THUANE CASTRO FRABEL DO NASCIMENTO

DESENVOLVIMENTO E AVALIAÇÃO FARMACOCINÉTICA DE NANOPARTÍCULAS DE PLGA E PLGA-PEG CONTENDO CURCUMINA

GUARAPUAVA

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Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Ciências Farmacêuticas, ao Curso de Pós-Graduação em Ciências Farmacêuticas, área de Concentração Fármacos, Medicamentos e Biociências Aplicadas à Farmácia, da UNICENTRO- PR.

Orientador: Profa. Dra. Rubiana Mara Mainardes

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TERMO DE APROVAÇÃO

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Guarapuava, 14 de dezembro de 2011

Não conheço ninguém que conseguiu realizar seu sonho,

sem sacrificar feriados e domingos pelo menos uma centena de vezes.

Da mesma forma, se você quiser construir uma relação amiga com seus filhos,

terá que se dedicar a isso, superar o cansaço, arrumar tempo para ficar com eles,

deixar de lado o orgulho e o comodismo. Se quiser um casamento gratificante, terá que

investir tempo, energia e sentimentos nesse objetivo.

O sucesso é construído à noite!

Durante o dia você faz o que todos fazem.

Mas, para obter um resultado diferente da maioria, você tem que ser especial.

Se fizer igual a todo mundo, obterá os mesmos resultados.

Não se compare à maioria, pois, infelizmente ela não é modelo de sucesso.

Se você quiser atingir uma meta especial,

terá que estudar no horário em que os outros estão tomando chopp com batatas fritas.

Terá de planejar, enquanto os outros permanecem à frente da televisão.

Terá de trabalhar enquanto os outros tomam sol à beira da piscina.

A realização de um sonho depende de dedicação.

Há muita gente que espera que o sonho se realize por mágica,

mas toda mágica é ilusão, e a ilusão não tira ninguém de onde está.

Em verdade, a ilusão é combustível dos perdedores.

Quem quer fazer alguma coisa, encontra um meio.

Quem não quer fazer nada, encontra uma desculpa.

Roberto Shinyashiki

DEDICATÓRIA

Aos meus pais, Aurora e Valdeci,

que primeiro me deram a vida,

me ensinaram os primeiros passos,

me ensinaram as primeiras palavras,

exemplos de caráter, honestidade e união.

Com eles pude comemorar as vitórias

e tive ombro nos momentos de desespero;

Obrigada por iluminarem meu caminho,

dando condições para que eu continuasse

nessa longa caminhada profissional;

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E ao meu filho Pedro Henrique,

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RESUMO

As nanopartículas poliméricas apresentam grande importância na área farmacêutica em virtude de serem sistemas coloidais que possuem interessantes propriedades físico-químicas, tais como o tamanho reduzido, a ampla área superficial, carga superficial, que as tornam eficientes sistemas para aplicação na liberação controlada de fármacos. A curcumina é um pigmento amarelo presente na *Curcuma longa* que possui baixa toxicidade e uma ampla faixa de atividades farmacológicas, estando entre os mais promissores e eficazes agentes quimiopreventivos e/ou antitumorais. Porém o seu uso terapêutico tem sido limitado devido a sua baixa solubilidade aquosa, sua alta velocidade de decomposição em pH neutro ou básico, além do rápido metabolismo e eliminação sistêmica, resultando em baixa biodisponibilidade. Neste trabalho obteve-se nanopartículas de ácido poli-(láctico-*co*-glicólico) (PLGA) e de blendas de PLGA com polietilenoglicol (PEG) contendo curcumina através da técnica de emulsificação-evaporação do solvente, com o objetivo de melhorar suas propriedades farmacocinéticas. Após a validação de um método por cromatografia líquida de alta eficiência (CLAE) para a quantificação da curcumina, as nanopartículas foram avaliadas quanto ao diâmetro médio e eficiência de encapsulação. Ambas as formulações obtiveram eficiência de encapsulação superior a 75% e o diâmetro médio não foi superior a 200 nm. O estudo de liberação *in vitro* mostrou que as nanopartículas sustentam a liberação da curcumina, e que a presença do PEG na formulação contribui para o aumento na velocidade de liberação da curcumina. Um método por cromatografia líquida acoplada a espectrometria de massas foi desenvolvido e validado e se mostrou altamente sensível, reprodutível e específico para análise de curcumina em plasma de rato. Após administração oral em ratos, as formulações de nanopartículas de PLGA e

blendas de PLGA-PEG foram capazes de manter uma liberação sustentada da curcumina, com resultados significativamente diferentes entre as formulações. As nanopartículas de PLGA e PLGA-PEG aumentaram o tempo de meia vida da curcumina em aproximadamente 4 e 6 h, respectivamente. Comparando-se com a suspensão aquosa de curcumina, o pico máximo de concentração plasmática da curcumina a partir das nanopartículas de PLGA e PLGA-PEG foi 2,9 e 7,4 vezes superior, respectivamente. A distribuição e o metabolismo da curcumina foi reduzido quando carreada pelas nanopartículas, principalmente pelas nanopartículas de PLGA-PEG. A biodisponibilidade da curcumina encapsulada em nanopartículas de PLGA-PEG foi 3,5 vezes superior em relação a encapsulada em nanopartículas de PLGA. Comparado com a suspensão aquosa de curcumina, as nanopartículas de PLGA e PLGA-PEG aumentaram a biodisponibilidade em 15,6 e 55,4 vezes, respectivamente. Estes resultados sugerem que nanopartículas de PLGA e principalmente de PLGA-PEG são promissores carreadores de curcumina para administração oral.

Palavras-chaves: Curcumina, Biodisponibilidade, LC-MS/MS, Nanopartículas, PLGA, PLGA-PEG.

ABSTRACT

The polymeric nanoparticles present great importance in the pharmaceutical field due to be colloidal systems, which have interesting physicochemical properties, such as the reduced size, the large superficial area and the superficial charge making them efficient systems for applying in the controlled releasing of drugs. The curcumin is a yellow pigment, which is present in the *Curcuma longa* having low toxicity and a large range of pharmacological activities. It is among the most promising effective chemopreventive agents and/or antitumorals. However, its therapeutical use has been limited owing to its low aqueous solubility, its high decomposition rate in low or neutral pH, besides the fast metabolism and systemic elimination resulting in low bioavailability. In this study poly(lactic-*co*-glycolic acid) (PLGA) and blends of PLGA with polyethylene glycol (PEG) nanoparticles containing curcumin were obtained through the solvent emulsification-evaporation technique aiming to improve its pharmacokinetic properties. After the method validation by high performance liquid chromatography (HPLC) for the quantitation of curcumin, the nanoparticles were assessed regarding the average diameter and the encapsulation efficiency. Both formulations obtained the encapsulation efficiency higher than 75% and the average diameter was not higher than 200 nm. The in vitro releasing study showed that the nanoparticles sustained the curcumin release and the PEG presence in the formulation contributes to the increased rate of curcumin release. A liquid chromatography mass spectrometry method was developed and validated and showed to be very sensitive, reproductive and specific for curcurmin in rat plasma. After oral administration in rats, the PLGA and the PLGA-PEG blends nanoparticles were able to keep a sustained release of curcumin, with significantly different results between formulations. The

PLGA and PLGA-PEG nanoparticles increased the half-life time of curcumin in approximately 4 and 6 h, respectively. Comparing the aqueous suspension of curcumin, the mean plasma concentration of curcumin from the PLGA and PLGA-PEG nanoparticles were 2.9 and 7.4 -fold higher, respectively. The distribution and the metabolism of curcumin were reduced when carried by the nanoparticles, mainly by the PLGA-PEG nanoparticles. The bioavailability of curcumin from the PLGA-PEG nanoparticles was 3.5 -fold greater than that of curcumin from PLGA nanoparticles. Compared to the curcumin aqueous suspension, the PLGA and PLGA-PEG nanoparticles increased the curcumin bioavailability in 15.6 and 55.4-fold, respectively. These results suggest that PLGA and mainly PLGA-PEG nanoparticles are promising carriers of curcumin for oral administration.

Keywords: Curcumin, Bioavailability, LC-MS/MS, Nanoparticles, PLGA, PLGA-PEG

SUMÁRIO

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

CLAE	Cromatografia Líquida de Alta Eficiência
LC/MS/MS	Cromatografia Líquida Acoplada a Espectrômetro de Massas
O/A	Óleo em água
PLA	Ácido poli-lático
PEG	Polietilenoglicol
PGA	Ácido poli-glicólico
A/O/A	Água em óleo em água
PLGA	Ácido poli-(láctico-*co*-glicólico)

INTRODUÇÃO

Nanotecnologia Farmacêutica

A nanotecnologia é uma área que se caracteriza pela ampla multidisciplinaridade e envolve a utilização de materiais das mais diversas origens, para o desenvolvimento de dispositivos e sistemas em escala nanométrica. Essa tecnologia tem demonstrado crescimento e inovação em diversas áreas, tais como na física, química, informática, eletrônica e apresenta relevante importância na área farmacêutica.

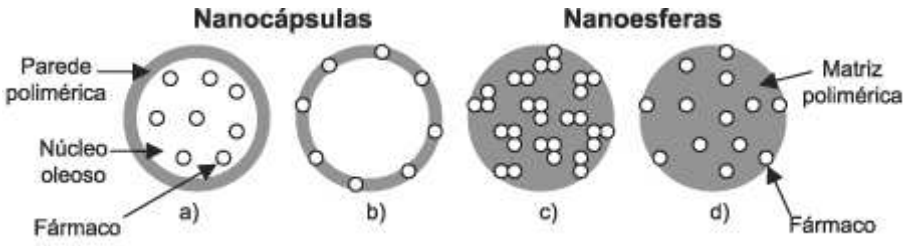
A nanotecnologia farmacêutica compreende a pesquisa e desenvolvimento de sistemas nanoestruturados, tais como, nanopartículas poliméricas, nanopartículas lipídicas sólidas, lipossomas e nanoemulsões, e a consequente aplicação desses sistemas como dispositivos carreadores de fármacos, proteínas, genes e vacinas. A versatilidade, flexibilidade e adaptabilidade dos sistemas de liberação nanoestruturados têm provado seus benefícios na área médica/farmacêutica e no aumento da adesão à terapêutica pelos pacientes. (LU *et al.*, 2004).

As nanopartículas poliméricas apresentam grande importancia na área farmacêutica em virtude de serem sistemas coloidais que possuem interessantes propriedades físico-químicas, tais como tamanho reduzido, ampla área superficial, diferentes características de carga superficial, que as tornam eficientes sistemas para aplicação na liberação controlada e/ou prolongada de fármacos. Modulando características como sua composição polimérica, tamanho e carga/composição superficial, pode-se conseguir diferentes perfis de liberação do fármaco por elas veiculado (AVGOUSTAKIS *et al*., 2003; SCHAFFAZICK *et al*., 2003).

O termo nanopartículas aplicado à liberação controlada de fármacos é amplo e

diz respeito a dois diferentes tipos de estruturas, as nanoesferas e as nanocápsulas (Fig. 1). O fármaco pode ser dissolvido, adsorvido, ligado ou encapsulado nas nanopartículas, dependendo do método de preparo e da estrutura da nanopartícula. No que diz respeito às nanocápsulas, estas se constituem de sistemas reservatórios, onde é possível identificar um núcleo diferenciado, que pode ser sólido ou líquido, envolto por uma membrana polimérica, isolando o núcleo do meio externo. Já as nanoesferas são sistemas em que o fármaco encontra-se homogeneamente disperso ou solubilizado na matriz polimérica, compondo um sistema monolítico, não sendo possível identificar-se um núcleo diferenciado (CHANDY & SHARMA, 1993; AVGOUSTAKIS *et al*., 2002).

Figura 1 – Representação esquemática de nanocápsulas e nanoesferas, a) fármaco dissolvido no núcleo oleoso da nanopartícula; b) fármaco adsorvido na parede polimérica da nanocápsula; c) fármaco aderido na matriz polimérica da nanoesfera; d) fármaco adsorvido ou disperso na matriz polimérica.



FONTE: SCHAFFAZICK *et al.*, 2003.

As nanopartículas são capazes de proteger o fármaco da degradação (estabilidade física durante o armazenamento e nos fluídos biológicos), bem como propiciam interessantes propriedades *in vivo*, como aumento da sua absorção, maior capacidade de ultrapassar barreiras biológicas, distribuição diferenciada, podendo ser

direcionadas para células e tecidos específicos (macrófagos, células tumorais, cérebro, entre outros), pois sua superfície e carga superficial podem ser modificadas pela inserção de determinados ligantes, como por exemplo, anticorpos, surfactantes, polímeros, entre outros e assim prolongar a liberação do fármaco (habilidade de liberação sustentada por um período de dias a semanas), consequentemente, aumentando o tempo de meia-vida no plasma do fármaco veiculado (OPPENHEIM, 1981; ALLÉMAN *et al*., 1993). Os parâmetros farmacocinéticos são alterados com as nanopartículas e a composição de sua superfície desempenha um papel importante na biodisponibilidade do fármaco (UBRICH *et al*., 2005; HOFFART *et al*., 2006). A resposta biológica de fármacos veiculados através de nanopartículas também pode ser ampliada, uma vez que as propriedades farmacocinéticas são melhoradas, possibilitando assim, administrações por diferentes vias (KREUTER, 1991; MAINARDES *et al*., 2005; MAINARDES *et al*., 2006a; MAINARDES *et al*., 2006b; MAINARDES *et al*., 2009; MAINARDES *et al*., 2010;). Com relação à administração oral de nanopartículas, as pesquisas têm sido direcionadas especialmente a: i) diminuição dos efeitos colaterais de certos fármacos, destacando-se os antiinflamatórios não-esteroides (diclofenaco, indometacina), os quais causam frequentemente irritação a mucosa gastrintestinal; ii) proteção de fármacos degradáveis no trato gastrintestinal, como peptídeos, proteínas e/ou hormônios, aumentando a biodisponibilidade dos mesmos e, iii) aumento da solubilidade de fármacos insolúveis ou pouco solúveis aumentando a biodisponibilidade dos mesmos (SCHAFFAZICK *et al*., 2003).

As propriedades das nanopartículas dependem do seu tamanho e de características da sua superfície, como potencial zeta, hidrofobicidade, presença de ligantes e morfologia. A escolha do polímero tem grande importância no desenvolvimento das nanopartículas e prediz sua biodistribuição (REIS *et al.*, 2006;

CHENG *et al.*, 2007).

A matriz polimérica das nanopartículas deve reunir uma série de requisitos como: biocompatibilidade, biodegradabilidade e resistência mecânica (MU & FENG, 2003). Vários tipos de polímeros biodegradáveis são utilizados na produção de nanopartículas, como: ácido poli-láctico – PLA (CAUCHETIER *et* al., 2003); polialquilcianoacrilatos; poli(ε-caprolactona) – PCL; ácido poli-(láctico-*co*-glicólico) – PLGA (MU & FENG, 2003).

Uma variedade de biopolímeros também tem sido estudada para utilização em sistemas de liberação de fármacos, tais como albumina bovina, albumina humana, gelatina e colágeno (MU & FENG, 2003), porém os polímeros sintéticos possuem uma pureza elevada e melhor reprodutibilidade do que os polímeros naturais (MORA-HUERTAS *et al.*, 2010; RAO & GECKELER, 2011).

Os polímeros e copolímeros do ácido lático são os de maior interesse para aplicação como carreadores de fármacos devido à sua completa biodegradabilidade gerando metabólitos não tóxicos e bem tolerados pelos tecidos (GUTERRES *et al.*, 1995).

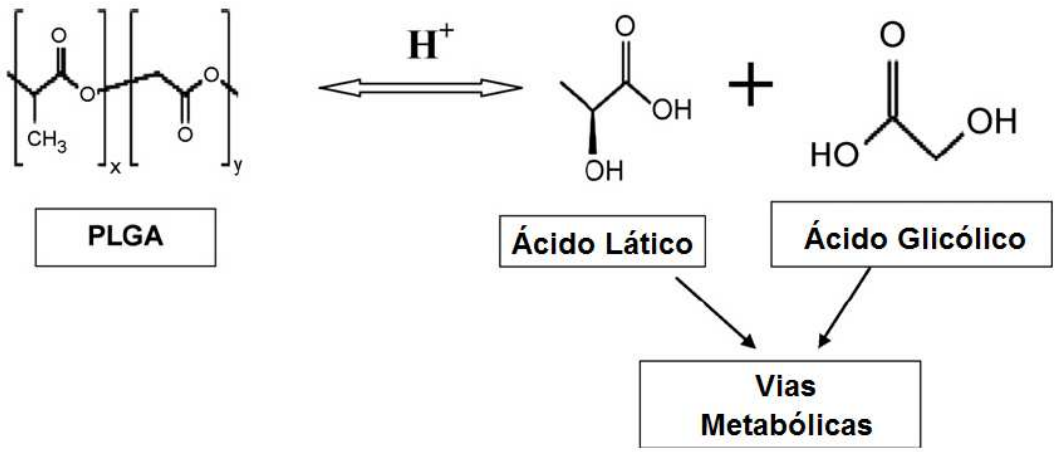
Polímeros de PLGA têm sido amplamente utilizados na indústria biomédica devido sua biocompatibilidade e biodegradação. O PLGA é aprovado pelo *Food and Drug Administration* (FDA) e tem sido utilizado para o uso humano como material reabsorvível para sutura. Nanopartículas de PLGA tem emergido como carreadores de fármacos devido sua relativa facilidade de encapsular fármacos hidrofóbicos (MANCHANDA *et al.*, 2010; SWARNAKAR, *et al.*, 2011).

O PLGA é um copolímero formado por dois monômeros, o ácido láctico e o ácido glicólico, em diferentes proporções. Os dímeros cíclicos destes ácidos são ligados randomicamente por meio de ligações do tipo éster, resultando em um poliéster de

cadeia alifática (PINTO *et al.*, 2006; BRANNON-PEPPAS, 1997). Seu interesse no desenvolvimento de nanosistemas é devido sua hidrólise no organismo produzindo metabólitos biodegradáveis, ou seja, monômeros de ácido lático e ácido glicólico (fig. 2). Terminada a hidrólise do material a degradação segue o processo de oxidação à ácido láctico (para o PLA) e conversão das unidades de PGA em glicina, que por sua vez são convertidos em ácido pirúvico. Na presença da acetil coenzima A, ocorre a liberação de CO_2 e, consequentemente, a decomposição em citrato. O citrato será então incorporado no Ciclo de Krebs, resultando em CO_2 e H_2O, podendo sua eliminação ser feita através da urina e da respiração (fig. 3) (BARBANTI, *et al*, 2005).

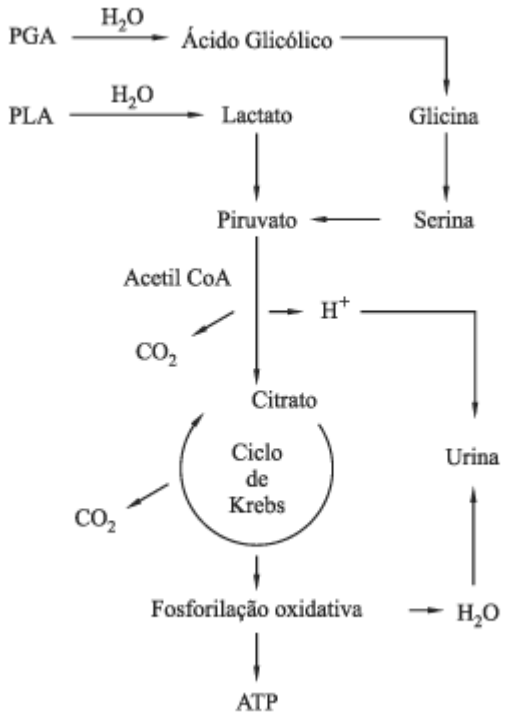
Uma vez que o organismo metaboliza efetivamente esses dois monômeros, há mínima toxicidade sistêmica associada à utilização do PLGA como matriz polimérica para liberação de fármacos ou na sua aplicação como biomateriais (KUMARI *et al.*, 2010).

Figura 2 – Hidrólise do PLGA: nanopartículas de PLGA são biologicamente hidrolisadas em meio ácido em ácido lático e glicólico.



FONTE: KUMARI *et al.*, 2010,modificado.

Figura 3 – Rota metabólica dos monômeros de ácido lático e glicólico.



FONTE: BARBANTI *et al.,* 2005, modificado.

A resistência mecânica, o comportamento de intumescimento, a capacidade de sofrer hidrólise e, posteriormente a taxa de biodegradação são diretamente influenciados pela cristalinidade do PLGA. A cristalinidade resultante do PLGA é dependente do tipo e da razão molar dos componentes individuais dos monômeros (ácido lático e glicólico) na cadeia do copolímero. Polímeros de PLGA contendo uma proporção de 50:50 de ácidos lático e glicólico são hidrolisados muito mais rápido do que aqueles que contêm uma proporção maior de qualquer um dos dois monômeros. O PGA é altamente cristalino porque lhe falta grupos metil. O ácido lático é mais hidrofóbico que o acido glicólico e, portanto, copolímeros de PLGA ricos em ácido lático são menos hidrofílicos, absorvem menos água e, posteriormente, degradam mais lentamente (MUTHU, 2009)

Para um sistema de liberação de fármacos ser considerado eficiente, ele deve estar presente na corrente sanguínea por um tempo suficiente para alcançar e reconhecer os sítios de ação terapêutica. No entanto, a opsonização e remoção das nanopartículas carreadoras do corpo pelo sistema fagocitário mononuclear (SFM) é a maior barreira para a realização desses objetivos (GREF, *et al.*, 1994; CHENG, *et al.*, 2007; KUMAR *et al.*, 2001).

Nanopartículas poliméricas com superfície não modificada são rapidamente removidas da circulação sanguínea após administração intravenosa, pelas células do SFM, principalmente pelas células do fígado e macrófagos do baço (GREF, *et al.*, 1994; AVGOUSTAKIS *et al.*, 2003; OWENS & PEPPAS, 2006). Estas células não tem a habilidade de identificar diretamente as partículas, mas, reconhecem proteínas específicas, as opsoninas, ligadas a superfície das partículas. A opsonização dessas partículas ocorre principalmente devido às suas características de superfície, considerando a polaridade, carga e presença de longas cadeias. Neste aspecto, partículas feitas com polímeros hidrofóbicos e com superfície apresentando alto valor de potencial zeta, são muito susceptíveis a opsonização pelas proteínas plasmáticas e depois sofrem a fagocitose. A opsonização ocorre devido a presença de interação eletrostática e hidrofóbica entre as opsoninas e a superfície das partículas. Estas partículas são chamadas nanopartículas convencionais, apresentam curto tempo de meia-vida na circulação sanguínea e tem menor possibilidade de chegar a outros órgãos. Devido a este fato, tem-se aumentado o interesse em desenvolver carreadores que apresentem tempo de circulação prolongado. Entre as estratégias com esse propósito, nanopartículas que apresentam polímeros hidrofílicos com longas e flexíveis cadeias em sua superfície tem mostrado resultados interessantes (GREF *et al.*, 1994, VERRECHIA *et al.*, 1995; BAZILE *et al.*, 1995).

A presença de uma cobertura feita com cadeias longas de polímeros hidrofílicos promove uma estabilização estérica das nanopartículas evitando a opsonização e a fagocitose.

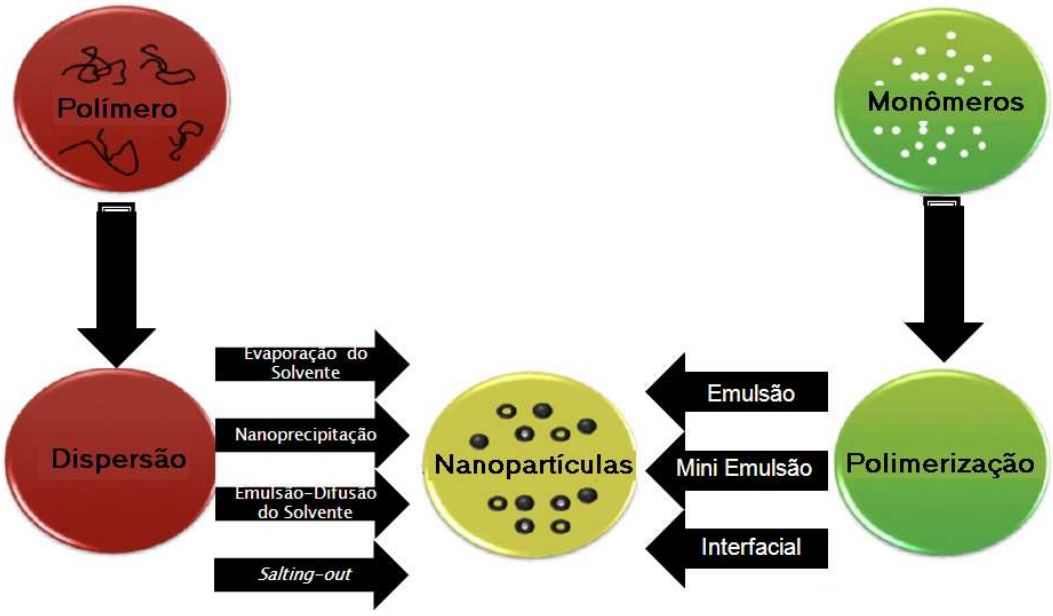
Entre os polímeros com a finalidade de estabilização estérica, o polietilenoglicol (PEG) é até o momento, o material mais utilizado. O modo de fixação deste polímero sobre a superfície das partículas envolve adsorção física ou ligação covalente com o polímero formador da matriz ou da parede polimérica. As características físico-químicas do PEG, como a alta hidrofilicidade, cadeias flexíveis, neutralidade elétrica e a ausência de grupos funcionais, são adequadas para prevenir interações com componentes biológicos, como o SFM (STORM *et al.*, 1995; GREF *et al.*, 1995). A repulsão estérica é resultado de uma perda de entropia conformacional das cadeias de PEG ligadas a uma superfície estranha e uma baixa energia livre interfacial do PEG em água e contribuem para uma importante propriedade fisiológica das nanopartículas revestidas com PEG (OTSUKA *et al.*, 2003). Além do comportamento de longa circulação na corrente sanguínea de nanopartículas contendo PEG, tem-se sugerido que esse revestimento pode afetar sua interação com superfícies biológicas, tais como a mucosa nasal e intestinal e, consequentemente, aumentar sua capacidade de funcionar como transportadores de fármacos através das membranas (TOBÍO *et al.*, 1998, 2000).

As propriedades das nanopartículas podem ser otimizadas de acordo com a finalidade da aplicação. A fim de alcançar propriedades de interesse, o modo de preparação tem um importante papel. A escolha do método de preparação é baseada em vários fatores, como tipo de polímero, finalidade pretendida tamanho de partícula requerido (RAO & GECKELER, 2011), solubilidade do fármaco e considerações a respeito de estabilidade molecular (COHEN-SELA *et al.*, 2009).

De forma geral, as técnicas de preparação de nanopartículas poliméricas podem

ser classificadas em dispersão de polímeros pré-formados ou polimerização de monômeros (fig. 4) (RAO & GECKELER, 2011).

Figura 4 – Representação esquemática das várias técnicas de preparação de nanopartículas poliméricas.



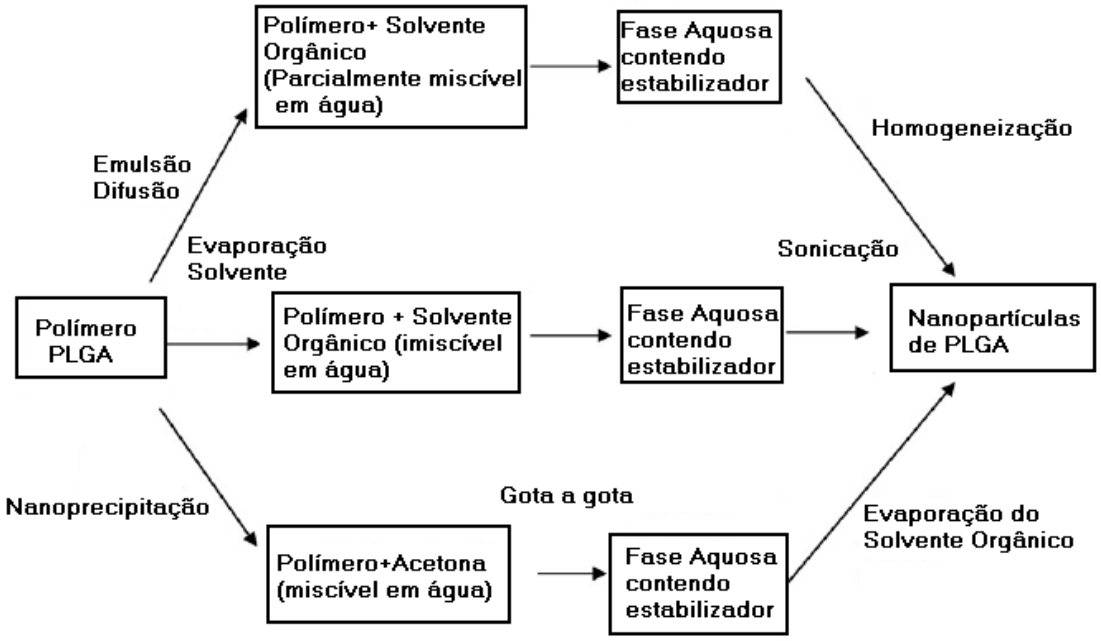
FONTE: RAO & GECKELER, 2011, modificado.

Os métodos a partir da polimerização interfacial de monômeros dispersos apresentam a desvantagem da presença de monômeros e oligômeros residuais, que por sua vez apresentam toxicidade e podem reagir com o fármaco, fato que pode dificultar o controle da massa molecular do polímero resultante, e também inviabilizar a liberação da substância ativa. Por estes motivos, as técnicas baseadas na utilização de polímeros pré-formados tem recebido mais atenção (QUINTANAR-GUERREIRO *et al.*, 1998b)

Incluídos na classificação de dispersão de polímeros pré-formados encontram-se os métodos de emulsificação-evaporação do solvente, emulsão-difusão do solvente e nanoprecipitação (fig. 5), e ainda a técnica de *salting out*. Geralmente, nessas técnicas,

durante a preparação, a solução orgânica constitui a fase interna das nanopartículas, e a fase aquosa externa apresenta agentes estabilizantes. Outra similaridade entre essas técnicas é a baixa capacidade de encapsular fármacos hidrossolúveis, sendo preferencialmente utilizadas para fármacos lipossolúveis (QUINTANAR-GUERRERO *et al.*, 1998a; SOPPIMATH *et al.*, 2001; RAO & GECKELER, 2011).

Figura 5 – Diferentes métodos para a obtenção de nanopartículas de PLGA.



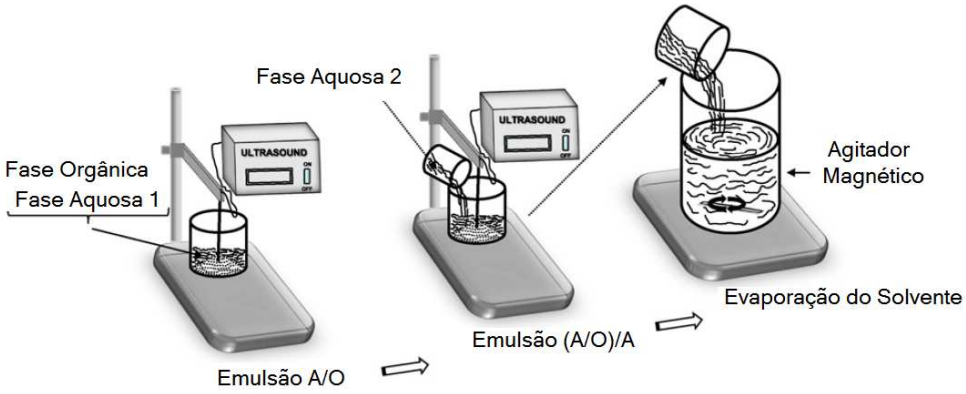
FONTE: KUMARI, *et al.*, 2010, modificado.

O método de emulsão evaporação do solvente tem a vantagem de poder ser utilizado para encapsular tanto fármacos hidrofóbicos quanto hidrofílicos pela formação de emulsões do tipo óleo-em-água (O/A) ou água-em-óleo-em-água (A/O/A), respectivamente. A escolha final do método de preparação de nanopartículas pode ser baseada nas características do componente ativo (fármaco de escolha) e suas interações com os solventes, polímeros, surfactantes e a finalidade das nanopartículas (MANCHANDA *et al.*, 2010).

Na técnica da emulsificação-evaporação, a encapsulação de fármacos lipofílicos é feita pelo preparo de uma emulsão do tipo simples (O/A), em que primeiramente o polímero e o fármaco são dissolvidos em um solvente orgânico imiscível em água, como diclorometano ou acetato de etila, e essa fase é emulsificada em um fase aquosa, geralmente contendo um estabilizador de emulsão (SAWALHA *et al.,* 2011). A formação da emulsão é o passo mais importante da preparação de nanopartículas, pois o tamanho das gotas da emulsão está diretamente relacionado com o tamanho final das nanopartículas. A emulsão é quebrada em gotículas aplicando-se uma energia externa (sonicação), e essas nano-gotículas levam a formação das nanopartículas após a evaporação do solvente orgânico (MANCHANDA *et al.,* 2010). Na literatura, a evaporação do solvente é a técnica mais amplamente empregada para preparar nanopartículas a partir de polímeros pré-formados. Posteriormente, o solidificado de nanopartículas pode ser recolhido por ultracentrifugação e lavado com água destilada para remover os aditivos, tais como surfactantes. Finalmente, o produto pode ou não ser liofilizado (RAO & GECKELER, 2011).

Emulsões duplas são geralmente preparadas em duas etapas de emulsificação, (fig. 6) utilizando-se dois surfactantes: um hidrofóbico designado para estabilizar a interface da emulsão interna (A/O) e um hidrofílico para estabilizar a interface externa dos glóbulos de óleo para a emulsão (A/O/A (MORA-HUERTAS *et al.,* 2010). O método da dupla emulsão-evaporação do solvente permite encapsular altas quantidades de fármacos hidrossolúveis quando comparado a outros métodos como a emulsão-difusão do solvente (CUN *et al.,* 2010).

Figura 6 – Esquema representativo do método de dupla emulsão.



FONTE: MORA-HUERTAS, *et al.,* 2010, modificado.

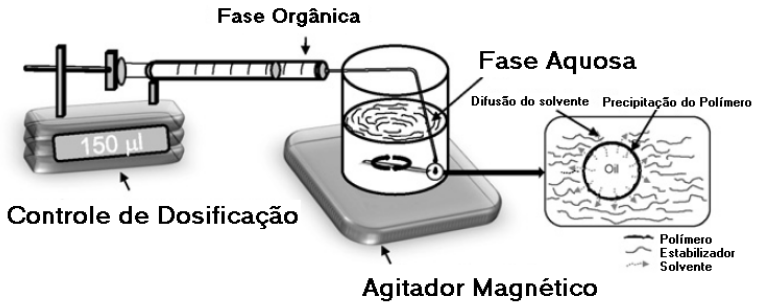
A técnica do *salting-out* é uma versão modificada do processo de emulsão-evaporação que evita a adição de surfactantes e solventes clorados. A emulsão é formulada utilizando-se um polímero e o fármaco solubilizado em um solvente de polaridade intermediária, que normalmente é totalmente miscível com água, como por exemplo, a acetona e o etanol (fase oleosa), assim a emulsificação da solução de polímero na fase aquosa é obtida utilizando-se um agente *salting-out* (cloreto de magnésio, sódio ou de cálcio), que satura a fase aquosa impedindo a precipitada difusão do solvente orgânico nesta fase. Depois de preparada a emulsão O/A, certa quantidade de água é adicionada para permitir a difusão completa do solvente orgânico para a fase aquosa, induzindo a formação de nanopartículas. Tanto o solvente quanto o agente *salting-out* são eliminados por filtração de fluxo cruzado. Esta técnica permite encapsular elevadas quantidades de fármaco, origina rendimentos elevados, não requer temperaturas elevadas e é facilmente transposta à escala industrial. A grande desvantagem é a sua aplicação exclusiva a fármacos lipofílicos e as indispensáveis etapas de lavagem das partículas (QUINTANAR-GUERRERO *et al.*, 1996, 1998; COUVREUR, *et al.*, 1995; REIS, *et al*, 2006; RAO & GECKELER, 2011).

A emulsificação-difusão do solvente é uma técnica mais recente e é uma modificação do *salting-out*, que apresenta a vantagem de evitar o uso de sais e, portanto, elimina a necessidade de purificação intensiva (QUINTANAR-GUERRERO *et al*, 1998a). O método baseia-se na formação inicial de uma emulsão O/A, a partir do polímero e o fármaco, em um solvente parcialmente miscível em água e uma dispersão aquosa contendo tensoativo ou outro estabilizador. Antes da formação da emulsão, ocorre a saturação mútua entre a agua e o solvente orgânico, para atingir o equilíbrio termodinâmico inicial de ambos os líquidos. Após a formação da emulsão, o solvente orgânico é deslocado para a fase aquosa pela adição de um excesso de água, formando nanoesferas. Esta fase de diluição da emulsão deve ter uma agitação suficiente para homogeneizar a mistura. Após o solvente pode ser eliminado por destilação ou filtração (QUINTANAR-GUERRERO *et al.*, 1998a)

Pela técnica da nanoprecipitação (fig. 7), as nanopartículas são formadas instantaneamente e todo o procedimento é realizado em apenas um passo (BILATI, *et al.*, 2005). O polímero (por exemplo, PLA, PCL, PLGA) e o fármaco são dissolvidos num solvente miscível em água (fase orgânica), como por exemplo a acetona ou o etanol. Esta fase é injetada ou vertida para uma solução aquosa contendo um agente estabilizador (por exemplo, álcool polivinílico ou poloxâmero 188), onde o polímero e o fármaco não são solúveis, sob agitação. A nanoprecipitação ocorre pela rápida dessolvatação do polímero quando a solução de polímero e fármaco é adicionada na fase aquosa. Assim que o solvente contendo o polímero é difundido no meio de dispersão, o polímero precipita, ocorrendo imediatamente a encapsulação do fármaco. Posteriormente o solvente é evaporado a pressão negativa. Este método forma nanoesféras, e com adição de um óleo na fase orgânica, é possível a produção de nanocápsulas. A formação das nanocápsulas baseia-se no fato de que quando a fase

orgânica, contendo o polímero e o óleo, é vertida na fase aquosa, as vesículas são formadas por emulsificação espontânea, enquanto o solvente difunde na fase aquosa (QUINTANAR-GUERRERO *et al.*, 1998; BILATI, *et al.*, 2005, RAO & GECKELER, 2011).

Figura 7 – Esquema representativo do método de nanoprecipitação.



FONTE: MORA-HUERTAS, *et al.*, 2010, modificado.

A liberação dos fármacos a partir de sistemas nanoparticulados e subsequente biodegradação dependem de diferentes fatores: a) da dessorção do fármaco da superfície das partículas; b) da difusão do fármaco através da matriz das nanoesferas; c) da difusão através da parede polimérica das nanocápsulas; d) da erosão da matriz polimérica ou e) da combinação dos processos de difusão e erosão. Portanto, difusão e biodegradação governam o processo de liberação da droga (SOPPIMATH *et al*, 2001). Métodos como a difusão em sacos de diálise e a separação baseada na ultracentrifugação, na filtração a baixa pressão ou na ultrafiltração-centrifugação têm sido utilizados para este fim (SCHAFFAZICK *et al,* 2003). Por este método uma suspensão de nanopartículas é

colocada em sacos de dialise e estes sacos são incubados no meio de dissolução, e mantidos sob agitação, após determinados períodos de tempo, alíquotas do meio de dissolução são coletadas e quantificadas. Outra técnica envolve o uso de uma célula de difusão que consiste em um compartimento doador e outro receptor, separados através de uma membrana biológica ou sintética. Esta técnica, porém, não é realizada em perfeitas condições *sink*, pois as nanopartículas não estão diretamente diluídas no meio de dissolução. Assim, a quantidade de fármaco liberado, não reflete a real quantidade de fármaco liberado (SOPPIMATH *et al*, 2001).

Ao longo do tempo, o princípio básico para o desenvolvimento de novas terapias medicamentosas tem residido na otimização da ação do fármaco. De fato, a intensidade do efeito farmacológico está diretamente relacionada à concentração de fármaco que atinge o local de ação desejado. Após administração do medicamento, o fármaco é liberado a partir da forma farmacêutica e então dissolvido nos fluidos biológicos. Após estas etapas, o destino do fármaco é determinado pela combinação dos seguintes processos farmacocinéticos: absorção, distribuição, metabolismo e eliminação, que dependem essencialmente das características estruturais e físico-químicas do mesmo (MAZZARINO, 2009). A tecnologia de nanopartículas tem mostrado elevado potencial para aumentar a biodisponibilidade de fármacos pouco solúveis, como é o caso da anfotericina B, nifedipina, curcumina, entre outros, podendo ser comprovado através de estudos de biodisponibilidade oral (COUVREUR *et al.*, 2002).

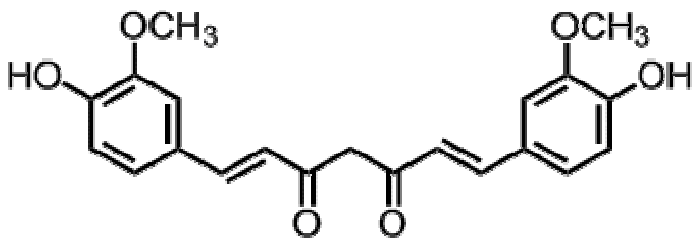
Curcumina

A curcumina é um pigmento amarelo presente na *Curcuma longa* planta da família *Zingiberaceae*, popularmente conhecida como açafrão da índia, açafrão da terra ou gengibre dourado. Trata-se de um arbusto perene, nativo do sul e sudoeste asiático e extensivamente cultivado na Índia, também na China, Taiwan, Japão, Burma, Indonésia, e no continente Africano (SCARTEZZINI & SPERONI, 2000).

Os princípios ativos contidos na cúrcuma são pigmentos curcuminóides (curcumina, desmetoxicurcumina e bisdesmetoxicurcumina). Estudos mostram que as atividades farmacológicas são decorrentes dos pigmentos curcuminóides, mais especificamente da curcumina, isolada pela primeira vez em 1815 (JAYAPRAKASHA *et al.*, 2005; KUNNUMAKKARA *et al.*, 2008; SONG *et al.*, 2011)

A curcumina, cuja estrutura química está representada na figura 8, é um polifenol de baixo peso molecular, 368,37 g/mol, apresenta ponto de fusão a 183 °C e fórmula molecular $C_{21}H_{20}O_6$. Possui baixa solubilidade em água e éter, apresentando solubilidade aparente de 348 µg/mL em tampão pH 7,4 (KUPPUSAMY *et. al.*,2009), mas apresenta-se solúvel em etanol, dimetilsulfóxido, acetona e outros solventes orgânicos (KUNNUMAKKARA *et al.*, 2008).

Figura 8 – Estrutura química da curcumina

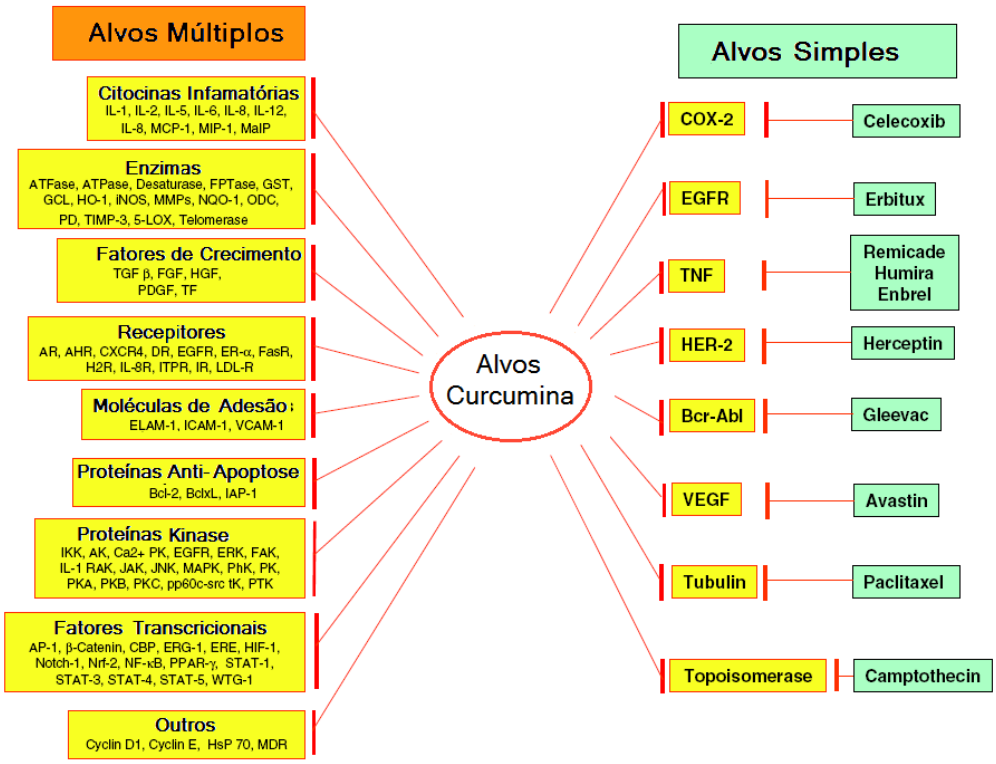


A curcumina vem sendo utilizada por centenas de anos como fármaco para diversas doenças, sendo muito popular na medicina asiática. Possui baixa toxicidade e uma ampla faixa de atividades farmacológicas (SAHU *et al*., 2008), estando entre os mais promissores e eficazes agentes quimiopreventivos e/ou antitumorais (SINGH & KHAR, 2006). Dentre as atividades farmacológicas mais bem estabelecidas e estudadas da curcumina na literatura está a sua propriedade como agente antitumoral. Já foram descritas atividades antiproliferativas sobre os tumores de fígado (CHUANG *et al*., 1992), cólon (RAO *et al*., 1995; KIM *et al*., 1999; KAWAMORI *et al*., 1999), de pele (LU *et al*., 1994; LIMTRAKUL *et al*., 1997; HUANG *et al*., 1997), pâncreas (LEV-ARI *et al*., 2007), células pituitárias (MILLER *et al*., 2008) e próstata (BARVE *et al*., 2008). Inclui-se ainda atividade antioxidante, antiinflamatória, antimicrobiana (ANAND *et al.*, 2008; SONG *et al*, 2011), antidepressiva, imunomodulatória, anti-convulsante e atividade neuroprotetora (REETA *et al*, 2011). Também tem sido reportado o uso da curcumina para tratamento de doenças respiratórias como a asma, desordens de fígado, anorexia, reumatismo, diabetes e sinusite (SHAIKH *et al*, 2009). Em várias doenças crônicas, em que a inflamação é conhecida por desempenhar um papel importante, a curcumina tem demonstrado efetividade terapêutica. Estas doenças incluem a de Alzheimer, o mal de Parkinson, esclerose múltipla, epilepsia, câncer, alergia, asma, bronquite, colites, artrite reumatóide, isquemia renal, psoríase, diabetes, obesidade, depressão, fadiga e AIDS (AGGARWAL & HARIKUMAR, 2009).

A curcumina é uma molécula que demonstrou-se suprimir múltiplas vias de sinalização e inibir a proliferação celular, invasão, metástase e angiogênese. Sugere-se que a curcumina tem uma variada gama de alvos moleculares, apoiando o conceito de que ela age sobre numerosas cascatas bioquímicas e moleculares. Este polifenol modula vários alvos através de interação direta ou através da expressão gênica. Vários alvos

moleculares incluem fatores de transcrição, fatores de crescimento e seus receptores, citocinas, enzimas e genes reguladores de proliferação celular e apoptose (fig. 9) (KUNNUMAKKARA *et al.*, 2008).

Figura 9 – Terapia de alvos simples *versus* terapia em alvos multiplos pela curcumina. Terapias modernas incluem alvos celecoxib, avastin, erbitux, enbrel, herceptin, gleevec, e avastin (COX2, EGFR, TNF, HER2, bcr-abl e VEGF, respectivamente) e também inibe outros alvos, incluindo fatores de transcrição, enzimas, fatores de crescimento e seus receptores, kinases e proteínas anti-apoptose.



FONTE: KUNNUMAKKARA *et al.*, 2008, modificado.

Os múltiplos efeitos terapêuticos da curcumina devem-se a sua habilidade de modular a atividade de diversas enzimas, a expressão gênica das células tumorais (WU

et al., 2007), e especialmente por ser um potente inibidor de NF-κB, um fator de transcrição implicado na patogênese de diversos tumores malignos (SINGH & AGGARWAL, 1995).

Embora o grande interesse farmacêutico relacionado à curcumina tenha aumentado consideravelmente, o uso terapêutico deste composto tem sido limitado devido a sua baixa solubilidade aquosa, a qual resulta em baixa absorção após administração oral ou impede a obtenção de soluções para administração intravenosa. Além disso, a curcumina exibe alta taxa de decomposição em pH neutro ou básico e susceptibilidade à degradação fotoquímica (TONNESEN, 2002; TONNESEN *et al*., 2002; TOMREN *et al*., 2007). Em conjunto com estes fatores, o rápido metabolismo e a rápida eliminação sistêmica também contribuem para os reduzidos níveis séricos e teciduais deste interessante composto fenólico e, portanto, para a sua baixa biodisponibilidade (ANAND *et al*., 2007; KUNNUMAKKARA *et al.*, 2008; AGGARWAL & HARIKUMAR, 2009).

A biodisponibilidade indica a velocidade e a extensão de absorção de um princípio ativo a partir de uma forma farmacêutica, a partir de sua curva de concentração *versus* tempo na circulação sistêmica ou na excreção urinária (BRASIL, 1999). A ação terapêutica de um fármaco depende da existência de uma concentração efetiva deste no seu local de ação durante um período de tempo desejável. Uma vez que a concentração de um fármaco no local de ação encontra-se em equilíbrio com a concentração do mesmo na corrente sanguínea, para a maioria dos fármacos, a determinação da sua concentração ao longo do tempo no sangue ou na urina tornam-se medidas indiretas, mas preditivas da concentração do fármaco no local de ação (INFARMED).

Um estudo realizado em ratos, foi encontrado quantidades insignificantes da curcumina no sangue dos animais após administração oral de 1g/kg de curcumina (KUNNUMAKKARA *et al.,* 2008).

Em outro estudo com animais, a curcumina apresentou rápido metabolismo, resultando em baixa biodisponibilidade sistêmica após administração oral. Em uma dose de 0,1 g/kg da curcumina livre administrada via oral para ratos, resultou em uma concentração máxima no plasma de apenas 2,25 µg/mL (PAN *et al.,* 1999). Em ratos, a curcumina desapareceu completamente do plasma após 1 h da administração de uma dose de 40 mg/kg pela via intravenosa. Quando administrada oralmente uma dose de 500 mg/kg, apresentou uma concentração máxima do plasma de 1,8 ng/mL (IRESON *et al.*,2001).

Em um estudo em humanos (fase I), utilizando-se doses de 500-8000 mg/dia, não foi encontrado nenhum traço de curcumina em nível sistêmico, e apenas traços do composto na minoria dos pacientes quando administrado 10-12g/dia. (BISHT *et al.*, 2007). Em um estudo piloto com 24 voluntários sadios, curcumina foi administrada até 12 g/dia e as amostras só puderam ser quantificadas no plasma a partir de 10 e 12 g/dia (AGGARWAL & HARIKUMAR, 2009).

Observa-se então que a curcumina apresenta limitações farmacotécnicas e farmacocinéticas que resultam em problemas biofarmacêuticos. Desta forma, o desenvolvimento farmacotécnico torna-se importante no sentido de se desenvolver uma nova formulação para veiculação de curcumina que permita que a solubilidade aquosa e estabilidade físico-química da molécula seja aumentada proporcionando maior biodisponibilidade oral.

Nos últimos anos, a busca por alternativas mais eficientes e menos tóxicas para a terapia de diversas doenças, tais como o câncer, despertou interesse em pesquisadores

de várias áreas, particularmente as que envolvem a utilização de sistemas lipídicos e polímeros biodegradáveis, no desenvolvimento de sistemas de liberação controlada de fármacos. Uma estratégia promissora para contornar os efeitos colaterais, limitações e inconveniências da quimioterapia do câncer tem sido o emprego de sistemas nanoestruturados na forma de lipossomas, nanopartículas, micelas, nanoemulsões e ciclodextrinas, como carreadores de fármacos antitumorais (MUSUMECI *et al.*, 2006; GRYPARIS *et al, 2007;* KUMARI *et al.*, 2010; LIANG *et al.*, 2011).

Diversos trabalhos mostram resultados promissores obtidos pela obtenção de sistemas nanoestruturados contendo curcumina. Micelas poliméricas compostas por conjugados de ácido láctico e polietilenoglicol (PLA-PEG) e palmitato contendo curcumina resultaram em aumento na solubilidade aquosa do fármaco e aumento na atividade antitumoral (SAHU *et al*., 2008). Nanopartículas lipídicas sólidas contendo curcumina demonstraram grande potencial como sistemas antioxidantes e antiinflamatórios, apresentando liberação sítio-específica em macrófagos (SOU *et al*., 2008). Micelas poliméricas constituídas por N-isopropilacrilamida com polivinilpirrolidona e polietilenoglicol-monoacrilato apresentaram aumento na solubilidade aquosa da curcumina e eficácia antitumoral e antiinflamatória *in vitro* equivalente à curcumina livre (BISHT *et al*., 2007). Curcumina carreada por micelas constituídas de um copolímero tribloco (PLGA-PEG-PLGA) mostrou uma diminuição da absorção das micelas contendo curcumina pelas células do fígado e do baço, e assim uma melhor distribuição da droga no cérebro e pulmão, mostrando as micelas como um potencial carreador de curcumina (SONG *et al.*, 2011). Um estudo que formulou nanopartículas de curcumina com N,O-carboximetil quitosana, mostrou que as nanopartículas exibiram toxicidade específica às células cancerosas e nenhuma toxicidade para as células normais (ANITHA *et al.*, 2011)

Recentemente, Saik e colaboradores (2009) demonstraram que nanopartículas de PLGA foram capazes de aumentar a biodisponibilidade da curcumina em no mínimo nove vezes quando comparado com a curcumina administrada em conjunto com a pepirina, um composto capaz de elevar sua absorção. Tsai e colaboradores (2011) desenvolveram nanopartículas de PLGA contendo curcumina e quando estas foram administradas via intravenosa em ratos, uma quantidade significativa de curcumina foi encontrada principalmente no baço, devido à absorção pelas células fagocitárias no sistema reticuloendotelial. Xie e colaboradores (2011) mostrou que após administração oral de nanopartículas de PLGA contendo curcumina, a biodisponibilidade relativa foi 5,6 vezes maior e aumentou-se o tempo de meia vida maior quando comparado com a curcumina livre. Um estudo similar demonstrou que a curcumina veiculada através de nanopartículas de PLGA aumentou a apoptose elevando a atividade biológica *in vitro* e obteve-se uma biodisponibilidade superior *in vivo* sobre a curcumina livre (ANAND *et al.*, 2010).

Deste modo, propõem-se o desenvolvimento de um sistema nanoestruturado polimérico que promova liberação prolongada da curcumina. Além de ser possível a obtenção de uma nova forma de veiculação da curcumina, busca-se melhorar as suas propriedades farmacocinéticas após a administração oral, visto que sua solubilidade é restrita resultando em baixa biodisponibilidade oral.

OBJETIVOS

Objetivo geral

Desenvolver e caracterizar nanopartículas poliméricas contendo curcumina e avaliar suas propriedades farmacocinéticas, principalmente biodisponibilidade oral, a qual é muito restrita quando utilizada na sua forma livre.

Objetivos específicos

- Obter nanopartículas de PLGA e de blendas de PLGA-PEG contendo curcumina
- Desenvolver e validar metodologia analítica por CLAE para análise quantitativa de curcumina em nanopartículas;
- Caracterizar os sistemas obtidos quanto à eficiência de encapsulação, diâmetro médio e distribuição de tamanho;
- Determinar o perfil de liberação *in vitro* da curcumina a partir das nanopartículas;
- Desenvolver e validar metodologia analítica por LC/MS/MS para análise quantitativa de curcumina em plasma de rato;
- Determinar parâmetros farmacocinéticos das nanopartículas contendo curcumina após administração oral em ratos e comparar com a curcumina não encapsulada.

CAPÍTULO I

ARTIGO CIENTÍFICO

DEVELOPMENT AND VALIDATION OF AN HPLC METHOD USING FLUORESCENCE DETECTION FOR THE QUANTITATIVE DETERMINATION OF CURCUMIN FROM PLGA AND PLGA-PEG NANOPARTICLES

DEVELOPMENT AND VALIDATION OF AN HPLC METHOD USING FLUORESCENCE DETECTION FOR THE QUANTITATIVE DETERMINATION OF CURCUMIN FROM PLGA AND PLGA-PEG NANOPARTICLES

Abstract

In this paper, a rapid and effective chromatographic procedure for determining the curcumin encapsulation efficiency in poly(lactic-*co*-glycolic acid) (PLGA) and poly(lactic-*co*-glycolic acid)-polyethyleneglycol (PLGA-PEG) nanoparticles via reversed-phase high-performance liquid chromatography (RP-HPLC) using a fluorescence detector and low flow rate is described. Chromatographic runs were performed on a RP C18 column (250 mm×4.6 mm, 5 µm) with a mixture of ethanol, water and acetonitrile (80:10:10, v/v/v) as the mobile phase and a flow rate of 0.8 mL/min in the isocratic mode. Curcumin was detected using a fluorescence detector operating at an excitation wavelength of 365 nm and an emission wavelength of 512 nm. This method was validated in terms of the selectivity, linearity, precision, accuracy, robustness, limit of detection and limit of quantitation. The analytical curve was linear over the concentration range of 1–50 µg/mL, and the limits of detection and quantitation were 9.65 and 50 ng/ml, respectively. The mean recovery for curcumin was 101.14 ± 2.8% (n = 9). The intra- and inter-assay coefficients of variation were less than 3.73%. The method was robust for changes in the mobile phase, column temperature and flow rate. The maximum relative standard deviation was 3.08%. The method was successfully used to determine the encapsulation efficiency of curcumin in PLGA and PLGA-PEG nanoparticles.

Keywords: biodegradable polymers, curcumin, encapsulation efficiency, fluorescence, HPLC, nanoparticles, particle size, PLGA, PLGA-PEG, validation.

1. Introduction

Curcumin is a polyphenol compound obtained from the roots (rhizomes) of the plant Curcuma longa (*Zingiberaceae*). It is a yellow compound that is the primary ingredient in the spices turmeric, curry and mustard and is also used as food coloring [1]. Because of its ability to interact with a diverse range of molecular targets, curcumin affects numerous different molecular and biochemical cascades. Some curcumin targets include transcription factors (e.g., NF-κβ, PPAR-γ), receptors (e.g., IL-8, CXCR-4), kinases (e.g., EGFR, JAK), cytokines (e.g., TNF, IL), enzymes (e.g., iNOS, COX-2, 5-LOX) and growth factors (e.g., NGF) [2]. Therefore, curcumin has potential therapeutic applications for the treatment of several diseases. Previous studies have reported anti-inflammatory, antimicrobial, antioxidant, antiparasitic, antimutagenic [3-6] and, primarily, antitumor effects caused by curcumin's ability to affect the cell cycle, apoptosis, proliferation, invasion, angiogenesis and metastasis via the down-regulation of targets involved in its signaling pathway [2,7].

Despite the great therapeutic potential of curcumin, it has several restrictive properties, such as low aqueous solubility, rapid metabolism, rapid systemic elimination and inadequate tissue absorption, which severely decreases its availability and consequently impairs its therapeutic application. Additionally, curcumin has stability drawbacks, such as degradation at alkaline pH and photodegradation [8-10]. Controlled drug delivery systems offer an alternative that can circumvent these drawbacks. Curcumin represents an interesting drug model for improvement by nanotechnology.

Polymeric nanoparticles have been extensively studied because of their unique and valuable physicochemical and biological properties. Indeed, nanoparticles can protect the drug from degradation, increase its aqueous solubility, enhance both its transport and distribution and prolong its release, thereby improving its plasma half-life [11-12]. The pharmacokinetic parameters of the drug increase with the use of nanoparticles, whose surface composition play an important role in drug bioavailability and depend on the polymer used. Polymeric nanoparticles with unmodified surfaces are quickly removed from the blood, primarily by Kupfer cells in the liver and macrophages in the spleen, after intravenous administration. These cells recognize specific opsonin proteins bound to the particle's surface, which are then phagocytosed. Nanoparticles with hydrophilic polymer chains on their surface, such as polyethyleneglycol (PEG), have a prolonged blood circulation time because this covering promotes the steric stabilization of the nanoparticles, which prevents their opsonization and phagocytosis [13-15].

One of the characterization steps for polymeric nanoparticles is the determination of their drug content. This parameter must be properly verified because the drug must be efficiently loaded into the nanoparticles to achieve the desired therapeutic efficacy. Therefore, a suitable and validated quantitation method is required to assess this parameter. Various methods have been described in the literature for determining curcumin content in samples, such as turmeric, using NMR with LC-MS/MS [16], HPLC-UV/Vis [17], HPLC-fluorescence [18] and thin-layer chromatography [19], and in biological matrices using HPLC-UV/Vis [20-21], HPLC-fluorescence [22] and LC-MS/MS [23-24]. However, there are few studies reporting the analytical determination of curcumin in pharmaceutical dosage forms, such as nanoparticles. Some UV-Vis spectrometric [25-27] and HPLC-UV/Vis methods [28-31]

were reported for such a determination, but these chromatographic methods have not been validated. Also, there are no reports of HPLC-fluorescence methods for the determination of curcumin in polymeric nanoparticles. Therefore, the objective of this work is to develop and validate a rapid, simple and optimized reversed-phase HPLC-fluorescence method to quantitatively determine the encapsulation efficiency of curcumin in poly(lactic-*co*-glycolic acid) (PLGA) and PLGA-PEG nanoparticles.

2. Experimental

2.1. Materials

Curcumin (code C1386), PLGA (Resomer RG 50:50 H; *M*w 40-75 kDa, inherent viscosity 0.45-0.6 dl/g), PEG (10 kDa) and polyvinyl alcohol (PVA, 31 KDa, 88% hydrolyzed) were purchased from Sigma-Aldrich (USA). Methylene chloride and ethylene acetate were purchased from FMaia® (Brazil). HPLC-grade ethanol and acetonitrile were purchased from JTBaker® (USA). Water was purified using a Milli-Q Plus system (Millipore®). All other solvents and chemicals were of either analytical or HPLC grade.

2.2. Equipment

A Waters 2695 Alliance HPLC system (Milford, MA, USA) was used for method development. This HPLC system was equipped with a column compartment with temperature control, an on-line degasser, a quaternary pump, an autosampler, a photodiode array wavelength detector (Waters 2998) and a fluorescence detector (Waters 2475). Data acquisition, analysis, and reporting were performed using the Empower chromatography software (Milford, MA, USA). HPLC analysis was conducted using a reverse phase C18 column (VertiSep GES, Vertical Chromatography

Co, Ltd), with a 5 µm particle size, 4.6 mm internal diameter, and 250 mm in length.

2.3. Chromatographic conditions

Chromatographic analysis was performed in the isocratic mode with a mobile phase consisting of an ethanol, acetonitrile and water mixture (80:10:10, v/v/v), pumped at a flow rate of 0.8 mL/min. The sample injection volume was 20 µL, and the fluorescence detector operated at an excitation wavelength of 365 nm and an emission wavelength of 512 nm. The method run time was 5 min, and all experiments were performed at 22°C.

2.4. Preparation of standard and sample solutions

A stock standard solution of 500 µg/mL of curcumin was prepared by weighing approximately 5mg of curcumin into a 10mL volumetric flask and filling with ethanol. Eight standard solutions (1, 5, 10, 15, 20, 30, 40 and 50 µg/mL) were obtained by mixing the required amount of this (500 µg/mL) working standard solution with a sufficient quantity of ethanol (up to 1.5 mL). Similarly, eight standard solutions (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1.0 µg/mL) were obtained to determine the limit of detection (LOD) and limit of quantitation (LOQ) of this method.

The standards and samples were previously filtered through a 0.22 µm pore size filter (Millipore, Bedford, USA) prior to injection.

2.5. Method validation

The HPLC method was validated according to the International Conference on Harmonization (ICH) guidelines [32]. The following characteristics were considered for validation: specificity, linearity, accuracy, precision, LOD, LOQ and robustness.

The linearity was determined by calculating a regression line from the plot of peak area vs. concentration for the eight standard solutions (1, 5, 10, 15, 20, 30, 40 and 50 µg/mL) using the linear least squares methodology.

The accuracy was tested by calculating the percent recoveries of the mean concentration of curcumin at three different concentration levels and determining their relative standard deviations (RSD). The mean concentration value obtained for each level was compared to the theoretical value, which was considered to be 100%.

Two factors of precision were assessed, the repeatability and intra-day variability, by testing three different standard solutions on the same day and three different standard samples on two different days, respectively. The results are reported as the standard deviation (SD) and RSD.

The LOD and LOQ were determined from the specific calibration curve obtained for the eight standard solutions (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1.0 µg/mL) that where closest to these concentration limits (ICH, 2005). The LOQ was defined as the lowest concentration on the standard calibration curve that could be quantitatively determined with precision and accuracy. The LOD was based on the response and slope of the calibration curve (ICH, 2005) using the following equation (eq. 1):

$$LOD = 3.3\sigma/S \qquad \text{Eq. 1}$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve.

The robustness was evaluated by deliberately varying the temperature of the

analytical column (35°C and 45°C), the flow rate (0.7 mL/min) and the proportions of the mobile phase (75:15:10) from the parameters described in sub-sections 2.2 and 2.3.

The specificity was evaluated by comparing representative chromatograms of samples containing possible interfering substances (excipient solutions used in nanoparticle production) to samples containing curcumin. Additionally, the specificity was demonstrated by performing stress studies (photostability).

2.6. Method applicability

2.6.1. Preparation of curcumin-loaded PLGA and PLGA-PEG nanoparticles

The nanoparticles were obtained using the single-emulsion solvent-evaporation technique, as described elsewhere [33]. Briefly, curcumin (5 mg) and PLGA (50 mg) were dissolved in a mixture of ethylene acetate (1.5 mL) and methylene chloride (0.5 mL) either with or without PEG (10 mg) at room temperature. This organic phase was poured rapidly into 10 mL of an aqueous PVA solution (0.5%, w/v) and emulsified by sonicating for 5 min (35% of 500 W, Unique® Ultrasonic Mixing, mod. DES 500, equipped with a 4 mm probe, Unique Group, Brazil), which resulted in an oil-in-water (O/A) emulsion. Next, the organic solvent was rapidly removed by evaporation under a vacuum at 37ºC (20 min). The particles were then recovered by ultracentrifugation (19,975 *g*, 30 min, 4°C, Cientec CT-15000R centrifuge, Brazil) and washed twice with water to remove the surfactant. The nanoparticles were dispersed in a cryoprotectant sucrose (5%, w/v), and the resulting nanosuspension was subsequently cooled to −18ºC and freeze-dried (Terroni®, Brazil).

The mean particle size, size distribution and polydispersity index were determined by dynamic light scattering (BIC 90 plus, Brookhaven Instruments Corp.). The analyses were performed at a scattering angle of 90° and a temperature of 25°C.

For each sample, the mean particle diameter, polydispersity and standard deviation for ten determinations were calculated.

2.6.2. Determination of curcumin encapsulation efficiency

The amount of curcumin incorporated into the nanoparticles was determined indirectly [34-35]. The analyte was the supernatant, which contained free curcumin separated from solid nanoparticles by ultracentrifugation, as described in 2.6.1.

After diluting with ethanol, 20 μL of the sample was injected into the HPLC system, and the drug concentration in the supernatant was obtained by comparing the obtained concentration to a previously constructed analytical curve. All solutions were filtered through a membrane filter (0.22 μm pore size, Millipore) before injection. The amount of curcumin trapped in the nanoparticles was obtained by subtracting the quantity in the supernatant from the total used during the preparation. These analyses were performed in triplicate.

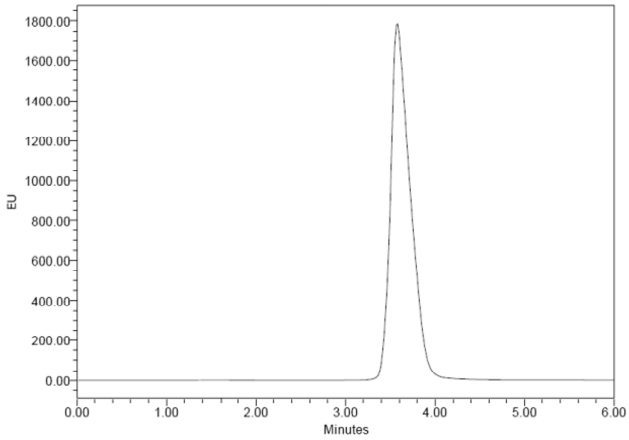
3. Results and discussion

3.1. Method development

Initial runs were performed using methanol and water in various proportions as the mobile phase in the isocratic mode. Irregular shaping and tailing of the curcumin peak was observed. Furthermore, acetic acid was added but did not improve the peak symmetry. When a methanol:water:acetic acid (65:35:0.1, v/v/v) mixture was used, a noticeable tailing was observed in the peak. Variations in the methanol, water and acetonitrile proportions were tested. It was observed that, when a methanol:water:acetonitrile ratio of 41:23:36 (v/v/v) was used, there was decrease in the tailing; however, the peak remained broad and was irregularly shaped. Due to the high

solubility of curcumin in ethanol, this solvent was used to reduce the affinity of the drug for the column and thus obtain short retention times. When ethanol and water were evaluated in the proportion of 85:15 (v/v, ethanol:water), the curcumin peak did not possess a tail, and a regular and symmetric shape was observed; however, the peak was still broad. This problem was solved by adding acetonitrile and modifying the flow rate. The most acceptable combination for the mobile phase was an ethanol:acetonitrile:water ratio of 80:10:10 at a flow rate of 0.8 mL/min. Increasing the flow rate resulted in peak tailing. Under these conditions, the curcumin peak was detected within approximately 3.7 min (Fig. 1).

Figure 1. Representative HPLC chromatograms of 30 µg/mL curcumin standard solution. Conditions: mobile phase, ethanol:acetonitrile:water (80:10:10, v/v/v); flow rate, 0.8 mL/min; fluorescence detection wavelength, 365 nm (excitation) and 512 nm (emission); column temperature, 22ºC; injection volume, 20 µL.



3.2. Method validation

3.2.1. System suitability

The system suitability of this method was evaluated by analyzing the retention time, peak symmetry and theoretical plates of the column during the run of the curcumin ethanolic solution over six repetitions. The system suitability results are

summarized in Table 1 and can be observed that the parameters analysed are in accordance with acceptance criteria.

Table 1. Chromatographic performance parameters for the chosen setup, as described in section 2.

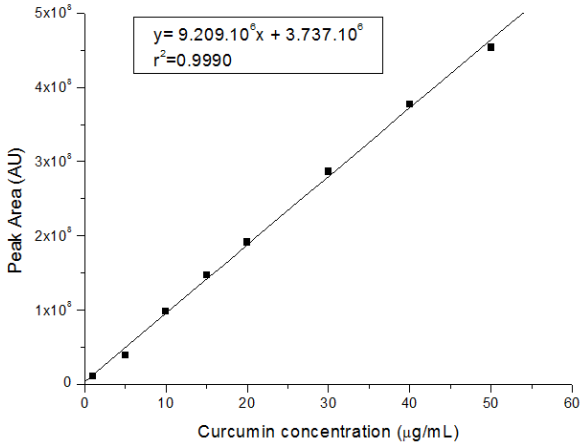
Chromatographic parameters	Result*	Acceptance criteria
Tailing factor (*T*)	1.26 ± 0.11	$T < 2$
Number of theoretical plates (*N*)	2177 ± 23	$N > 2000$

* Presented as mean value ± standard deviation

3.2.2. Linearity

The linearity was evaluated at eight concentration levels ranging from 1 to 50 µg/mL by calculating the regression equation and correlation coefficient (r^2) by the least squares method. Figure 2 shows the plot of the response factor vs. the standard sample concentration. The relationship between the theoretical and measured concentrations is clearly linear with an r^2 value of nearly 1. This result indicates the linearity of the peak area ratio for curcumin over the concentration range of 1–50 µg/mL.

Figure 2. Calibration curve obtained with curcumin ethanolic solutions in the range of 1-50 µg/mL (n = 3).



3.2.3. Accuracy

The accuracy was assessed by calculating the percent recovery and RSD of the mean concentration of the analyte at three different concentrations. Three standard solutions (5, 15 and 30 µg/mL) were carefully prepared in triplicate and analyzed by the proposed method. Detailed results for these three tested concentration levels are presented in Table 2. The mean recovery of curcumin from the samples was 100.027% (RSD = 1.24%, n = 9), which indicates an agreement between the experimental and theoretical values.

Table 2. Accuracy results for the curcumin concentrations in the standard solutions.

Standard solution (µg/mL)*	Recovery (%)	RSD (%)
5	101.21	1.57
15	102.28	0.64
30	96.59	1.52

*n=3; RSD = relative standard deviation

3.2.4. Precision

Precision is a measure of the relative error for the method and is expressed as the RSD for the repeatability and intra-day variability. Three concentrations of curcumin (5, 15 and 30 µg/mL) were prepared in triplicate and analyzed over either one or two different days to evaluate the intra- and inter-day variations, respectively. The RSD of the responses was calculated for each case and are shown in Table 3, which indicates that a good precision was obtained because a maximal RSD of 3.85% was obtained.

Table 3. Precision results for the different levels of curcumin in the standard solutions.

Standard solution (µg/mL)*	Measured concentration ± SD (µg/mL)	RSD (%)
Repeatability (n=3)		
5	5.73 ± 0.09	1.65
15	17.98 ± 0.44	2.45
30	34.42 ± 1.32	3.85
Intermediate precision (n=3)		
Day 1		
5	5.06 ± 0.07	1.58
15	15.34 ± 0.09	0.63
30	28.98 ± 0.43	1.52
Day 2		
5	4.80 ± 0.17	3.72
15	15.97 ± 0.13	0.87
30	30.23 ± 0.58	1.92

*n=3; SD = Standard deviation; RSD = relative standard deviation

3.2.5. Robustness

Robustness is a measure of the influence of small changes to the analytical procedures/parameters on the response. The robustness was evaluated based on the percent recovery and RSD values obtained using different parameters for the mobile phase flow rate, column temperature and mobile phase proportions (Table 4). The method is robust with regards to these alterations in the chromatographic parameters. The maximum RSD obtained was 3.08%. (The RSD cannot be greater than 5% for the method to be considered robust.)

Table 4. Robustness results for the different flow rates, column temperatures and mobile phase proportions.

Changes to original method*	Percentage of Recovery ± RSD (n=3)			
	5 µg/mL	15 µg/mL	30 µg/mL	Mean
None	96.09 ± 3.72	106.44 ± 0.87	100.75 ± 1.93	101.09 ± 2.17
Flow rate: 0.7 mL/min	101.21 ± 0.85	98.99 ± 0.71	100.43 ± 0.31	100.21 ± 0.62
Column Temperature: 35°C	103.46 ± 2.41	101.44 ± 1.02	104.45 ± 3.08	103.12 ± 2.17
Column Temperature: 45°C	102.27 ± 1.59	101.15 ± 0.81	103.63 ± 2.52	102.35 ± 1.64
Mobile phase proportion: ethanol:acetonitrile: water (75:15:10)	100.85 ± 0.60	97.01 ±2.14	100.24 ± 0.18	99.37 ± 0.97

*0.8 mL/min, column at 22°C and mobile phase (ethanol:acetonitrile:water 80:10:10 v/v/v).

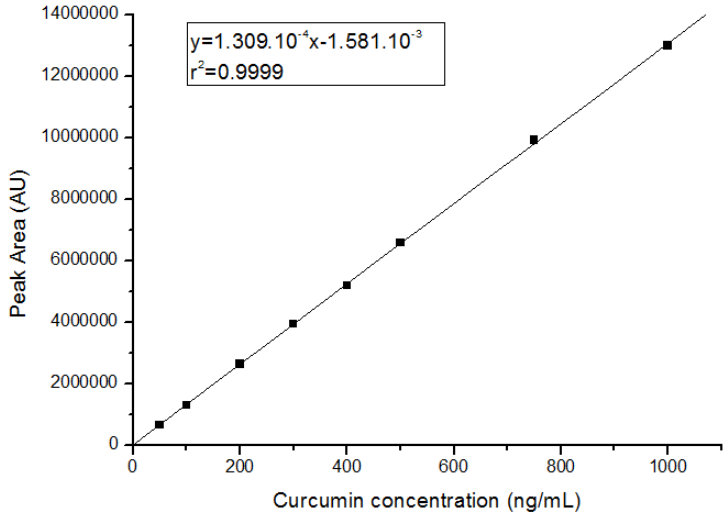
RSD=relative standard deviation

3.2.6. Limit of quantitation and limit of detection

The lowest concentration at which an analyte can be detected (LOD) or quantified with acceptable precision and accuracy (LOQ) was calculated based on the

specific calibration curve obtained from the standard samples (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1.0 µg/mL) in the low end of the proposed range (ICH, 2005). Figure 3 shows the plot of the response factors vs. the standard sample concentrations and indicates that the method was linear over this range. The LOD and LOQ were found to be 9.65 and 50 ng/mL, respectively.

Figure 3. Calibration curve obtained with curcumin ethanolic solutions in the range of 50-1000 ng/mL (n = 3).



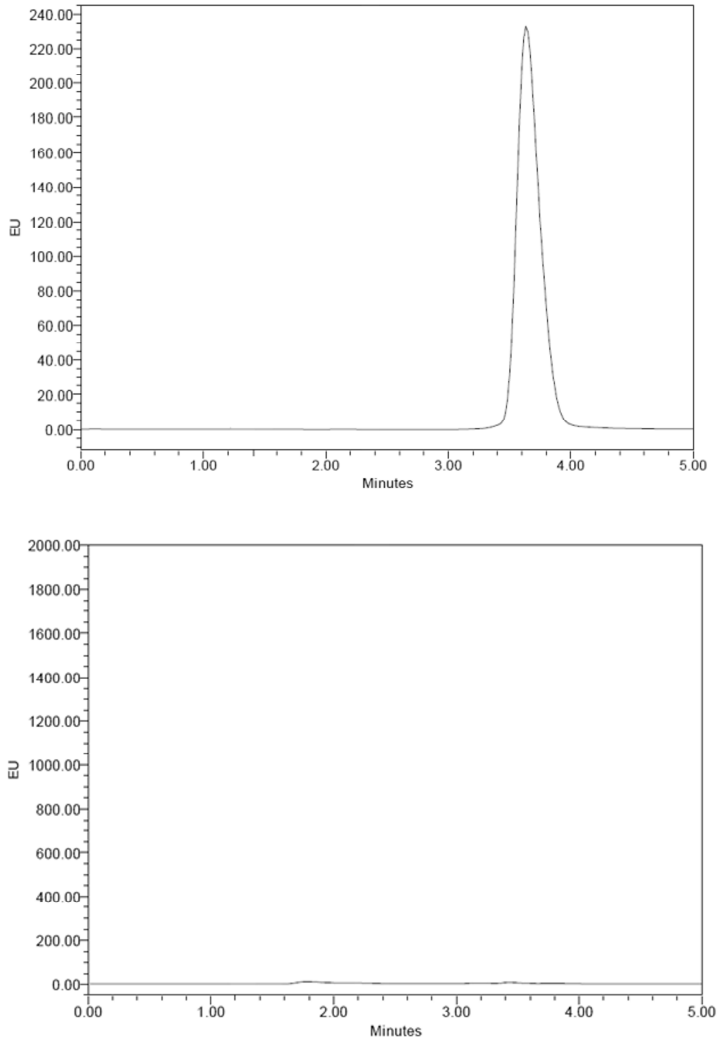
3.2.7. Range

The working range of the method, defined as the range that possessed the required versatility, linearity, accuracy and precision, was between the LOQ and 50 µg/ml. Therefore, samples containing these concentration levels may be assayed by the proposed HPLC method.

3.2.8. Specificity

The specificity of the method was evaluated by comparing the chromatograms of both curcumin standards and samples to those of potential interfering formulation components. For this study, blank nanoparticles (without curcumin) were prepared as described in section 2.6.1, and the supernatant obtained after their ultracentrifugation was diluted with ethanol and analyzed by the described HPLC method. The representative chromatogram of a curcumin sample (with curcumin in the supernatant) (Fig. 4A) showed a curcumin peak at approximately 3.7 min, which was in agreement with that obtained for the curcumin standard (Fig. 1). No peaks at this retention time were observed in the chromatogram of the supernatant from the blank nanoparticles (Fig. 4B), which indicates there was no interference in the quantitative determination of curcumin from the formulation components. Tests were also performed under visible light (stress condition). The standards were exposed to visible light for 24 h to identify any occurrences of possible interfering peaks from curcumin degradation. The results showed no alterations in the curcumin retention time; however, a decrease in curcumin recovery caused by photodegradation was observed. The percent recoveries of curcumin from the standard solutions exposed to visible light are presented in Table 5. Also, no peaks for curcumin degradation products were observed. This method can be regarded as specific because no potential interfering peak was observed.

Figure 4. Representative HPLC chromatograms of curcumin sample (curcumin in supernatant from nanoparticles) (A) and supernatant from blank nanoparticles (B). Conditions: mobile phase, ethanol:acetonitrile:water (80:10:10, v/v/v); flow rate, 0.8 mL/min; fluorescence detection wavelength, 365 nm (excitation) and 512 nm (emission); column temperature, 22ºC; injection volume, 20 µL.



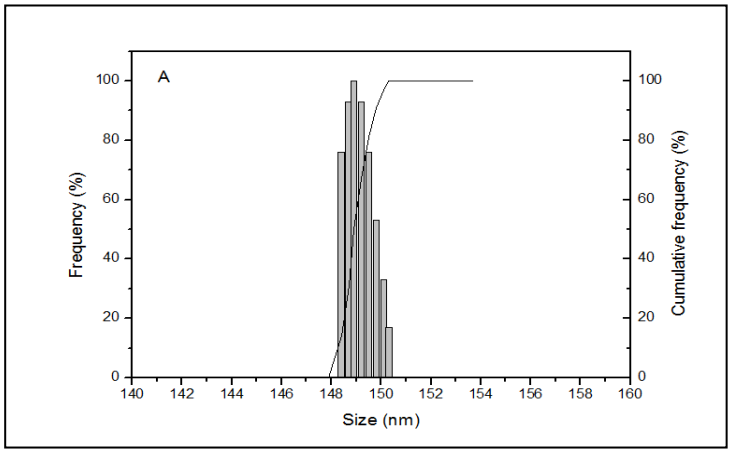
3.3. Method applicability

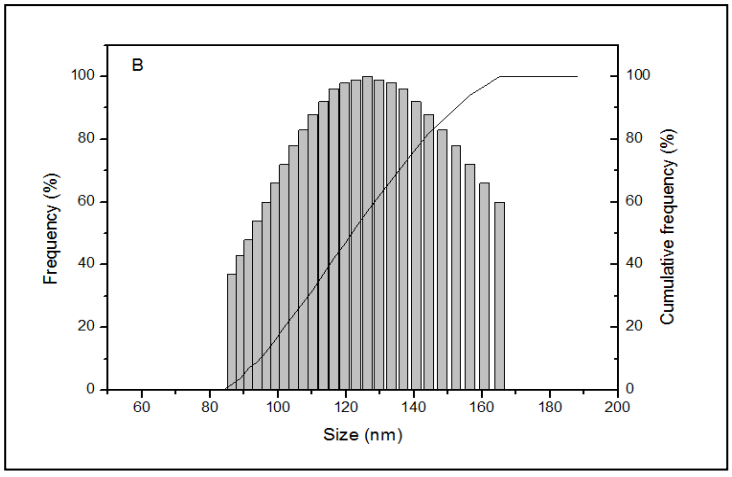
The proposed analytical method was used to evaluate the encapsulation efficiency of curcumin in PLGA and PLGA-PEG nanoparticles. An indirect method was used, which involved analyzing the supernatant containing the free curcumin, which was separated from the solid nanoparticles by ultracentrifugation. As shown in the

specificity test, no alterations or unusual peaks were observed in the chromatograms during the drug quantitation.

The single emulsion-solvent evaporation method was adequate for obtaining curcumin-loaded nanoparticles. The PLGA and PLGA-PEG nanoparticles possessed diameters of approximately 148.4 ± 5.4 and 122.6 ± 7.8 nm, respectively, and monomodal distribution profiles (Fig. 5A and 5B). The encapsulation efficiency of curcumin was 81.16 ± 1.21% (n = 3) for the PLGA nanoparticles and 70.83 ± 6.50% (n = 3) for the PLGA-PEG nanoparticles. These values are considered high and indicate that the PLGA and PLGA-PEG nanoparticles are potential colloidal carriers for curcumin that can be used to improve its oral bioavailability.

Figure 5. Size distribution profile of the nanoparticles containing curcumin determined by dynamic light scattering: A) PLGA nanoparticles; B) PLGA-PEG nanoparticles.





3.4. Considerations about the HPLC-fluorescence method developed and validated

Our objective was to develop a fast, simple and effective HPLC-fluorescence method to quantitatively analyze curcumin in polymeric nanoparticles. The literature describes mainly spectrophotometric methods for curcumin quantitation in nanoformulations [25-27, 36-38], but these methods are not so convenient as HPLC methods considering the sensitivity. HPLC coupled to UV-Vis detector has been used for curcumin detection and quantitation in nanoformulations, but it can be observed a restriction considering the run time of analysis. One example is the method proposed by Tsai *et al* (2011) [39] that used a mobile phase composed of acetonitrile:10 mM monosodium phosphate (pH 3.5) (40:60, v/v) at a flow-rate of 0.8 mL/min and detection wavelength was set at 425 nm for curcumin quantitation in nanoformulations. But in this method the run time for analysis was 27 min. Also has been observed that in the most studies using HPLC-UV/Vis detection the authors only cites the chromatographic parameters and does not give any information about the method validation and even the retention time of curcumin. Mulik *et al* (2009) [40] used an HPLC method with UV-Vis detection for the stability study of curcuminoids-loaded nanoparticles. The mobile phase consisted of 0.1% (v/v) trifuroacetic acid and acetonitrile (1:1, v/v) (adjusted to pH 3.0 with an ammonia) at flow rate of 1.5 mL. Mohanty and Sahoo (2010) [29] used acetonitrile and citric buffer (60:40, v/v) at flow rate of 1 mL/min for curcumin quantitation in the nanoformulation. Yen *et al* (2010) [41] cited the following parameters for curcumin detection in nanoparticles: mobile phase consisted of acetic acid, 10 mM phosphate buffer, and acetonitrile (1:50:50, v/v/v), and phosphoric acid was also included to adjust the pH value to 2.5. The flow rate was set at 0.8 mL/min. The main difference of this method is that the wavelength of the UV detector was operated at 250 nm and not in the maximun wavelength of curcumin absorption that is

in visible region (420-450 nm). In all these works the authors did not provide any information about the curcumin retention time or validation results.

Fluorescence-HPLC methods also has been described in the literature for the analysis of curcumin mainly in turmeric. Generally these methods are time-consuming and/or can use gradient elution for the separation of curcuminoids, thus, can not be used for our objectives. One example is the method of Zhang *et al* (2009) [18], which used a mixture of acetate buffer (pH 4.0) and acetonitrile (57:43, v/v) as mobile phase that proved to be very sensitive; however, the curcumin (from turmeric) was only detected after 25 min.

The HPLC-fluorescence method developed and validated in this work represents an alternative to spectrophotomeric and HPLC-UV/Vis methods for analysis of curcumin in nanoparticles. The retention time of curcumin was short allowing the analysis of a large number of samples in a short period of time and with reduced costs with solvents and absence of buffer in composition of mobile phase. The method fulfill all the requirements to be considered reliable and feasible and could applied not only for the determination of encapsulation efficiency of curcumin in nanoparticles, but for others assays involving curcumin-loaded nanoparticles, such as in vitro curcumin release profile and stability studies.

Conclusion

A fast, simple and reliable reversed-phase HPLC method using fluorescence detection for determining the encapsulation efficiency of curcumin in PLGA and PLGA-PEG nanoparticles has been developed and validated according to the ICH guidelines. This method fulfilled the requirements to be considered a reliable and feasible method, which includes specificity, linearity, precision, accuracy, robustness,

LOD and LOQ. This method was found to be suitable for the determination of curcumin encapsulation efficiency in polymeric nanoparticles.

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CAPÍTULO II

ARTIGO CIENTÍFICO

PHARMACOKINETICS OF CURCUMIN FROM PLGA AND PLGA-PEG BLEND NANOPARTICLES AFTER ORAL ADMINISTRATION IN RATS

PHARMACOKINETICS OF CURCUMIN FROM PLGA AND PLGA-PEG BLEND NANOPARTICLES AFTER ORAL ADMINISTRATION IN RATS

Abstract

The aim of this study was to assess the potential of nanoparticles to improve the pharmacokinetics of curcumin, with a primary goal of enhancing its bioavailability. Poly (lactide-co-glycolide) (PLGA) and PLGA-polyethylene glycol (PEG) (PLGA-PEG) nanoparticles containing curcumin were obtained by a single-emulsion solvent-evaporation technique, resulting in particles sizes smaller than 200 nm. The encapsulation efficiency was over 70% for both formulations. The in vitro release study showed that curcumin was released more slowly from the PLGA nanoparticles than from the PLGA-PEG nanoparticles. After oral administration in rats, both nanoparticles formulations were able to sustain curcumin delivery over time, but greater efficiency was obtained with the PLGA–PEG nanoparticles, which showed better results in all of the pharmacokinetic parameters analyzed. The PLGA and PLGA–PEG nanoparticles increased the curcumin mean half-life by approximately 4 and 6 h, respectively, and the C_{max} of curcumin was increased 2.9- and 7.4-fold, respectively. The distribution and metabolism of curcumin was decreased when it was carried by nanoparticles, particularly PLGA-PEG nanoparticles. The bioavailability of curcumin-loaded PLGA-PEG nanoparticles was 3.5-fold greater than that of curcumin from PLGA nanoparticles. Compared to a curcumin aqueous suspension, the PLGA and PLGA-PEG nanoparticles increased the curcumin bioavailability by 15.6- and 55.4-fold, respectively. These results suggest that PLGA and, in particular, PLGA-PEG blend nanoparticles are potential carriers for the oral delivery of curcumin.

Keywords: curcumin, LC-MS/MS, bioavailability, nanoparticles

1. Introduction

Curcumin is a polyphenol compound extracted from the root of *Curcuma longa* Linn, commonly known as turmeric. Chemically, curcumin is 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, commonly called diferuloylmethane. Curcumin has been used for centuries in Chinese and Indian medicine to treat a variety of disorders (Goel et al. 2008). Several studies have shown that curcumin presents antiinflammatory (Brouet and Oshima, 1995; Gao et al. 2004), antioxidant (Lakshmanan et al., 2011; Masuda et al. 1999) and antimicrobial activities (Mazumder et al. 1995), but the most important effect is its potential use against cancer due to its ability to suppresses the proliferation of a wide variety of tumor cells (Aggarwal et al. 2003; Aggarwal and Harikumar, 2009; Shao et al. 2002). Curcumin is able to modulate numerous targets including transcription factors, receptors, kinases, cytokines, enzymes and growth factors, affecting numerous molecular and biochemical cascades (Anand et al. 2008; Goel et al. 2008).

The great pharmacological potential of curcumin and its therapeutic applications are restricted because curcumin presents some drawbacks, including low aqueous solubility at acidic and physiological pH conditions, rapid hydrolysis in alkaline media and photo instability, inherent to its chemical composition. The hydrophobic character of curcumin results in pharmacokinetic restrictions such as low absorption and bioavailability by an oral route, extensive metabolism and rapid elimination (Anand et al. 2007; Sharma et al. 2007). The main strategies used to overcome the

physicochemical limitations of curcumin and to increase its bioavailability are based on loading the compound in nanocarriers, such as liposomes (Kunwar et al. 2006), cyclodextrins (Yadav et al. 2010), solid lipids (Kakkar et al. 2010; Tiyaboonchai et al. 2007) and polymeric nanoparticles (Anand et al. 2010; Shaikh et al. 2009; Tsai et al. 2011).

Biodegradable polymeric nanoparticles are extensively used to improve the therapeutic properties of various drugs and bioactive compounds. Nanoencapsulation protects the molecules from premature degradation, improves their solubility, and promotes controlled drug release and drug targeting. Nanoparticles present a low risk of toxicity, and drug efficacy, specificity, tolerability and the therapeutic index are enhanced with their use. The pharmacokinetic parameters of the drug are modified when it is loaded in nanoparticles. Specifically, there are improvements in absorption, bioavailability, and plasma circulation time, with a reduction of clearance, consequently increasing the drug's mean half-life (Frank et al. 2008; Khalil and Mainardes, 2009; Khalil et al. 2011; Kumari et al. 2010; Leroux et al. 1996). The physicochemical parameters of nanoparticles such as particle size, surface modification charge, and hydrophobicity influence the drug's pharmacokinetics, impacting the drug's bioavailability and biodistribution in particular. It is well documented that nanoparticles presenting a hydrophobic surface, like the surface of poly (lactide-co-glycolide) (PLGA) nanoparticles, present short circulation times because they are rapidly recognized by antigen-presenting cells and cleared by cells of the mononuclear phagocytic system (MPS). The process of opsonization is one of the most important biological barriers to nanoparticle-based controlled drug delivery. Coating the surface of nanoparticles with hydrophilic polymers, such as polyethylene glycol (PEG), polysorbates or poloxamers, sterically stabilizes the particles, i.e., they are able to repel

the absorption of opsonin proteins via steric repulsion forces, and thus, the particles become “invisible” to MPS cells, increasing their plasmatic circulation time and resulting in an improvement in drug bioavailability and half-life. Also, longer plasmatic circulation times increase the probability of the nanoparticles reaching their target (Mainardes et al. 2009; Owens and Peppas, 2006, Stolnik and Illum, 1995; Torchilin and Trubetskoy, 1995).

Some recent works have demonstrated that PLGA nanoparticles are able to improve the bioavailability of curcumin after oral administration (Anand et al. 2010, Shaik et al. 2009, Xie et al. 2001). PLGA–PEG nanoparticles have been developed because of their great potential for having long circulation times. Also, the potential advantage provided by the hydrophilic character of PEG can improve the biocompatibility of the delivery system (Avgoustakis, 2004). However, to the best of our knowledge, there has not yet been a demonstrated report about the use of PLGA-PEG nanoparticles as carriers for curcumin. Thus, polymeric nanoparticles, especially long-circulating nanoparticles, were evaluated as potential carriers for curcumin oral delivery.

In this work, PLGA and PLGA-PEG blend nanoparticles were obtained for curcumin loading. An analytical method based on LC-MS/MS was developed and validated to quantify curcumin in rat plasma. The nanoparticles were orally administered at a single dose in rats, and the pharmacokinetic parameters were evaluated and compared with those of a curcumin aqueous suspension.

2. Materials and methods

2.1. Materials

Curcumin (code C1386), PLGA (Resomer RG 50:50 H; Mw 40-75 kDa, inherent viscosity 0.45-0.6 dl/g), PEG (10 kDa) and polyvinyl alcohol (PVA, 31 KDa, 88% hydrolyzed) were purchased from Sigma-Aldrich (USA). The internal standard, salbutamol (99%), was obtained from European Pharmacopeia. Methylene chloride and ethylene acetate were purchased from FMaia® (Brazil). Analytical HPLC-grade ethanol, acetonitrile, methanol and acetic acid were purchased from J.T. Baker (USA). All other solvents and chemicals were of analytical or HPLC grade.

2.2. Preparation of curcumin-loaded PLGA and PLGA-PEG blend nanoparticles

The nanoparticles were obtained by the single-emulsion solvent-evaporation technique, as previously described (Li et al. 2008). Briefly, curcumin (5 mg) and PLGA (50 mg) were dissolved in a mixture of ethylene acetate (1.5 mL) and methylene chloride (0.5 mL) with or without PEG (10 mg) at room temperature. This organic phase was rapidly poured into 10 mL of PVA aqueous solution (0.5 %, w/v) and emulsified by sonication for 5 min (35% of 500 W, Unique® Ultrasonic Mixing, Brazil), resulting in an oil-in-water (o/w) emulsion. Next, the organic solvent was rapidly eliminated by evaporation under vacuum (20 min) at 37°C. The particles were then recovered by centrifugation (19,975 x g, 30 min, 4°C, Cientec CT-15000R centrifuge, Brazil) and washed twice with water to remove the surfactant. The nanoparticles were dispersed in the cryoprotectant sucrose (5%, w/v), and the resulting nanosuspension was cooled to −18ºC and freeze-dried (Terroni®, Brazil).

2.3. Particle size

The mean particle size and polydispersity index were determined by dynamic light scattering (BIC 90 plus - Brookhaven Instruments Corp., USA). The analyses were performed at a scattering angle of 90° and a temperature of 25°C. For each sample, the mean particle diameter, polydispersity and standard deviation of ten measurements were calculated.

2.4. Drug entrapment efficiency

A Waters 2695 Alliance HPLC system (Milford, MA, USA) was used for curcumin quantitation. The chromatographic analysis was performed in isocratic mode using a reverse phase C18 column (VertiSep GES,Vertical Chromatography Co, Ltd) with a 5 µm particle size, 4.6 mm internal diameter and 250 mm length. The mobile phase consisted of a mixture of ethanol, acetonitrile and water (80:10:10, v/v/v), pumped at a flow rate of 0.8 mL/min. The sample injection volume was 20 µL, and the fluorescence detector was operated at an excitation wavelength of 365 nm and an emission wavelength of 512 nm.

The amount of curcumin incorporated into the nanoparticles was determined indirectly Àrbos et al. 2002; das Neves et al. 2010). The supernatant, containing free curcumin separated from the solid nanoparticles by ultracentrifugation, was analyzed as described in section 2.2. After the appropriate dilutions in ethanol, 20 µL of the sample was injected into the HPLC system, and the drug concentration in the supernatant was obtained by comparison with a previously constructed analytical curve. Before

injection, all of the solutions were filtered through a membrane filter (0.45-μm pore size, Millipore). The amount of curcumin entrapped in the nanoparticles was obtained by subtracting the quantity of drug in the supernatant from the total amount used for the preparation. The analyses were performed in triplicate.

2.5. In vitro release profile

The release of curcumin from nanoparticles was conducted by dispersing the nanoparticles (containing 1.5 mg of curcumin) in 12 mL of phosphate saline buffer (PBS - 0.01 M, pH 7.4), and the solution was divided among eight Eppendorf tubes. The experiment was performed in triplicate. The tubes were kept in a shaker at 37ºC at 150 rpm. At predetermined time intervals, the suspension was centrifuged at 14,000 rpm for 15 min to separate the released curcumin from the nanoparticles (Das et al. 2010; Mohanty and Sahoo, 2010). The released curcumin was diluted in ethanol, and 20 µL of this solution was injected in the HPLC to determine the amount of curcumin released at different time intervals.

2.6. Chromatograph system and conditions for curcumin quantitation in plasma

The LC-MS/MS analysis was conducted in positive ion ESI mode on a Quattro Micro API – Waters hexapole mass spectrometer connected to a liquid chromatograph (Waters Alliance). The analysis was conducted on a Phenomenex Luna C18(2) 100A column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of methanol and 0.05% acetic acid solution (80:20, v/v) at a flow rate of 1.0 mL/min. The sheath gas and

auxiliary gas were tuned to give an optimum response as necessary. The needle voltage was 4.5 kV. Argon was used as the collision gas at collision energy of 15 eV (curcumin) and 18 eV (salbutamol). The collision energy was individually tuned for each analyte to obtain an optimum value. The analytes were quantified using multiple-reaction monitoring (MRM). The ion transitions *m/z* 369.3→285.0 and *m/z* 240.0→147.7 were used for the determination of curcumin and salbutamol, respectively. The autosampler cooler was maintained at 4ºC.

2.7. Preparation of curcumin standards and quality control

A concentrated stock standard of curcumin and salbutamol (IS, internal standard) were prepared by dissolving 4 mg of each in 20 mL of methanol, generating a 200 µg/mL stock solution. Eight point calibration curves were prepared by a serial dilution of the curcumin stock solution (200 µg/mL in methanol) in the range of 25–2500 ng/mL. The calibration curve was prepared daily using 2.45 mL of blank plasma with 50 µL of the appropriate working solution, resulting in concentrations of 0.5, 10, 25, 50, 100, 200, 350 and 500 ng/mL. Three quality controls (QC) were prepared at 1.5 (low concentration), 225 (medium concentration) and 450 ng/mL (high concentration).

2.8. Plasma sample preparation

To 100 µL of rat plasma sample (or a calibration standard or a QC sample) were added 100 µL of internal standard (IS) salbutamol and 100 µL of 0.5 M sodium hydroxide (to assist in the extraction of curcumin). The mixture was vortexed for 1 min. After the curcumin was extracted with 1300 µL of ethyl acetate (liquid-liquid extraction), followed by agitation in a shaker (10 min), it was centrifuged at 4°C for 10

min at 10,000 rpm. The supernatant was evaporated using nitrogen gas in a sample concentrator. The obtained residue was reconstituted with the mobile phase and vortexed for 20 s. The samples were subjected to LC-MS/MS analysis.

2.9. Bioanalytical method validation

The specificity of the method was investigated by comparing the chromatogram of blank plasma with the blank plasma spiked with standard solutions and with the samples collected from rats after curcumin administration.

The linearity of the bioanalytical assay was evaluated with a total of eight calibration standards over the concentration range of 0.5-500 ng/mL. Calibration curves were constructed by linear least-squares regression analysis by plotting the peak-area ratios versus the drug concentrations.

The limit of quantitation (LOQ) was defined as the lowest concentration of the analyte in the calibration curve that could be detected with a variation of less than 15%.

The intra-day precision and accuracy were determined within one day by analyzing ten replicates of the QC samples at concentrations of 1.5, 225 and 450 ng/mL of curcumin. The inter-day precision and accuracy were determined on two separate occasions using replicates (n=10) of each concentration used. The intra- and inter-day precision was defined as the relative standard deviation (RSD). The accuracy was expressed using the following formula:

[measured concentration/nominal spiked concentration]×100 eq. 1

The freeze–thaw stability of the plasma samples was evaluated by exposing QC samples at low and high concentrations to four freeze–thaw (−20ºC to room temperature) cycles before sample preparation. The stability of the samples in the

autosampler was evaluated by analyzing the extracted QC samples after being placed in the autosampler at 20ºC for 6 h, at which time the samples were analyzed. The long-term stability was verified by freezing (−20ºC) the QC samples for 250 days. Freshly processed standard samples were used to quantitate all of the QC samples. All of the stability QC samples were analyzed in quintuplicate.

2.10. Pharmacokinetic study

Male adult Wistar rats with a mean body weight of 200–300 g were fasted overnight prior to the experiments, with free access to water. The experimental protocols were approved by the Institutional Animal Ethics committee of the Universidade Estadual de Ponta Grossa, Brazil (Registration no. 06/2010). The rats were divided randomly into three groups (n=5). The formulations (curcumin aqueous suspension, the curcumin-loaded PLGA nanoparticles and the curcumin-loaded PLGA-PEG blend nanoparticles) were administered by oral gavage at a single dose of 50 mg/kg. The nanoparticles were dispersed in ultrapure water.

Blood samples (500 µL) were withdrawn from the tail vein into heparinized microtubes at the following times: 0.25, 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h after dosing. The blood samples were centrifuged at 3020×g for 10 min. The supernatant was collected, transferred to tightly sealed plastic tubes and stored at −20ºC until analysis by LC-MS/MS.

2.11. Data analysis and statistics

All of the in vitro results were expressed as the mean ± standard deviation (SD)

of three replicates. The in vivo results were presented as the mean ± SD of five replicates. Pharmacokinetic parameters were estimated using the model-independent method. The terminal elimination rate constant (K_e) was estimated by a linear regression analysis of the terminal portion of the log-linear blood concentration–time profile of curcumin. The terminal elimination half-life ($t_{1/2}$) was calculated from K_e using the formula $t_{1/2} = 0.693/K_e$. The maximum observed plasma concentration (C_{max}) and the time taken to reach it (T_{max}) were obtained from the curve plotting curcumin concentration vs. time. The area under each drug concentration time curve (AUC, $ng.ml^{-1}.h$) to the last data point was calculated by the linear trapezoidal rule and extrapolated to time infinity by the addition of C_{Last}/K_e, where C_{Last} is the concentration of the last measured plasma sample. The apparent body clearance (Cl) was calculated using the equation Cl = dose/AUC. The apparent volume of distribution (V_d) was calculated by the equation V_d = dose/K_e.AUC. Statistical analysis of the data was performed via one-way analysis of variance (ANOVA). The level of confidence was 95%.

3. Results and discussion

3.1. Preparation of the curcumin-loaded PLGA and PLGA-PEG blend nanoparticles

The nanoparticles containing curcumin were successfully obtained by the single-emulsion solvent-evaporation method. The choice of a nanoencapsulation method is based on the drug solubility, and because curcumin is hydrophobic, the method of reducing the size of the emulsion oil-in-water (o/w) is adequate for this molecule. The

ultrasonication was crucial to reduce the emulsion globules to nanometer size. Table 1 illustrates the size characteristics of the obtained nanoparticles. Both formulations, the PLGA and PLGA-PEG blend nanoparticles containing curcumin, presented monodisperse profiles and narrow size distributions. The presence of PEG did not influence the mean particle size, approximately 147 and 168 nm, respectively, but the polydispersity index was superior ($p<0.05$) than that obtained from PLGA nanoparticles, while maintaining a monomodal profile.

The encapsulation efficiency was determined indirectly, and the results are presented in Table 1. The method used for nanoencapsulation resulted in significant enclosure of curcumin, and the process was found to be highly reproducible. The PEG did not influence the encapsulation, as the values between batches were not significantly different ($p>0.05$). Indeed, the hydrophilic character of PEG kept it directed to aqueous phase, while the hydrophobic core of PLGA can entrap the hydrophobic drugs.

Table 1. Curcumin_Nanoparticle characteristics

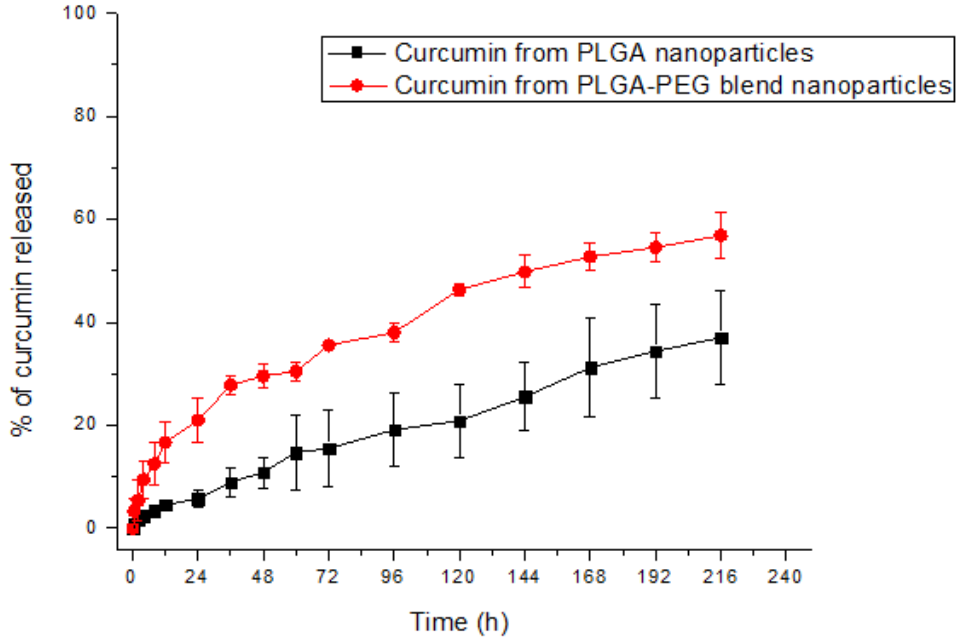
Polymer	Particle size (nm)[a]	Polydispersity index[a]	Size distribution[a]	Encapsulation efficiency (%)[b]
PLGA	161.93 ± 6.7	0.042 ± 0.01	146.2 - 200.7 nm (100%)	77.07 ± 8.16
PLGA-PEG	152.37 ± 4.5	0.077 ± 0.01	109.9 - 185.1 nm (100%)	73.22 ± 9.77

Values reported as mean ± S.D. [a](n = 3), [b](n = 30).

3.2. In vitro release profile

The in vitro release of curcumin from nanoparticles was studied simulating physiological conditions (37ºC, PBS buffer pH 7.4). The in vitro release profiles of curcumin were obtained by graphing the cumulative percentage of the drug release with respect to the amount of curcumin encapsulated as a function of time. The experiment was performed over nine days. Figure 1 illustrates the curcumin release profiles from the PLGA and PLGA-PEG blend nanoparticles and indicates that there was a pronounced time prolongation of the drug release. It is evident that the PEG influenced the curcumin release because there was a great difference between the release profiles of curcumin from nanoparticles of different compositions. A biphasic release pattern of curcumin was observed from the PLGA-PEG nanoparticles, where the initial 24 h period released approximately 21% of drug, followed by a sustained release to a total of 56.9% over nine days of observation. This initial burst release may be due to drug desorption from the particle surface, and the sustained release can be characterized by the drug diffusion through the polymeric matrix and subsequent diffusion/erosion of the polymeric matrix. The curcumin release from the PLGA nanoparticles was slower than that from the PLGA-PEG blend nanoparticles ($p<0.05$), and the release was progressive because it did not have a biphasic profile. After 24 h, only 5.8% of the drug had been released, and in nine days, 37% of the curcumin had been released.

Figure 1. In vitro release profile of curcumin from PLGA and PLGA-PEG blend nanoparticles in PBS (0.01 M, pH 7.4) at 37ºC. Values reported as the mean ± SD (n = 3).



In general, it can be affirmed that the drug release depends upon the solubility, diffusion and biodegradation of the matrix materials. Thus, the drug release mechanisms can be modified by the choice of polymer matrices. Drug release also depends upon the loading efficiency of the drug and the size of the nanoparticles (Kumari et al. 2010). In our case, because the size and curcumin loading are similar for the PLGA and PLGA-PEG nanoparticles, we can attest that the difference between the amount of drug released from the two nanoparticles is due to the presence of PEG, as it has a hydrophilic character and can enhance the water permeation and drug diffusion through the polymeric matrix (Perrachia et al. 2007). It is possible that curcumin strongly interacts with the PLGA matrix, thus retarding the release capability, and that the PEG

can increase the wettability of the polymeric surface and matrix, contributing to the increase in drug release. The results show that the PLA-PEG nanoparticles released more curcumin than the PLGA nanoparticles (by approximately 1.5-fold; $p<0.05$) during the period analyzed.

3.3. Bioanalytical method development and validation

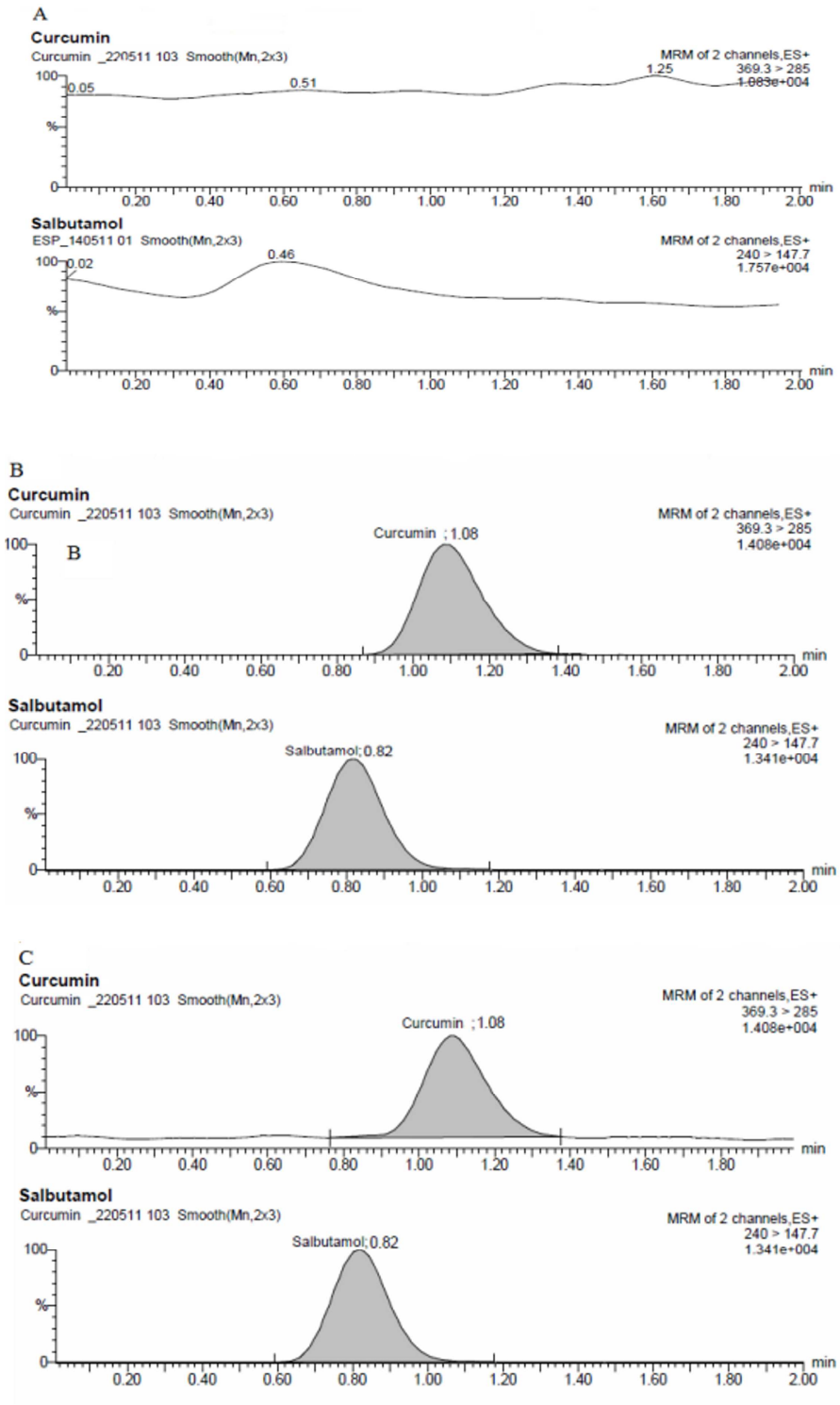
An LC-MS/MS method for the determination and quantitation of curcumin in rat plasma has been developed and validated. Initial runs were conducted with mobile phases composed of acetonitrile:0.2% formic acid solution (40:60, v/v), acetonitrile:1% formic acid solution (70:30, v/v), acetonitrile:0.1% acetic acid solution (70:30, v/v) and acetonitrile:0.005% acetic acid solution (70:30, v/v). In all of these combinations, the curcumin peak resulted in tailing, and the signal was slow.

Testing several ratios of methanol and acetic acid, the combination that resulted in a sharp peak with a sufficient response area was using methanol and 0.05% acetic acid solution (80:20, v/v) as the mobile phase at a flow rate of 1 mL/min. The retention times were approximately 1.08 min and 0.82 min for curcumin and IS, respectively, and the total run time was 2 min.

The specificity of the method was evaluated by comparing the chromatograms of curcumin in plasma (standard and sample) and those of potentially interfering plasma components. Representative chromatograms are shown in Fig. 2, including a blank plasma sample (Fig. 2A), plasma curcumin and salbutamol standard (Fig. 2B) and a plasma sample obtained 30 min after the oral administration of 50 mg/kg of curcumin-loaded PLGA nanoparticles (Fig. 2C). The resulting chromatograms show the assay specificity, as there were no endogenous plasma components eluted at the retention time

of curcumin or IS.

Figure 2. Representative chromatograms of (A) a blank plasma sample, (B) a curcumin standard, and (C) a curcumin sample. The upper peak of the figure is representative of curcumin, while that the lower are indicative of the peak of the internal standard.



The method was validated over a wide concentration range, and the results were directly obtained and extrapolated on the calibration curve. The calibration lines were shown to be linear from 0.5 to 500 ng/mL (r^2=0.9941). The method was sensitive, and the LOQ was low (0.5 ng/ml). Other LC-MS/MS methods described in the literature for curcumin determination in plasma showed a LOQ of 10 ng/mL (Kakkar et al., 2010) and 2.5 ng/mL (Yang et al., 2007).

Table 2 shows a summary of intra- and inter-day precision and accuracy for curcumin detection in rat plasma. The intra-day accuracy of curcumin for rat plasma samples was 101.72–110.54% for QC samples with a RSD of less than 6.70%. The inter-day accuracy of curcumin for rat plasma samples ranged from 96.34% to 107.94% for QC samples with an RSD of less than 4.34%. These results were within the limits established by the FDA guidelines for the validation of bioanalytical methods (2011).

Table 2. Intra-day and inter-day precision and accuracy of curcumin in rat plasma (n = 10).

Nominal concentration (ng/mL)	Measured Concentration (ng/mL)	R.S.D. (%)	Accuracy (%)
Intra-day[a]			
1.5	1.66 ± 0.04	2.33	110.54
225	224.66 ± 8.00	3.56	99.85
450	457.76 ± 30.66	6.70	101.72
Inter-day[b]			
1.5	1.62 ± 0.05	3.39	107.94
225	216.77 ± 9.41	4.34	96.34
450	436.59 ± 18.74	4.29	97.02

[a] The analyses were performed in the same day; R.S.D.=relative standard deviation

[b] The analyses were performed in two different days within one month;

Table 3 lists the data from the stability tests. No significant loss of curcumin (≤ 1.4%) was observed after storage of the plasma at room temperature on the bench top for at least 6 h. The plasma samples were stable over at least four freeze/thaw cycles and were stable at −20ºC for at least 250 days, with no significant loss of curcumin (≤1.8%). These results suggested that the plasma samples could be stored at -20ºC for long periods, could be thawed and refrozen and could be maintained at room temperature for 6 h without compromising the integrity and accuracy of the samples.

Table 3. Stability of curcumin in rat plasma.

Sample condition	Curcumin nominal concentration 1.5 ng/mL			450 ng/mL		
	Concentration measured (ng/mL)[a]	R.S.D (%)[b]	Accuracy (%)	Concentration measured (ng/mL)[a]	R.S.D (%)[b]	Accuracy (%)
6h at room temperature	1.62 ± 0.1	1.42	108.31	474.50 ± 13.36	0.47	105.44
Freeze-thaw four cycles	1.63 ± 0.04	0.7	109.00	481.07 ± 18.52	0.56	106.9
250 days at -20ºC	1.6 ± 0.05	1.1	107.25	483.99 ± 5.6	1.8	107.55

[a] Values reported as mean ± S.D. (n=5)

[b] Relative standard deviation, calculated comparing with CQ freshly prepared.

The sensitivity of this LC-MS/MS method offered advantages over other LC-MS/MS methods and conventional HPLC-UV methods applied for curcumin pharmacokinetics. Also, the excellent specificity and short run time analysis make this method efficient for curcumin pharmacokinetic applications.

3.4. Pharmacokinetic study

The curcumin plasma concentration–time profiles after oral administrations of 50 mg/kg of curcumin in different formulations, curcumin aqueous suspension,

curcumin-loaded PLGA nanoparticles and curcumin-loaded PLGA-PEG blend nanoparticles, are expressed in Fig. 3. Table 4 summarizes the relevant pharmacokinetic parameters.

Figure 3. Comparison of in vivo plasma concentration vs. time profiles of the different curcumin formulations. All values reported are the mean ± SD (n = 5).

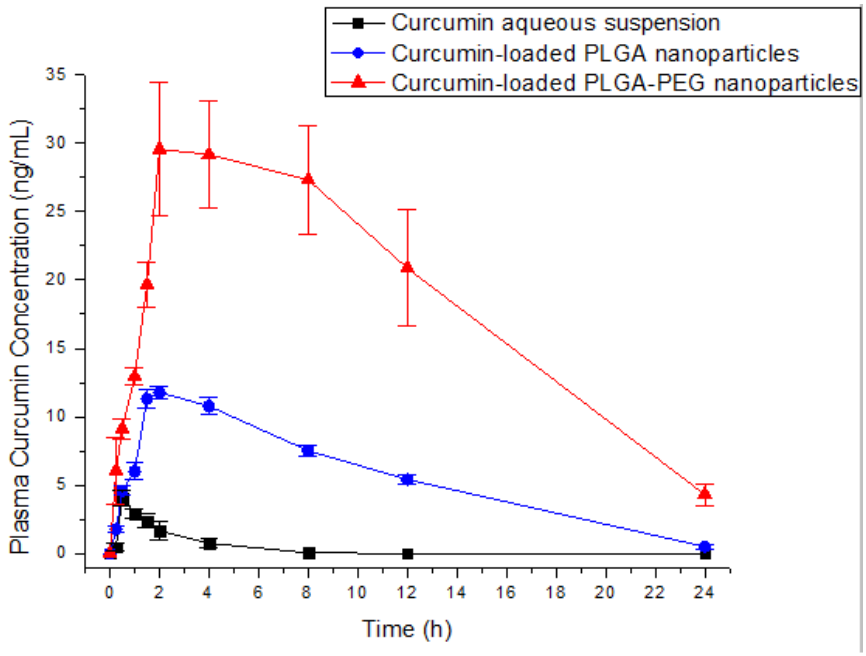


Table 4. Pharmacokinetic parameters of curcumin following single oral administration of curcumin aqueous suspension, curcumin-loaded PLGA nanoparticles and curcumin-loaded PLGA-PEG nanoparticles, in rats (n=5).

Pharmacokinetic Parameters	Formulations		
	Curcumin Aqueous suspension	Curcumin_PLGA nanoparticles	Curcumin_PLGA-PEG nanoparticles
Dose (mg/Kg)	50	50	50
AUC_{0-t} (h.ng/mL)	8.695 ± 1.872	134.251 ± 3.446*#	447.80 ± 64.028*
AUC_{0-inf} (h.ng/mL)	8.762 ± 1.862	137.162 ± 3.694*#	485.941 ± 54.663*
C_{max} (ng/mL)	4.066 ± 0.564	11.783 ± 0.454*#	29.778 ± 4.632*
T_{max} (h)	0.5	2*#	3*
K_e (L/h)	0.631 ± 0.072	0.178 ± 0.021*#	0.119 ± 0.021*
$t_{1/2}$ (h)	1.109 ± 0.124	3.929 ± 0.451*#	5.979 ± 1.126*
V_d (L/Kg)	9432.536 ± 2511.617	2073.664 ± 352.612*#	900.544 ± 225.772*
Cl (L/h/Kg)	5859.700 ± 1399.927	365.191 ± 37.351*#	103.679 ± 10.903*

Values reported as mean ± S.D. (n = 5). *AUC*: area under the plasma concentration-time curve. C_{max}: Peak concentration. T_{max}: Time to reach peak concentration. K_e: constant of elimination. $t_{1/2}$: mean half-life. V_d: apparent volume of distribution. Cl: clearance. * significantly different of free curcumin ($p<0.01$); # significantly different of curcumin from PLGA-PEG nanoparticles ($p<0.01$).

After the oral administration of a curcumin aqueous suspension, the drug was absorbed quickly, and a maximum plasma concentration (C_{max}) of approximately 4.066 ± 0.564 ng/mL was reached in 30 min. Thereafter, the curcumin plasma concentration decreased abruptly, as the drug was distributed and rapidly metabolized, resulting in a high K_e and short $t_{1/2}$, approximately 1.1 h. The curcumin was detected up to 8 h after administration. A sustained release of curcumin over 24 h was observed when it was carried by the two nanoparticles formulations. Thirty minutes after oral administration of the curcumin-loaded PLGA nanoparticles, the mean plasma concentration was 4.57 ± 0.35 ng/mL, and for the curcumin-loaded PLGA-PEG nanoparticles, the plasma concentration was 9.1 ± 0.95 ng/mL. There was a significant increase ($p<0.01$) in curcumin absorption from the PLGA-PEG nanoparticles in the first 30 min compared to free curcumin and curcumin from the PLGA nanoparticles. The curcumin concentration increased to 11.783 ± 0.454 ng/mL, C_{max}, after 2 h (T_{max}), and to 29.778 ± 4.632 ng/mL, C_{max}, after 3h (T_{max}), with the PLGA and PLGA-PEG nanoparticles, respectively. Compared to free curcumin, the C_{max} of curcumin from PLGA nanoparticles and PLGA-PEG nanoparticles was increased 2.9- and 7.4-fold, respectively. The increase in C_{max} indicates that the nanoparticles were effective in increasing drug absorption, and the delayed T_{max} demonstrates an obvious sustained release of curcumin. The distribution and metabolism of curcumin was decreased when it was carried by nanoparticles. The clearance of curcumin from the PLGA and PLGA-PEG nanoparticles was 16.3- and 61.6-fold lower than that of free curcumin, respectively. The PLGA-PEG nanoparticles and PLGA nanoparticles decreased the curcumin volume of distribution by 11.6- and 4.6-fold compared to free curcumin. Thus, the $t_{1/2}$ of curcumin from the PLGA increased to 4 h, and that from the PLGA-PEG nanoparticles was increased to 6 h, while for free curcumin, the $t_{1/2}$ was 1 h. There was a significant difference in the AUC_{0-inf}

between the curcumin aqueous suspension, the curcumin-PLGA nanoparticles and the curcumin-PLGA-PEG nanoparticles ($p < 0.01$). Between the two nanoparticle formulations, the curcumin from PLGA-PEG presented a relative bioavailability 3.5-fold superior to that of the curcumin from PLGA nanoparticles. Compared to the curcumin aqueous suspension, the PLGA and PLGA-PEG blend nanoparticles increased the curcumin bioavailability 15.6- and 55.4-fold, respectively.

Recently, Shaik and co-workers (2009) demonstrated that the PLGA nanoparticles were able to increase the curcumin bioavailability at least 9-fold when compared to curcumin administered with an absorption enhancer. Tsai et al. (2011) developed curcumin-loaded PLGA nanoparticles. When these particles were intravenously administrated in rats, a significant amount of curcumin was found mainly in the spleen due to phagocytic cell uptake in the reticuloendothelial system. Xie et al. (2011) showed that, after the oral administration of curcumin-loaded PLGA nanoparticles, the curcumin had a 5.6-fold higher relative bioavailability and had a longer half-life than that of native curcumin. In a similar work, Anand et al. (2010) demonstrated that curcumin-loaded PLGA nanoparticles have enhanced cellular uptake, increased bioactivity in vitro and superior bioavailability in vivo relative to free curcumin. To date, no study has compared the pharmacokinetics of curcumin loaded in PLGA and PLGA-PEG blend nanoparticles.

In our study, the significant difference in pharmacokinetic parameters, mainly bioavailability and half-life, between the free curcumin aqueous suspension and the curcumin-loaded PLGA and PLGA-PEG nanoparticle dispersions is explained by the inherent properties of colloidal nanoparticles in biological media, which prolong drug release and its in vivo trajectory. The in vitro release profile demonstrated that curcumin is released more rapidly from PLGA-PEG nanoparticles than from PLGA nanoparticles

and could be more quickly available in blood. It is well supported that pharmacokinetic parameters are altered depending upon the nanoparticles used, and their surface composition plays an important role in drug bioavailability (Hoffart et al. 2006; Ubrich et al. 2005). PEG is frequently used for the surface modification of various polymeric nanoparticles because it exhibits excellent biocompatibility and is able to improve the long-term systemic circulation of the nanoparticles. The PEG coating on the surface of the polymer reduces the interactions between the nanoparticles and the enzymes of the digestive fluids and increases the uptake of the drug in the blood stream and lymphatic tissue (Tobio et al. 2000). This effect can explain the difference between the curcumin pharmacokinetics from PLGA and PLGA-PEG blend nanoparticles. The ability of the PEG to make the coated nanoparticles invisible to recognition by macrophages and circulating monocytes gives the particles long circulation times. Consequently, the drug half-life and bioavailability are higher than those of a drug carried in uncoated nanoparticles (Owens and Peppas, 2006). We recently demonstrated that the presence of PEG in PLA nanoparticles containing zidovudine was essential in promoting the increase in drug bioavailability after intranasal administration in rats (Mainardes et al. 2010).

The increased curcumin bioavailability obtained with the nanoparticulate systems confirms the excellent abilities of the nanoparticles to modulate the physicochemical properties of drugs, resulting in improved pharmacokinetics profiles. Because the poor water solubility and low oral bioavailability of curcumin are the major drawbacks in its medicinal application, the studied nanoparticles represent an important initial step in the development of a medicine containing curcumin, using nanotechnology as a tool.

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CONCLUSÕES

- Obteve-se nanopartículas poliméricas de PLGA e de blendas de PLGA com PEG contendo curcumina através do método da emulsificação-evaporação do solvente;
- Ambas as formulações apresentaram perfil monomodal de distribuição de tamanho, na faixa de 85 a 165 nm;
- O método por CLAE com detecção por fluorescência desenvolvido para análise quantitativa de curcumina em nanopartículas mostrou ser específico, linear, preciso, exato e robusto;
- A eficiência de encapsulação da curcumina nas nanopartículas foi de aproximadamente 80% para as nanopartículas de PLGA e 70% para as nanopartículas de blendas de PLGA-PEG.
- O perfil de liberação *in vitro* da curcumina foi sustentado a partir das nanopartículas, sendo que ao longo de 9 dias, 37% e 56,9% da curcumina foi liberada a partir das nanopartículas de PLGA e PLGA-PEG, respectivamente.
- A metodologia desenvolvida e validada para quantificação da curcumina em plasma de rato mostrou-se específica, sensível, linear, precisa e exata, através da técnica de LC-MS/MS;
- A biodisponibilidade oral da curcumina foi melhorada através do seu carreamento por ambas as formulações de nanopartículas em relação à administração da suspensão aquosa de curcumina. A concentração máxima obtida *(C_{max})* da curcumina foi 2,9 e 7,4 vezes maior para as formulações de nanopartículas de PLGA e PLGA-PEG respectivamente, em relação a suspensão

aquosa de curcumina. O clearance foi 16,3 vezes menor para as nanopartículas de PLGA e 61,6 vezes menor para a formulação que continha o PEG. O volume de distribuição foi 4,6 vezes menor para a formulação de PLGA e 11,6 vezes menor para a formulação de PLGA- PEG. O tempo de meia vida ($T_{1/2}$) foi de 4h para a formulação de PLGA e 6h para a formulação de PLGA-PEG, indicando um maior tempo de circulação quando comparada com a curcumina em sua forma livre que foi de 1,1h. A biodisponibilidade da curcumina foi 15,6 vezes maior para as nanopartículas de PLGA e 55,4 vezes maior para a formulação de PLGA-PEG em relação a suspensão aquosa de curcumina livre, e quando comparada as duas formulações, as nanopartículas que continham PEG aumentaram 3,5 vezes a biodisponibilidade da curcumina.

CONSIDERAÇÕES FINAIS

As nanopartículas tem sido alvo de pesquisas no campo farmacêutico, representando avanços no desenvolvimento de sistemas de liberação de fármacos, minimização de efeitos colaterais, aumento da biodisponibilidade e direcionamento a sítios alvo. Neste trabalho, preparou-se nanopartículas de PLGA e PLGA-PEG contendo curcumina a fim de aumentar sua biodisponibilidade. A curcumina é um composto natural com várias propriedades terapêuticas, tais como antiinflamatória, antimicrobiana, antioxidante, destacando-se a atividade antitumoral. Porém suas propriedades farmacológicas estão restritas devido sua baixa solubilidade em soluções aquosas, sendo de difícil veiculação e administração, além de exibir alta taxa de degradação em pH neutro a básico e na presença de luz, em conjunto com um rápido metabolismo e rápida eliminação sistêmica, que contribuem para uma baixa biodisponibilidade. As nanopartículas de PLGA e PLGA-PEG preparadas pela técnica emulsão-evaporação do solvente, que após administração via oral a ratos *wistar*, demonstraram aumentar a biodisponibilidade da curcumina, em comparação com a curcumina administrada em sua forma livre, sendo mais eficaz quando preparadas adicionando o polímero polietilenoglicol (PEG), que através de suas propriedades hidrofílicas, formam uma barreira estérica sobre as nanopartículas, aumentando ainda mais a absorção do fármaco e tornando o tempo de circulação na corrente sanguínea maior.

Como a baixa solubilidade em água e baixa biodiponibilidade oral da curcumina são os maiores obstáculos para sua aplicação medicinal, o desenvolvimento de nanopartículas representa um importante passo inical para originar medicamentos contendo curcumina, utilizando a nanotecnologia como ferramenta.

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