que pode ter ocorrido solubilização micelar do fármaco acima da concentração micelar crítica, porém, uma redução da permeabilidade também deveria ter sido observada. Nesta situação, sugere-se que as micelas seriam capazes de “capturar” moléculas de

[13]RIAD, L.; SAWCHUK, R. Effect of polyethylene glycol 400 on the intestinal permeability of carbamazepine in the rabbit. **Pharmaceutical Research**, v.8, p.491-497, 1991.

[14]MILLER, J.M.; BEIG, A.; CARR, R.A.; WEBSTER, G.K.; DAHAN, A. The solubilitypermeability interplay when using cosolvents for solubilization: revising the way we use solubility-enabling formulations. **Molecular Pharmaceutics**, v. 9, p. 581-590, 2012.

fármaco livre, reduzindo a quantidade disponível para a absorção. Desta forma, diferentes concentrações do tensoativo devem ser consideradas, quando da realização deste tipo de experimento, bem como o efeito na formulação mucoadesiva final. Eventos de precipitação/agregação com a utilização da combinação de polietilenoglicol e ácido oleico também promoveram redução ainda maior da permeabilidade bucal do fármaco, e isto poderia estar relacionado com a provável complexação iônica dos grupamentos carboxílicos do ácido oleico com a amina terciária do fármaco.

Ainda que estes resultados tenham sido diferentes daqueles esperados, esta redução da absorção bucal do cloridrato de donepezila, na presença destes reforçadores químicos de absorção, poderia levar a certo controle da liberação do fármaco, reduzindo os intervalos entre as tomadas do medicamento, o que seria valorável tendo em vista que os pacientes com Doença de Alzheimer se esquecem da medicação frequentemente. Com isto, sistemas mucoadesivos de liberação prolongada, incluindo alguns destes agentes reforçadores, poderiam ser desenvolvidos, contribuindo para uma maior adesão ao tratamento em função da redução da frequência de administração do medicamento.

6. CONSIDERAÇÕES FINAIS

- A mucosa bucal não mostrou ser uma rota promissora para a administração do MS, uma vez que não foi detectada a presença deste fármaco no compartimento receptor após 4 h de ensaio, o que pode estar relacionado com seu alto peso molecular.

- O pré-tratamento com 50% de Azone^{TM} ou com tensoativos, tais como dodecil sulfato de sódio ou taurodeoxicolato de sódio (ambos a 1%), não promoveram qualquer aumento da absorção bucal do MS.

- A co-administração do MS com 0,5% do ácido deoxicólico ou com a combinação de 1% de ácido oleico e 5% de polietilenoglicol também não reforçaram a permeabilidade bucal do fármaco.

- A permeabilidade bucal do DPZ foi superior à permeabilidade intestinal e transdérmica do mesmo, a qual não foi afetada pelo pré-tratamento com 50% de Azone^{TM}.

- A co-administração do DPZ com 0,6% do ácido deoxicólico reduziu significativamente a absorção bucal do DPZ, o que não ocorreu com a concentração abaixo da concentrçaão micelar crítica (0,01%), a qual não afetou a permeabilidade bucal deste fármaco.

- A absorção bucal do DPZ foi reduzida de modo concentração-dependente após o co-tratamento com 5% e 50% de PEG, reduzindo-a em 1,6 e 1,8 vezes, respectivamente.

- O co-tratamento com a combinação de 1% de ácido oleico e 5% de PEG 400 reduziu a permeabilidade bucal do DPZ em 5,5 vezes, o que poderia estar associado com uma precipitação do fármaco (observada por mciroscopia), induzida pelo excipiente.