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DESENVOLVIMENTO DE MÉTODOS ANALÍTICOS RÁPIDOS E DE BAIXO CUSTO UTILIZANDO CICLODEXTRINAS PARA O CONTROLE DE QUALIDADE DE FÁRMACOS

PABYTON GONÇALVES CADENA

RECIFE
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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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PABYTON GONÇALVES CADENA

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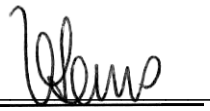

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Tudo tem o seu tempo determinado, e há tempo para todo o propósito debaixo do céu;
Há tempo de nascer, e tempo de morrer; tempo de plantar, e tempo de arrancar o que se plantou;
Tempo de matar, e tempo de curar; tempo de derrubar, e tempo de edificar;
Tempo de chorar, e tempo de rir; tempo de prantear, e tempo de dançar;
Tempo de espalhar pedras, e tempo de ajuntar pedras; tempo de abraçar, e tempo de afastar-se de abraçar;
Tempo de buscar, e tempo de perder; tempo de guardar, e tempo de lançar fora;
Tempo de rasgar, e tempo de coser; tempo de estar calado, e tempo de falar;
Tempo de amar, e tempo de odiar; tempo de guerra, e tempo de paz.
(Eclesiastes 3.1-8)

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LISTA DE ABREVIAÇÕES

Abreviação	*Português*	*English*
a	Coeficiente de Absorção molar	Molar absorption coefficient
A	Absorbância	Absorbance
A_0	Absorbância inicial	Initial absorbance
ANOVA	Análise de Variância	Analysis of variance
ANVISA	Agência Nacional de Vigilância Sanitária	Brazilian National Health Surveillance Agency
CA	Ácido Cólico	Cholic acid
CD(s)	Ciclodextrina	Cyclodextrin
CDCA	Ácido quenodesoxicólico	Chenodeoxycholic acid
CGTase	Ciclodextrina-glicosil-transferase	Cyclodextrin glucosyl transferase
C_L	Limite de detecção	Limit of detection
C_{LQ}	Limite de quantificação	Limit of quantitation
cm	Centímetro	Centimetre
CYP7a1	Colesterol 7α-hidroxilase	Cholesterol 7α-hydroxylase
CYP8b1	Esterol 12α-hidroxilase	Sterol 12α-hydroxylase
DCA	Ácido desoxicólico	Deoxycholic acid
EMEA	Agência européia de medicamentos	European medicine agency
FDA	Administração de medicamentos e alimentos dos Estados Unidos	U.S. Food and drug administration
FE/PHP	Fenolftaleína	Phenolphthalein
HPLC	Cromatografia líquida de alta eficiência	High performance liquid chromatography
K_c	Constante de equilíbrio	Equilibrium constant
Ki	Constante de inativação	Inactivation constant
L	Litro	Litre
LCA	Ácido litocólico	Lithocholic acid
M	Declive	Slope
MEKC	Cromatografia micelar eletrocinética	Micellar electrokinetic chromatography
mg	Miligrama	Milligram
mL	Mililitro	Millilitre
mmol	Milimol	Millimol
nm	Nanomol	Nanomol

PHP_0	Concentração inicial de Fenolftaleína	Initial phenolphthalein concentration
pKa	Cologaritmo da constante de dissociação ácida	Cologarithm of Acid dissociation constant
R	Constante dos gases ideais	Ideal gas constant
RGB	Vermelho, Verde, Azul	Red, Green, Blue
RMN	Ressonância magnética nuclear	Nuclear magnetic resonance
S_B	Desvio padrão	Standard deviation
SFC	Cromatografia de Fluído Supercrítico	Supercritical fluid chromatography
SLCA	Ácido sulfolitocólico	Sulpholithocholic acid
T	Temperatura absoluta	Absolute Temperature
t	Tempo	Time
$t_{½}$	Tempo de meia-vida	Half-life time
UDCA	Ácido ursodesoxicólico	Ursodeoxycholic acid
UV-Vis	Ultravioleta-Visível	UV-Visible
w/v	Peso/Volume	Weigh/Volume
%	Por cento	Per cent

Letras Gregas

Abreviação	*Português*	*English*
α-CD	Alfa-Ciclodextrina	Alpha-Cyclodexrin
β-CD	Beta-Ciclodextrina	Beta-Cyclodextrin
$\Delta G°$	Variação da energia livre padrão	Standard free energy change
$\Delta H°$	Variação da entalpia padrão	Standard enthalpy change
$\Delta S°$	Variação da entropia padrão	Standard entropy change
γ-CD	Gama-Ciclodextrina	Gamma- Cyclodextrin

RESUMO

O ácido desoxicólico (DCA) e o ácido ursodesoxicólico (UDCA) são ácidos biliares com inúmeras aplicações farmacêuticas, no entanto, as metodologias empregadas para determinação de suas concentrações são demoradas e onerosas. Neste trabalho foram usadas as ciclodextrinas, oligossacarídeos cíclicos, que têm a capacidade de formar complexos de inclusão no desenvolvimento de uma metodologia colorimétrica, rápida e de baixo custo. Este método é baseado numa reação de complexação competitiva das ciclodextrinas, que tendem a formar complexos de inclusão com moléculas, como os ácidos biliares e indicadores, estes últimos quando expulsos da cavidade da ciclodextrina produzem alterações colorimétricas que podem ser facilmente detectadas. Esta tecnologia de baixo custo poderá ser empregada no SUS. Dentre os indicadores testados a fenolftaleína (FE) mostrou a melhor interação com a β-ciclodextrina (β-CD) com um rendimento de inclusão superior a 95%. A melhor concentração de β-ciclodextrina para formar complexos de inclusão foi de $1,24x10^{-3} mol \cdot L^{-1}$ e $6,2x10^{-4} mol \cdot L^{-1}$ para o pH 9,5 e 10,5, respectivamente. A análise estatística dos resultados mostrou que o pH teve um efeito significativo sobre a determinação do DCA e que altas concentrações do complexo de inclusão β-CD-FE tiveram um efeito negativo significativo sobre a determinação do UDCA ($p<0.05$). No entanto, para complexos na proporção de $3,1x10^{-4}$:$7,75x10^{-5} mol \cdot L^{-1}$, a sensibilidade para a determinação do UDCA aumentou em 43,2%. O aumento da temperatura causou variações nas absorbâncias em todos os complexos de inclusão, entretanto, 20-30°C foi encontrado o melhor intervalo para a determinação dos ácidos biliares. A temperatura causou um efeito negativo na constante de equilíbrio resultando em valores altamente negativos de entalpia (β-CD-FE: -15,62±1,05, β-CD-DCA: -10,25±1,48 e β-CD-UDCA: -12,47±0,96 $kJ \cdot mol^{-1}$) e valores positivos de entropia (β-CD-FE: 25,56±3,35, β-CD-DCA: 50,31±4,74 e β-CD-UDCA: 43,42±3,12 $J \cdot mol^{-1}$). Em todos os casos, as reações de complexação competitiva foram espontâneas. Os complexos de inclusão foram estáveis por 12 dias tendo um tempo de meia vida de 68,71 dias para o DCA e 294,71 dias para a determinação do UDCA. O método foi validado pela metodologia da ANVISA e EMEA apresentando

limites de detecção e de quantificação de $3{,}94 \times 10^{-5}$ mol·L^{-1} e $1{,}31 \times 10^{-4}$ mol·L^{-1} para o DCA e $4{,}08 \times 10^{-5}$ mol·L^{-1} e $1{,}36 \times 10^{-4}$ mol·L^{-1} para o UDCA, respectivamente. Amostras dos ácidos biliares foram determinadas em formulações farmacêuticas com variação de 4% para o DCA e 1% para o UDCA. A reação de complexação competitiva também foi aplicada na construção de sensores químicos. Baseado nestes resultados, este método mostrou alta estabilidade, bom intervalo de temperatura, reação instantânea, baixo custo de reagentes e instrumentação.

Palavras-chave: Ciclodextrina, Complexos de inclusão, Indicadores ácido-base, ácido desoxicólico, ácido ursodesoxicólico.

ABSTRACT

The deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) are bile acids with numerous pharmaceutical applications; however, the methods used to determine their concentrations are slow and expensive. In this work were used cyclodextrins, cyclic oligosaccharides, which have the ability to form inclusion complexes with indicators useful to the development of fast and low cost colorimetric method. This method is based on the competitive complexation reaction of cyclodextrins, which tend to form inclusion complexes with molecules such as bile acids and indicators and when they are expelled from the cavity of cyclodextrin, produce colorimetric changes that can be easily detected. This low-cost technology could be employed in the SUS. Several pH colour indicators were tested, but the phenolphthalein (PHP) showed the best interaction with the β-cyclodextrin (β-CD) with an inclusion yield higher than 95%. The best concentrations of β-cyclodextrin to form inclusion complexes were $1.24x10^{-3}mol{\cdot}L^{-1}$ and $6.2x10^{-4}mol{\cdot}L^{-1}$ at the pH of 9.5 and 10.5, respectively. Statistical analysis of the results showed that pH had a significant effect on the DCA determination and that high β-CD-PHP inclusion complex concentrations had a significant negative effect on the UDCA determination ($p<0.05$). However, for the β-CD-PHP concentrations ratio of $3.1x10^{-4}$:$7.75x10^{-5}mol{\cdot}L^{-1}$, the sensitivity of UDCA determinations increases in 43.2%. The increase in temperature caused absorbance variation for all inclusion complexes, however, 20-30°C was the best range for the bile acids determination. The temperature had a negative effect on the equilibrium constant resulting in high negative values of enthalpy (β-CD-FE: -15.62±1.05, β-CD-DCA: -10.25±1.48 and β-CD-UDCA: -12.47±0.96 $kJ{\cdot}mol^{-1}$) and positive values of entropy (β-CD-FE: 25.56±3.35, β-CD-DCA: 50.31±4.74 and β-CD-UDCA: 43.42±3.12 $J{\cdot}mol^{-1}$), in all cases the competitive complexation reactions were spontaneous. The inclusion complexes were stable for 12 days with a half-life of 68.71 days to DCA determination and 294.71 days for the UDCA. The methods were validated by ANVISA and EMEA methodologies, showing limits of detection and quantification of $3.94x10^{-5}mol{\cdot}L^{-1}$ and $1.31x10^{-4}mol{\cdot}L^{-1}$ for the DCA, $4.08x10^{-5}mol{\cdot}L^{-1}$ and $1.36x10^{-4}mol.L^{-1}$ for UDCA, respectively. Samples of bile

acids were determined in pharmaceutical formulations with variation of 4% for the DCA and 1% for the UDCA. The competitive complexation reaction was also applied in the chemical sensors construction. Based on these results, this method showed high stability, good temperature range, instantaneous reaction, low cost for reagents and instrumentation.

Keywords: Cyclodextrin, Inclusion complexes, Acid-base indicators, deoxycholic acid, Ursodeoxycholic acid.

INTRODUÇÃO

1. Introdução

Os ácidos biliares são esteróides, surfactantes e agentes terapêuticos (MARPLES e STRETTON, 1987), dentre estes, encontra-se o ácido desoxicólico (DCA), um ácido biliar secundário (FERNANDEZ-LEYES *et al.*, 2007), formado a partir de formas conjugadas do ácido cólico e quenodesoxicólico (DEBRUYNE *et al.*, 2001). É bastante utilizado como agente colerético associado a outros componentes em terapias de disfunções do fígado (RODRIGUEZ *et al.*, 2000), formulações para cosméticos (VALENTA *et al.*, 1999), antibióticos antifúngicos (MARPLES e STRETTON, 1987), e formulações para dissolução de gorduras localizadas (ROTUNDA *et al.*, 2004). Esta última utilização está suspensa no Brasil desde outubro de 2005 (Resolução 2.682) devido à falta de estudos sobre a segurança e eficácia do tratamento (RUBINSTEIN, 2008).

Outro importante ácido biliar, o ácido ursodesoxicólico (UDCA), o menor constituinte da bile humana (SOLA *et al.*, 2006) e ácido biliar terciário, é o epímero 7β do ácido quenodesoxicólico (CDCA) sendo encontrado como um pó cristalino branco, inodoro e de sabor amargo (ORIENTI *et al.*, 1999). O UDCA é utilizado como medicamento para dissolver cálculos biliares, no trato de cirrose biliar, gastrite com refluxo da bile, tratamento de câncer coloretal e em várias outras condições colestáticas em adultos (SETCHELL *et al.*, 2005; TAY *et al.*, 2007).

Os métodos utilizados para a análise de ácidos biliares em solução ou em preparações farmacêuticas são geralmente muito onerosos, demandam grande tempo de preparação e apresentam dificuldade de extração dos componentes (KANG *et al.*, 2007; YOO, 2007; SOLA *et al.*, 2006; LIN *et al.*, 2003; MOMOSE *et al.*, 1998). Devido à grande aplicabilidade destes ácidos biliares, torna-se necessário o estudo de métodos alternativos rápidos, de baixo custo e com boa sensibilidade para a análise em formulações farmacêuticas.

Neste sentido, a utilização de ciclodextrinas (CDs) no desenvolvimento de novas metodologias analíticas tem se mostrado uma alternativa atraente, as CDs são oligossacarídeos cíclicos consistindo de 6 (α-ciclodextrina), 7 (β-ciclodextrina), 8 (γ-ciclodextrina) ou mais unidades de glicose unidas por ligações α-(1,4) (ABAY *et al.*,

2005; AL-SHERBINI, 2005; DE LEON-RODRIGUEZ e BASUIL-TOBIAS, 2005; CONSTANTIN *et al.*, 2004; DEL VALLE, 2004). Em solução aquosa possuem uma estrutura cilíndrica com exterior hidrofílico e interior hidrofóbico (AFKHAMI e KHALAFI, 2006) capaz de formar complexos de inclusão com uma grande variedade de moléculas dentro de sua cavidade hidrofóbica, tais como indicadores, drogas, pequenos ânions, ácidos carboxílicos, álcoois (TAWARAH e KHOURI, 2000). Estas moléculas ficam ligadas por forças intermoleculares não covalentes (AFKHAMI *et al.*, 2006; DEL VALLE, 2004).

A análise dos complexos de inclusão é realizada por uma grande variedade de técnicas: espectroscópicas (UV-Vis, fluorescência), HPLC, eletroquímica e calorimetria (ZHU *et al.*, 2007). As técnicas espectroscópicas utilizam CDs para aumentar a sensibilidade da técnica, no entanto, trabalhos utilizando análises UV-Vis com CDs são escassos. A utilização das ciclodextrinas em espectros de absorção UV-Vis tem como objetivos melhorar a solubilidade e estabilidade das substâncias colorimétricas, aumentar a seletividade e a sensibilidade da técnica (DE LEON-RODRIGUEZ e BASUIL-TOBIAS, 2005). Dentro deste contexto, complexos de inclusão com indicadores têm sido usados para detecção de analítos de interesse biológico, tais como fármacos e aminoácidos (AFKHAMI *et al.*, 2006; GLAZYRIN *et al.*, 2004).

A fenolftaleína (FE), indicador ácido-base, é incolor em solução ácida e rósea em soluções básicas, podendo formar complexos de inclusão 1:1 com a β-ciclodextrina. A formação desses complexos de inclusão entre a ciclodextrina e o indicador permite a determinação da concentração de substâncias com caráter ácido ou básico, uma vez que ocorre competição entre essas substâncias e o indicador pela cavidade cilíndrica hidrofóbica da ciclodextrina, gerando assim, uma diferença de leitura óptica (AFKHAMI *et al.*, 2006; GLAZYRIN *et al.*, 2004).

Complexos de inclusão têm sido empregados na construção de sensores químicos (ALARCÓN-ANGELES *et al.* 2008), estes são dispositivos pequenos, robustos, portáteis, de fácil manipulação com transdução eletroquímica ou óptica (LEI *et al.*, 2006; ALFAYA e KUBOTA, 2001; MEADOWS, 1996). Dentre estes transdutores, merece destaque os de origem óptica que constituem ferramentas poderosas imunes a interferências eletromagnéticas, sendo usados para a determinação e análise em várias áreas como ciências biomédicas, saúde, produtos farmacêuticos e monitoramento ambiental (FAN *et al.*, 2008).

2. Revisão da literatura

2.1. Ácidos biliares

Os ácidos biliares são ácidos orgânicos derivados do colesterol e surfactantes biológicos com um papel importante na absorção, transporte e secreção de lipídeos (YOO, 2007), são sintetizados no fígado a partir do colesterol, armazenados na vesícula biliar e liberados no intestino delgado ajudando a converter as gorduras alimentares em micelas mistas de ácidos biliares e triacilgliceróis (NELSON *et al.*, 2002).

Existem diferentes tipos de ácidos biliares. Os ácidos biliares primários como o ácido cólico (CA) e o ácido quenodesoxicólico (CDCA), são derivados do colesterol por duas rotas metabólicas diferentes (Figura 1) no fígado e conjugados com glicina e taurina (C-24) (DEBRUYNE *et al.*, 2001; SCALIA, 1995). Nas condições fisiológicas, as enzimas que participam da síntese de CA e CDCA são a colesterol 7α-hidroxilase (CYP7a1) e esterol 12α-hidroxilase (CYP8b1). A atividade da CYP8b1 pode determinar a relação de CA e CDCA e conseqüentemente a hidrofobicidade do pool de ácidos biliares que serão secretados pela vesícula biliar (VLAHCEVIC *et al.*, 2000; DEBRUYNE *et al.*, 2001).

Aproximadamente 85% dos ácidos biliares secretados são absorvidos no intestino delgado. A difusão passiva de micelas contendo ácidos biliares, ácidos graxos e monoglicerídeos ao longo do intestino delgado é maior para ácidos biliares hidrofóbicos na porção do Duodeno. No Íleo, esta ocorre por co-transporte ativo acoplado a bomba de sódio e potássio na superfície da borda-em-escova da membrana basolateral. Cerca de 15% dos ácidos biliares secretados atingem o cólon e retornam ao fígado pela veia porta hepática (DEBRUYNE *et al.*, 2001).

Os ácidos biliares secundários como o ácido desoxicólico (DCA) e o ácido litocólico (LCA), são constituídos de formas conjugadas de CA e CDCA, respectivamente, através da desconjugação da 7-αdehidroxilação pelas bactérias anaeróbicas da flora intestinal. Os ácidos biliares terciários são o ácido ursodesoxicólico (UDCA) e o sulfolitocólico (SLCA), formados através da epimerização do CDCA ou sulfatação do LCA, respectivamente. No cólon, cerca de 10% dos ácidos biliares secretados são reabsorvidos através de difusão passiva. O resultado é que 2-5% dos ácidos biliares secretados são eliminados nas fezes (DEBRUYNE *et al.*, 2001).

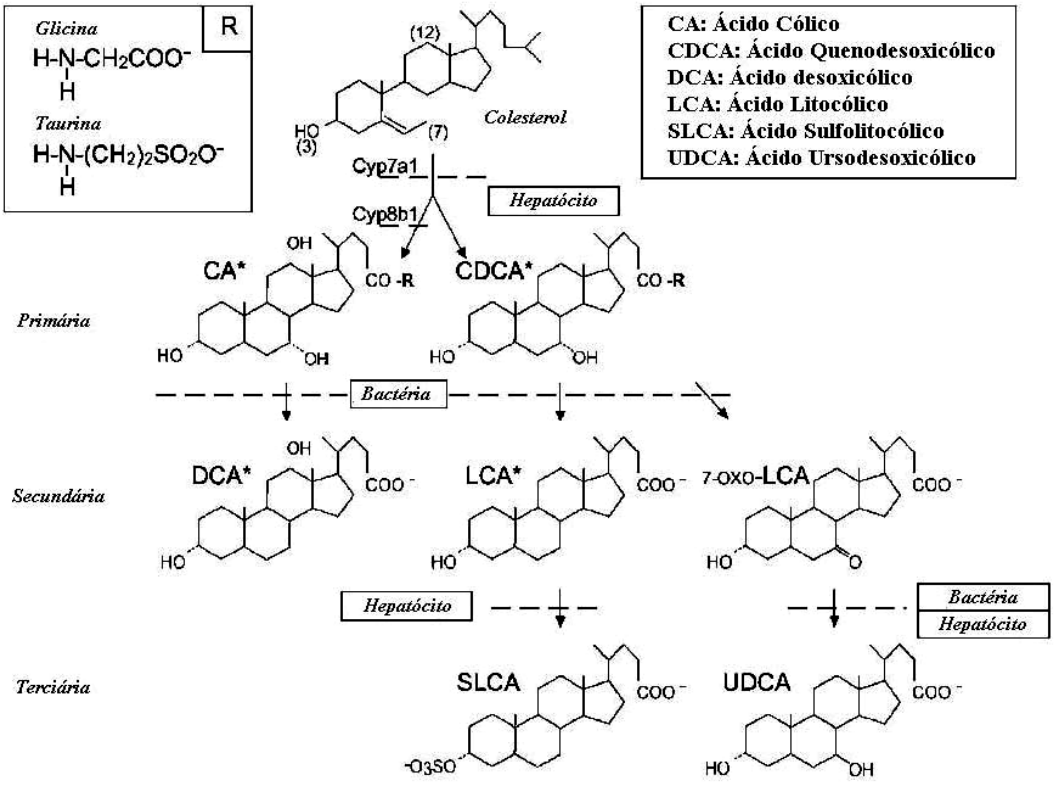


Figura 1: Estrutura química dos ácidos biliares primários, secundários, terciários e seus aminoácidos (R) conjugados. Linhas pontilhadas indicam locais de metabolismo dos hepatócitos ou das bactérias intestinais. A posição na via metabólica das enzimas colesterol 7α-hidroxilase (CYP7a1) e esterol 12α-hidroxilase (CYP8b1) são indicadas através de setas.

Nota: 7-oxo-LCA não é estável sendo imediatamente convertido em UDCA (Fonte: DEBRUYNE *et al.*, 2001).

Durante as últimas décadas alguns pesquisadores demonstraram o interesse pela síntese e estudos de surfactantes catiônicos derivados de ácidos biliares. Bernheim e Lack (1967) apud Mukhopadhyay e Maitra (2004) estudaram uma série de derivados do ácido cólico e mostraram que os sais catiônicos da bile podem acelerar a turgescência em células bacterianas, inibindo a síntese protéica e apresentando propriedades antimicrobianas.

Ácidos biliares também são usados para melhorar a absorção de medicamentos como peptídeos e seus derivados catiônicos e aumentando a absorção de DNA celular (MUKHOPADHYAY e MAITRA, 2004). Os ácidos biliares são usados terapeuticamente para diminuir a saturação do colesterol na bile, redução da

citotoxicidade na doença colestática hepática (HOFMANN e HAGEY, 2008) e dissolução de cálculos biliares (PETRONI *et al.*, 2001).

Diversos métodos têm sido utilizados para a análise de ácidos biliares em solução ou em preparações farmacêuticas, incluindo a potenciometria, alcalimetria, eletroquímica, cromatografia micelar eletrocinética (MEKC), cromatografia de fluido supercrítico e cromatografia gasosa, entretanto, estes métodos são muito onerosos, demandam grande tempo de preparação e dificuldade de extração dos componentes (KANG *et al.*, 2007; YOO, 2007; SOLA *et al.*, 2006; LIN *et al.*, 2003; MOMOSE *et al.*, 1998).

2.1.1. Ácido desoxicólico (DCA)

O ácido desoxicólico (3α, 5β, 12α)-3,12-dihidroxi-5-colano-24-oico (DCA – Figura 2), é um ácido biliar secundário constituído de formas conjugadas do ácido cólico (FERNANDEZ-LEYES *et al.*, 2007). O mesmo apresenta forma cristalina, *pKa* 6,58, é solúvel a 15°C em água (0,24g/L) e em álcool (220,7g/L) e quando na forma de sal de sódio tem sua solubilidade em água aumentada para valores maiores que 333g/L (BUDAVARI *et al.*, 1989).

O DCA é bastante usado como agente colerético associado a outros componentes em terapias e disfunções do fígado (RODRIGUEZ *et al.*, 2000); em hidrogéis aumentando a permeabilidade das membranas celulares (VALENTA *et al.*, 1999); em antibióticos com ação inibidora contra *Candida albicans* (MARPLES e STRETTON, 1987); é um componente dos derivados do N-(2-dimetilamino)etil que apresenta ação antimalárica (TERZIC *et al.*, 2007). Aumenta a biodisponibilidade de nanopartículas do copolímero de ácido lático e glicólico, usado no encapsulamento de medicamentos, protegendo-o durante seu transporte através do trato gastrointestinal e aumentando sua absorção no epitélio intestinal (SAMSTEIN *et al.*, 2008).

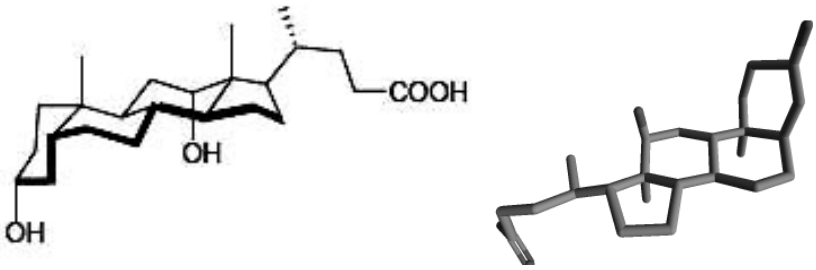


Figura 2: Estrutura química do ácido desoxicólico (Fonte: MUKHOPADHYAY e MAITRA, 2004).

O DCA é um dos componentes da injeção de fosfatidilcolina, que atua na dissolução de gorduras localizadas. Inicialmente o princípio ativo desta formulação era atribuído a fosfatidilcolina, mas Rotunda *et al.* (2004) observou que o DCA administrado isoladamente possuía o mesmo efeito que a primeira. O método utilizando injeções para a dissolução de gorduras localizadas é conhecido como Mesoterapia, e foi desenvolvido na Europa para posteriormente ser difundido para os Estados Unidos e Brasil, esta técnica foi inicialmente utilizada na remoção de celulite (BRYANT, 2004). O DCA também pode ser usado no tratamento de lipomas, lipodistrofia e obesidade, no entanto o emprego do DCA para fins estéticos está suspenso no Brasil desde outubro de 2005 (Resolução 2.682) e também na Europa e Estados Unidos (ROTUNDA e KOLODNEY, 2006) devido à falta de estudos sobre a segurança e eficácia do tratamento, mas continua sendo utilizado de forma clandestina em diversas clínicas de estética (RUBINSTEIN, 2008).

2.1.2. Ácido Ursodesoxicólico (UDCA)

O ácido ursodesoxicólico, (3α, 5β, 7β)-3,7-dihidroxi-5-colano-24-oico (UDCA, Figura 3) é um ácido biliar terciário formado pela epimerização do CDCA (DEBRUYNE *et al.*, 2001) e menor constituinte da bile humana (SOLA *et al.*, 2006). O mesmo é encontrado na forma de pó branco, cristalino, inodoro, de sabor amargo, pouco solúvel em água, apresentando boa solubilidade em etanol e ácido acético glacial e *pKa* de 5,10 (NAKASHIMA *et al.*, 2002; ORIENTI *et al.*, 1999; BUDAVARI *et al.*, 1989).

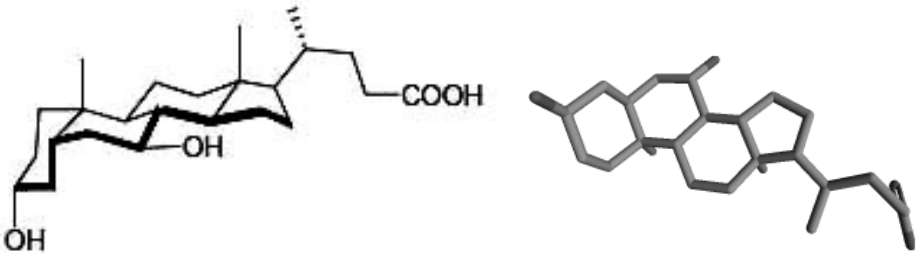


Figura 3: Estrutura química do ácido ursodesoxicólico (Fonte: MUKHOPADHYAY e MAITRA, 2004).

O UDCA é usado para dissolver cálculos biliares - colelitíase, tratar cirrose biliar, refluxo de bile na gastrite, no tratamento do câncer coloretal (TAY *et al.*, 2007; SETCHELL *et al.*, 2005, PETRONI *et al.*, 2001), colangite esclerosante primária (LINDOR, 1997), prevenção da pancreatite causada por microlitíases em pacientes com

a vesícula biliar intacta (OKORO *et al.*, 2008) e também em pacientes com hepatite C aumentando o nível da enzima alanina aminotransferase (IKEGAMI e MATSUZAKI, 2008).

2.2. Ciclodextrinas (CDs)

Em 1891, A. Villiers, um cientista francês, isolou uma substância produzida por *Bacilus amylobacter* contendo amido que o mesmo denominou de "celulosina". Depois, um microbiologista austríaco Franz Schardinger entre 1903 e 1911 descreveu dois compostos cristalinos – α-dextrina e β-dextrina isolado de sobrenadante bacteriano produzido em amido de batata. A celulosina descrita por Villers é a β-dextrina descrita por Schardinger. Em 1935, Freudenberg e Jacobi foram os primeiros a descrever as ciclodextrinas (CDs) como são conhecidas hoje: α-ciclodextrina (α-CD), β-ciclodextrina (β-CD) e γ-ciclodetrina (γ-CD). De 1935 a 1955, Freudenberg, Cramer e colaboradores identificaram a estrutura das CDs, suas propriedades físico-químicas e sua habilidade de formar complexos de inclusão. Na década de 1970, com o avanço dos processos biotecnológicos, foi possível a produção de CDs com alto grau de pureza e baixo custo (BREWSTER e LOFTSSON, 2007; LOFTSSON e DUCHËNE, 2007; DEL VALLE, 2004).

Ao longo do último século houve um aumento na utilização das CDs devido ao fato de serem produtos seminaturais decorrentes de fontes renováveis (amido) através de conversões enzimáticas simples. Milhares de toneladas são produzidos ao ano por tecnologias não agressivas sob o ponto de vista ambiental; baixo preço; capacidade de formar complexos de inclusão; baixos efeitos secundários e dependendo da aplicação, pode ser ingerida pelo homem em produtos indústrias com aplicações farmacêuticas (solubilizantes e estabilizantes), alimentícia (alterando o odor e o sabor) e cosmética (estabilizantes) (LOFTSSON e DUCHËNE, 2007; VEIGA *et al.*, 2006).

As ciclodextrinas são obtidas através da reação de transglicosilação intramolecular onde o amido é degradado pela ciclodextrina-glicosil-transferase (Figura 4) (CGTase) produzindo dextrinas cíclicas e acíclicas (SZEJTLI, 1998). Essa enzima possui peso molecular na ordem de 70-75kD e apresenta uma seqüência de aminoácidos com similaridade estrutural a α-amilase (VAN DER VEEN *et al.*, 2001).

Vários microorganismos catalisam esta reação enzimática como o *Bacillus megaterium*, *B. circulans*, *B. stearothermophilus, B. subtilis* e *Klebsiella pneumoniae*. As CDs que são obtidas em maiores porcentagens são a α-CD, β-CD e a γ-CD, cujas quantidades relativas dependem do tipo de microorganismo e das condições de reação (VEIGA *et al.*, 2006).

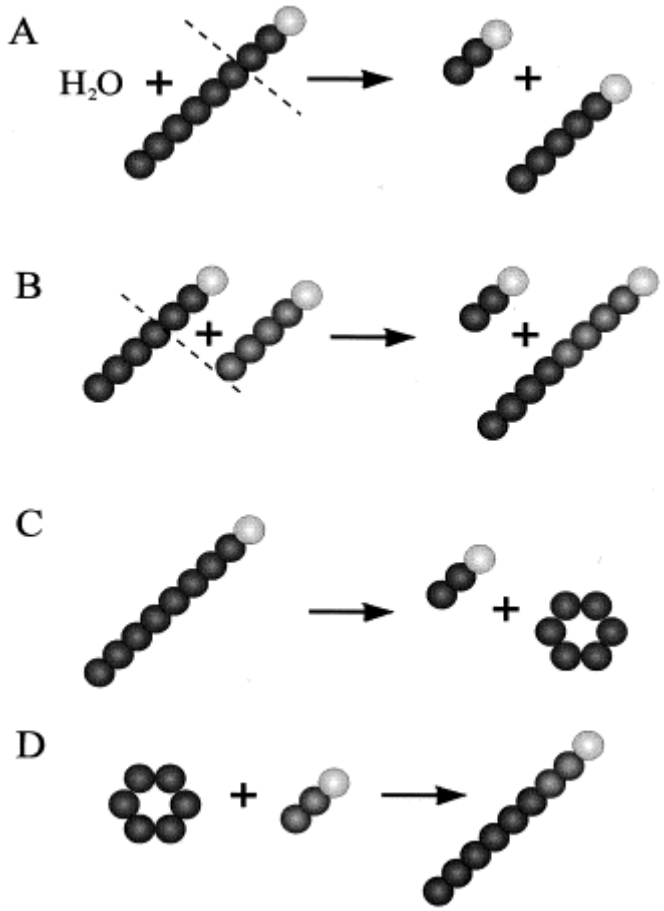


Figura 4: Representação esquemática de uma reação catalisada pela CGTase (ciclodextrina-glicosil-transferase). Os círculos escuros representam resíduos de glicose; os círculos claros representam o terminal redutor do açúcar. (A) Hidrólise; (B) Desproporção; (C) Ciclização; (D) Ligação (Fonte: VAN DER VEEN *et al.*, 2001).

As ciclodextrinas são oligossacarídeos cíclicos (Figura 5) derivados do amido com 6 (α-ciclodextrina), 7 (β-ciclodextrina), 8 (γ-ciclodextrina) ou mais unidades de glicose unidas por ligações α-(1,4) (ZHANG *et al.*, 2009; YUAN *et al.*, 2008; ABAY *et al.*, 2005; AL-SHERBINI, 2005; DE LEON-RODRIGUEZ e BASUIL-TOBIAS, 2005; CONSTANTIN *et al.*, 2004; DEL VALLE, 2004). CDs com número inferior a 6

moléculas de glicose não existem por razões estereoquímicas, mas CDs com mais de 8 moléculas de glicose já foram produzidas (VEIGA *et al.*, 2006). Devido à ausência de rotação livre nas ligações glicosídicas e da conformação em cadeira apresentadas pelas moléculas de glicose, as CDs apresentam uma forma tronco-cônica onde os radicais hidroxilas secundários ligados aos carbonos 2 e 3 e ocupam a base de maior diâmetro do tronco, enquanto que as hidroxilas primárias ligadas ao carbono 6 localizam-se na menor base do tronco. Esta conformação faz com que as ciclodextrinas em solução aquosa apresentem um exterior hidrofílico e interior hidrofóbico (AFKHAMI e KHALAFI, 2006; DEL VALLE, 2004).

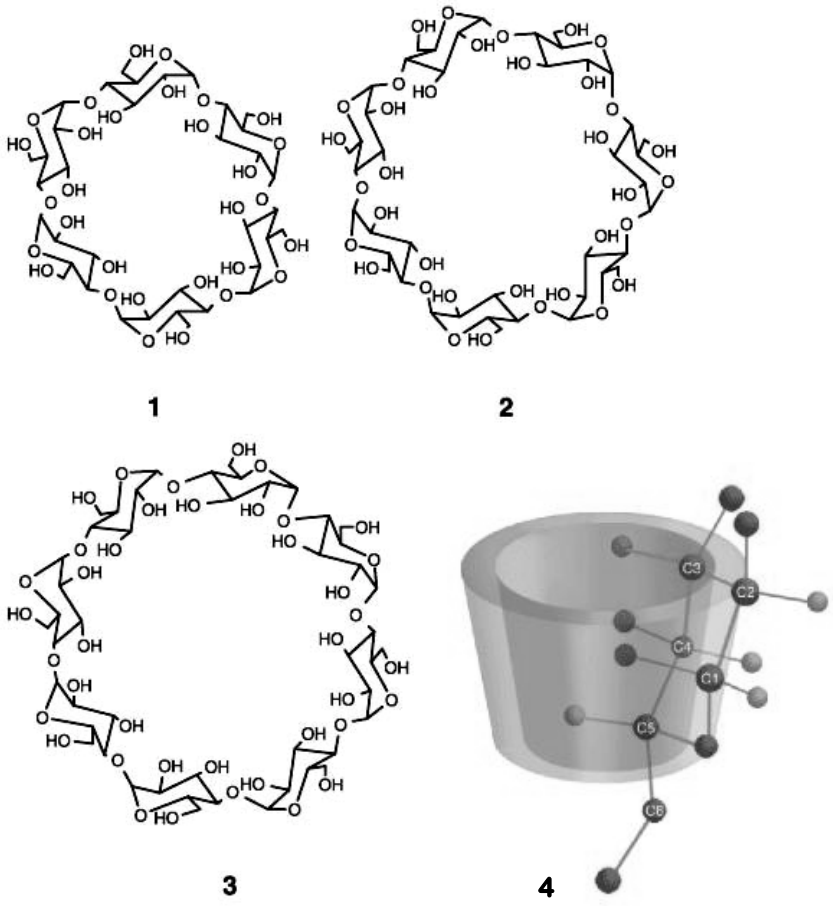


Figura 5: Estrutura da α-ciclodextrina (1), β-ciclodextrina (2), γ-ciclodetrina (3), estrutura tridimensional (4) (Fonte: VEIGA *et al.*, 2006).

Todas as ciclodextrinas apresentam estrutura cristalina, não-higroscópica e reduzida solubilidade aquosa, são bastante resistentes em meio alcalino, mas, hidrolisam-se facilmente em meio ácido (DEL VALLE, 2004). A Tabela 1 apresenta as propriedades físico-químicas das principais CDs.

Tabela 1: Propriedades físico-químicas das principais ciclodextrinas (Fonte: VEIGA *et al.*, 2006).

Propriedades	*α-CD*	*β-CD*	*γ-CD*
Unidades de Glicose	6	7	8
Massa molecular ($g \cdot mol^{-1}$)	972	1135	1297
Solubilidade aquosa (g/100mL a 25°C)	14,5	1,85	23,2
Diâmetro interno da cavidade (Å)	4,7-5,3	6,0-6,5	7,5-8,3
Altura da estrutura tronco-cônica (Å)	7,9±0,1	7,9±0,1	7,9±0,1
Volume aproximado da cavidade ($Å^3$)	174	262	427
Formas dos cristais	Lâminas hexagonais	Paralelogramas monoclínicos	Prismas quadráticos
pKa (a 25°C)	12,333	12,202	12,081
Constante de difusão a 40°C (m^2/s)	3,443	3,223	3,000

Em geral, as ciclodextrinas mais comuns apresentam baixa toxicidade sendo capazes apenas de permear membranas biológicas, tais como a córnea, se ingeridas por via oral, estas não são absorvidas no trato gastrointestinal e não apresentam toxicidade, quando administradas por via parentérica também apresentam baixa toxicidade (BREWSTER e LOFTSSON, 2007; LOFTSSON e DUCHËNE, 2007; DEL VALLE, 2004)

2.2.1. Complexos de inclusão

Devido a sua estrutura, as CDs podem ser consideradas cápsulas vazias cilíndricas abertas em ambas às extremidades, o que lhe permite a inclusão de uma enorme variedade de moléculas orgânicas em sua cavidade hidrofóbica. Neste complexo de inclusão a molécula que entra na cavidade é chamada de hospedeira enquanto a ciclodextrina é a molécula hóspede (BREWSTER e LOFTSSON, 2007; DEL VALLE, 2004; KUWABARA *et al.*, 1994).

Uma grande variedade de moléculas pode formar complexos de inclusão, tais como indicadores, drogas, pequenos ânions, ácidos carboxílicos e alcoóis (TAWARAH e KHOURI, 2000) (Tabela 2). Estas moléculas ficam ligadas por forças intermoleculares não covalentes (ANSELMI *et al.*, 2008; AFKHAMI *et al.*, 2006; DEL VALLE, 2004), além destas forças, o tamanho e estrutura interferem na inclusão destes

compostos (AL-SHERBINI, 2005). Devido a estas interações, as propriedades físico-químicas das moléculas hóspedes podem ser drasticamente modificadas (HERNÁNDEZ BALBOA *et al.*, 2008; LI *et al.*, 2008; YANEZ *et al.*, 2004).

Tabela 2: Algumas moléculas de importância biológica capazes de formar complexos de inclusão com ciclodextrinas e suas respectivas constantes de equilíbrio (K_c).

Biomolécula	*Tipo de CD*	K_c $(L \cdot mol^{-1})$	Referência
Fluoxetina (Antidepressivo)	β-CD	$8{,}20 \times 10^3$	AFKHAMI *et al.*, 2006
Ácido ferúlico (composto vegetal)	α-CD	$1{,}16 \times 10^3$	ANSELMI *et al.*, 2008
Esteroídes	β-CD e γ-CD	Variável	CAI *et al.*, 2005
Sais de alcalóides	β-CD e γ-CD	Variável	CSERNAK *et al.*, 2006
Clorambucil (Tratamento da Leucemia)	Tri-metil-β-CD	-	HERNÁNDEZ BALBOA *et al.*, 2008
Carvedilol (Tratamento da hipertenção)	Metil-β-CD	$2{,}77 \times 10^3$	HIRLEKAR e KADAM, 2008
Derivados de N-sulfamoiloxazolidinona (ação antimicrobiana)	β-CD	$\approx 10^4$	KADRI *et al.*, 2005
Norfloxacina (bactericida)	Metil-β-CD	$2{,}08 \times 10^4$	LI *et al.*, 2008
L-Tirosina	β-CD	$\approx 10^1$	SHANMUGAM *et al.*, 2008
Frunidipina (tratamento de doenças cardiovasculares)	β-CD	$1{,}56 \times 10^2$	YANEZ *et al.*, 2004

Em solução aquosa, a cavidade da ciclodextrina está ocupada por moléculas de água, isto é termodinamicamente desfavorável devido à interação polar-apolar. A formação do complexo de inclusão ocorre através do deslocamento destas moléculas de água da cavidade da CD pela molécula hóspede ou por grupos hidrofóbicos (Figura 6). Este processo é termodinamicamente favorável e contribui para a formação do complexo. As principais interações que ocorrem para a formação e estabilização do complexo são as interações de Van der Waals, pontes de hidrogênio e interações hidrofóbicas dependendo da molécula hóspede (GUNARATNE e CORKE, 2008; JADHAV e VAVIA, 2008; SHANMUGAM *et al.*, 2008; BREWSTER e LOFTSSON, 2007; AFKHAMI e KHALAFI, 2006; CHEN *et al.*, 2004; DEL VALLE, 2004).

Veiga *et al.* (2006) e Del Valle (2004) descreveram o mecanismo de formação de um complexo de inclusão: 1. Ocorre a aproximação da molécula hóspede à molécula de CD; 2. Acontece a quebra da estrutura da água existente no interior da cavidade da

CD e remoção de algumas das moléculas de água para a solução; 3. Em seguida há quebra da estrutura da água em volta da molécula hóspede; 4. Ocorre a interação dos substituintes da molécula hóspede com os grupos existentes na superfície ou no interior da CD; 5. Possívelmente há formação das pontes de hidrogênio entre a molécula hóspede e a CD; 6. Finalmete ocorre a reconstrução da estrutura da água em volta das partes expostas da molécula hóspede, após o processo de inclusão.

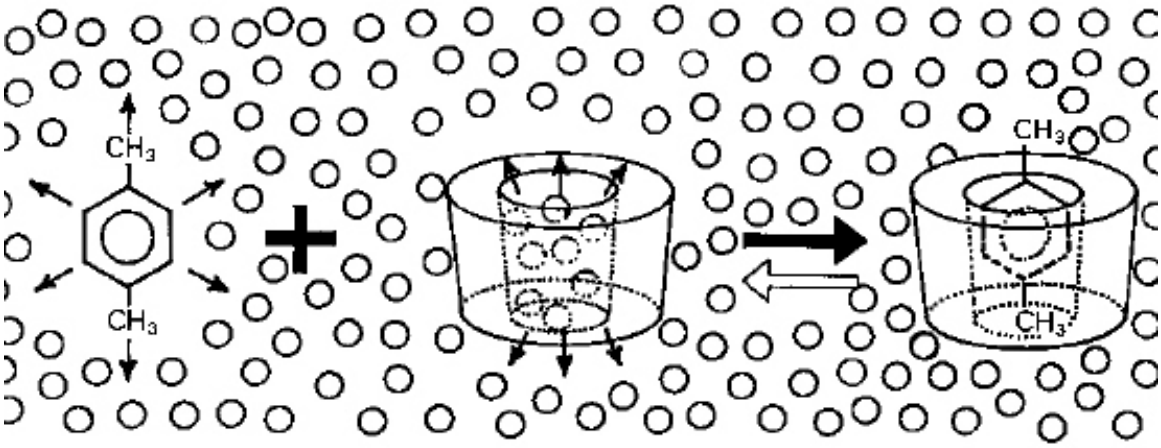


Figura 6: Formação de um complexo de inclusão entre a molécula hóspede e a ciclodextrina (Fonte: SZEJTLI, 1998).

Diversas técnicas são utilizadas para a produção de complexos de inclusão, as quais por sua vez têm grande influência no composto final obtido (MURA *et al.*, 1999): a co-precipitação é a técnica mais usada em laboratório e consiste na adição da molécula hóspede a uma solução aquosa de CD (ANSELMI *et al.*, 2008); a complexação fluída ocorre pela adição do hóspede a uma solução de CD 50-60% em massa; a complexação pastosa é semelhante a complexação fluída com a diferença de que a CD está praticamente sólida; na co-evaporação, a solução aquosa de CD é misturada à molécula hóspede e depois aquecida a 100°C para a eliminação da água; a extrusão é uma técnica onde o hóspede e a CD são misturadas e aquecidas continuamente (CHALLA *et al.*, 2005); por fim, na Mistura a Seco, o hóspede e a CD são diretamente combinados (VEIGA *et al.*, 2006; DEL VALLE, 2004).

A análise dos complexos de inclusão é realizada por uma grande variedade de técnicas: espectrofotométricas (UV-Vis, fluorescência), cromatográficas (HPLC), eletroquímica e calorimetria (GHOREISHI *et al.*, 2008; ZHU *et al.*, 2007; GEORGIOU

et al., 1995). Algumas técnicas espectrofotométricas utilizando CDs foram extensamente utilizadas para aumentar a sensibilidade do analito, no entando trabalhos utilizando análises UV-Vis com CDs são excassos. O emprego de ciclodextrinas em técnicas UV-Vis tem como objetivo melhorar a solubilidade e estabilidade das substâncias colorimétricas, além de aumentar a seletividade e sensibilidade desta técnica (DE LEON-RODRIGUEZ e BASUIL-TOBIAS, 2005).

Os complexos de inclusão também podem ser utilizados para a determinação indireta de substâncias. Para isso são utilizadas técnicas como a Ressonância Magnética Nuclear (RMN) (ABDEL-SHAFI, 2007; BREWSTER e LOFTSSON, 2007), a Fluorescência (LI *et al.*, 2008; LIU *et al.*, 2007) e a Espectrofotometria UV-Vis (JADHAV e VAVIA, 2008; ABAY *et al.*, 2005; KADRI *et al.*, 2005). Este último método, em associação com substâncias coloridas, tais como indicadores, apresenta-se como uma alternativa para a determinação de substâncias com baixa absortividade molar, onde a substância compete com o indicador pela cavidade da CD, sendo esta liberada em solução e em seguida quantificada. Esta técnica é conhecida como Reação de Complexação Competitiva (AFKHAMI *et al.*, 2006; GLAZYRIN *et al.*, 2004).

A determinação da constante de equilíbrio (K_c) da molécula hóspede-CD é de suma importância, pois esta serve como um parâmetro para medir as alterações físico-químicas ocasionadas pela complexação, tais como solubilidade aquosa, reatividade química, absortividade molar e outras propriedades ópticas (dispersão rotatória óptica), valores típicos para K_c foram apresentados na Tabela 2. Existem vários métodos para se determinar K_c: O método de solubilidade de fases de Higuchi e Connors, Espectrofotometria UV-Vis de Hildebrand e Benesi, RMN e Modelação molecular. Também é possível, através de K_c determinar os parâmetros termodinâmicos – variação da energia livre de Gibbs padrão ($\Delta G°$), variação da entalpia padrão ($\Delta H°$) e a variação da entropia padrão ($\Delta S°$) (BREWSTER e LOFTSSON, 2007; VEIGA *et al.*, 2006; DEL VALLE, 2004; BENESI e HILDEBRAND, 1949).

2.3. Indicadores Ácido-Base

O uso de indicadores de pH é uma prática bem antiga que foi introduzida no século XVII por Robert Boyle. Estas substâncias orgânicas são fracamente ácidas (indicadores ácidos) ou fracamente básicas (indicadores básicos) apresentam cores

diferentes para suas formas protonadas e desprotonadas dependendo das características físico-químicas da solução na qual estão contidos, em função de diversos fatores, tais como pH, potencial elétrico, complexação com íons metálicos e adsorção em sólidos (TERCI e ROSSI, 2002; ATKINS e JONES, 2001). A Tabela 3 apresenta uma lista com indicadores típicos.

Tabela 3: Indicadores e suas alterações pelo pH.

Indicador	*Cor da forma ácida*	*Faixa de pH da mudança de cor*	*Cor da forma básica*
Alaranjado de metila	Vermelho	3,2 a 4,4	Amarelo
Azul de Timol	Vermelho	1,2 a 2,8	Amarelo
	Amarelo	8,0 a 9,6	Azul
Azul de Bromofenol	Amarelo	3,0 a 4,6	Azul
Azul de Bromocresol	Amarelo	5,2 a 6,8	Azul
Fenolftaleína	Incolor	8,2 a 10,0	Rosa
Vermelho de fenol	Amarelo	6,6 a 8,0	Vermelho
Vermelho de metila	Vermelho	4,8 a 6,0	Amarelo

Os indicadores podem formar complexos de inclusão com as ciclodextrinas onde através da Reação de Complexação Competitiva permite a determinação de substâncias com caráter ácido ou básico, pois ocorre competição entre essas substâncias e o indicador pela cavidade cilíndrica hidrofóbica da ciclodextrina, gerando assim uma diferença de leitura óptica (AFKHAMI *et al.*, 2006; GLAZYRIN *et al.*, 2004). Desta forma, Afkhami *et al.* (2006) usou o complexo de inclusão β-CD-Fenolftaleína para a determinação da Fluoxetina; ZARZYCKI e LAMPARCZYK (1998) utilizou o mesmo complexo para a determinação de tetrahidrofurano, e; Yuexian *et al.* (2005) determinou aminoácidos aromáticos através da reação de complexação competitiva com o complexo α-CD-Alaranjado de metila.

2.4. Validação de procedimentos analíticos

Os laboratórios devem dispor de meios para demonstrar que os métodos de ensaio que executam, conduzem a resultados confiáveis e adequados à qualidade pretendida, cabendo também ao mesmo assegurar que estas características de desempenho do método atendam aos requisitos para as operações analíticas pretendidas. Desta forma, existem entidades responsáveis por determinar as regras para essa normalização como a ANVISA (Agência Nacional de Vigilância Sanitária - Resolução - RE nº 899, de 29 de maio de 2003), o INMETRO (Instituto Nacional de Metrologia, Normalização e Qualidade Industrial - DOQ-CGCRE-008), EMEA (*European Medicines Agency* - CPMP/ICH/381/95) e FDA (*Food and Drug Administration* - VICH GL2).

Ao validar-se uma metodologia alguns parâmetros devem ser avaliados, tais como: especificidade e seletividade - capacidade que o método possui de medir exatamente um composto em presença de outros componentes como impurezas e produtos de degradação; linearidade - capacidade de demonstrar que os resultados obtidos são diretamente proporcionais à concentração do analito na amostra, dentro de um intervalo especificado; intervalo - faixa entre os limites de quantificação superior e inferior de um método analítico; precisão - avaliação da proximidade dos resultados obtidos em uma série de medidas de uma amostragem múltipla de uma mesma amostra; limite de detecção - menor quantidade do analito presente em uma amostra que pode ser detectado; limite de quantificação - menor quantidade do analito em uma amostra que pode ser determinada com precisão e exatidão aceitáveis sob as condições experimentais estabelecidas; exatidão - proximidade dos resultados obtidos pelo método em estudo em relação ao valor verdadeiro; robustez - medida de sua capacidade em resistir a pequenas e deliberadas variações dos parâmetros analíticos (RUMEL, 2008, EMEA, 2008).

2.5. Sensores químicos

Os sensores químicos são dispositivos pequenos, robustos, portáteis, de fácil manipulação e não necessitam da adição contínua de reagentes para a sua operação podendo, assim, fornecer informações confiáveis continuamente. Portanto, o sensor químico tem sido um elemento chave na instrumentação analítica dispensando, em

muitos casos, a utilização de aparelhos complexos e a necessidade de uma enorme infra-estrutura de suporte (ALFAYA e KUBOTA, 2001).

A construção de um sensor baseia-se na comunicação entre uma reação química e um transdutor. Esta comunicação pode resultar de uma alteração na concentração de prótons, liberação ou absorção de gases, emissão de luz, entre outros. O transdutor converte esse sinal químico em um sinal de resposta mensurável podendo ainda ser ampliado, transformado e armazenado para posterior análise (LEI *et al.*, 2006). Estes sensores podem ter aplicações diretas em análises clínicas, controle de bioprocessos *on-line*, detecção de substâncias tóxicas no meio ambiente e controle de qualidade na indústria farmacêutica (LEI *et al.*, 2006; MEADOWS, 1996).

De acordo com o transdutor utilizado, o sensor pode ser classificado como eletroquímico (potenciométrico, amperométrico, condutométrico, eletrodos íons seletivos (ISE) e transistor de efeito de campo sensível a íon (ISFET)); óptico (elipsometria, luminescência, fosforescência, fluorescência, Raman); fibra óptica e guias de onda; interferometria (interferometria de luz branca, interferometria modal) e ressonância de plasma de superfície (SPR); calorimétricos; piezoelétricos (relaciona a oscilação da freqüência dos cristais de quartzo com variação da massa) e acústicos (FAN *et al.*, 2008; SILVA, 2008; RICCARDI *et al.*, 2002; THÉVENOT *et al.,* 2001).

Alguns artigos já descrevem o uso das ciclodextrinas em sensores associadas a enzimas como a glicose oxidase (ZHENG *et al.*, 2008) e a peroxidase (ZHU *et al.*, 2000). Por outro lado, sensores baseados na interação hóspede-hospedeiro com as ciclodextrinas (CD) são escassos. Alarcón-Angeles *et al.* (2008) construiu um sensor eletroquímico para determinação da dopamina cujo sinal era proveniente da formação do complexo de inclusão entre o analito e a CD.

2.5.1. Sensores químicos ópticos

Existem diversos tipos de transdutores, dentre eles os de origem óptica constituem ferramentas poderosas imunes a interferências eletromagnéticas, sendo usados para a determinação e análise em várias áreas como ciências biomédicas, saúde, produtos farmacêuticos e monitoramento ambiental (FAN *et al.*, 2008).

Os recentes avanços na tecnologia de aquisição de imagens digitais estão oferecendo uma nova forma de transdução óptica onde câmeras digitais ou *scanners* podem facilmente capturar imagens de reações colorimétricas combinado com

softwares que podem decompor e analisar estas imagens (GAIAO *et al.*, 2006; ZAITSEV *et al.*, 2008). Dal Grande *et al.* (2008) demonstrou uma relação entre o sistema RGB (sistema de definição de cores – Figura 7) e concentrações do analito.

As imagens formadas nas telas dos computadores, normalmente, utilizam o sistema RGB para a definição de cores. Neste padrão, cada tom de cor é definido por três canais: R (vermelho), G (verde) e B (azul), que variam como índices inteiros entre 0 e 255, permitindo uma combinação de 256^3 (= 16.777.216) tonalidades de cor em cada pixel (GODINHO *et al.*, 2008). Desta forma, é possível analisar quantitativamente a intensidade da variação de cor proporcional às concentrações do analito e gerar curvas de calibração (DAL GRANDE *et al.*, 2008). Trabalhos contendo sensores químicos com análise através do sistema RGB são escassos: Raja e Sankaranarayanan (2007) construíram um sensor contendo a glicose oxidase imobilizada onde as reações colorimétricas foram analisadas pelo RGB; Rosse e Walter (2008) utilizaram o referido sistema para a análise de um sensor químico utilizado para a determinação de carbono orgânico.

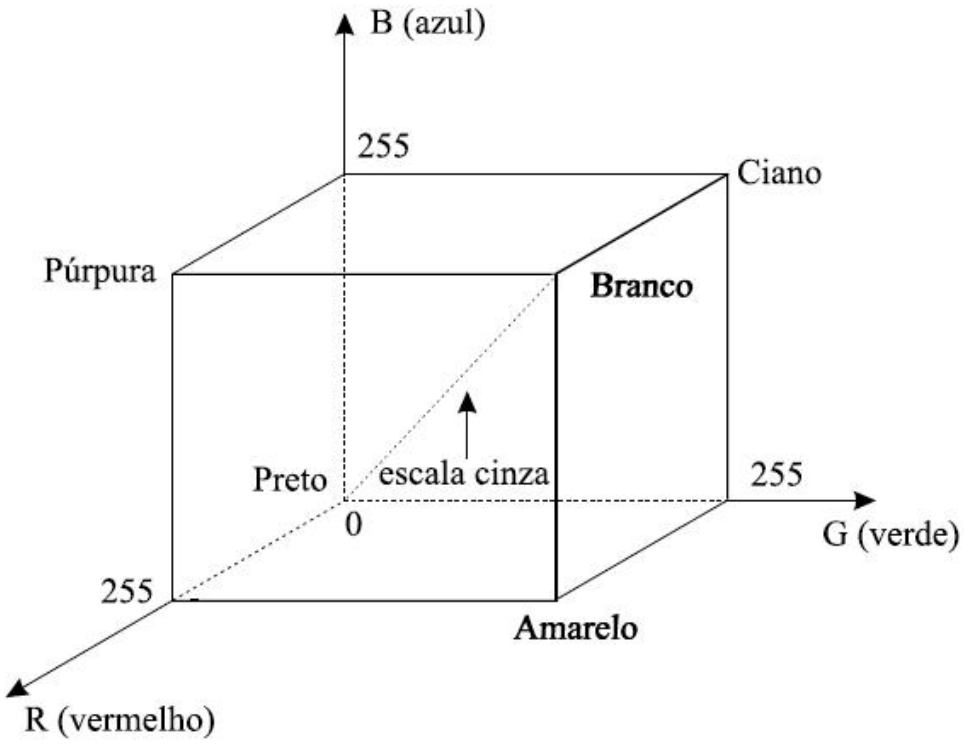


Figura 7: Cubo de cores RGB (R: vermelho; G: verde; B: azul). Cada eixo varia de 0 a 255 índices de cor. Nas extremidades do cubo estão as cores características (Fonte: GODINHO *et al.*, 2008).

3. Objetivos

3.1. Geral

Este projeto teve por objetivo o desenvolvimento de metodologias de baixo custo empregando ciclodextrinas para o controle de qualidade de fármacos visando o emprego no Sistema Único de Saúde - SUS.

3.2. Específicos

- Produção de um complexo de inclusão ciclodextrina-indicador;
- Otimização dos métodos analíticos para determinação dos ácidos desoxicólico e ursodesoxicólico;
- Aplicação desta metodologia em formulações farmacêuticas;
- Validação da metodologia para determinação dos ácidos biliares propostos;
- Avaliação de suportes poliméricos de baixo custo para imobilização do complexo de inclusão visando à construção de sensores químicos;
- Construção e avaliação de sensores químicos com transdução óptica para o controle de qualidade de fármacos.

4. Referências bibliográficas

ABAY, I.; DENIZLI, A.; BISKIN, E.; SALIH, B. Removal and pre-concentration of phenolic species onto β-cyclodextrin modified poly(hydroxyethylmethacrylate-ethyleneglycoldimethacrylate) microbeads. *Chemosphere*, v. 61, n. 9, p. 1263-1272, 2005.

ABDEL-SHAFI, A. A. Spectroscopic studies on the inclusion complex of 2-naphthol-6-sulfonate with β-cyclodextrin. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, v. 66, n. 3, p. 732-738, 2007.

AFKHAMI, A.; KHALAFI, L. Spectrophotometric determination of conditional acidity constant as a function of β-cyclodextrin concentration for some organic acids using rank annihilation factor analysis. *Analytica Chimica Acta*, v. 569, n. 1-2, p. 267-274, 2006.

AFKHAMI, A.; MADRAKIAN, T.; KHALAFI, L. Spectrophotometric determination of fluoxetine by batch and flow injection methods. *Chemical and Pharmaceutical Bulletin*, v. 54, n. 12, p. 1642-1646, 2006.

ALARCÓN-ANGELES, G.; PÉREZ-LÓPEZ, B.; PALOMAR-PARDAVE, M.; RAMÍREZ-SILVA, M. T.; ALEGRET, S.; MERKOÇI, A. Enhanced host-guest electrochemical recognition of dopamine using cyclodextrin in the presence of carbon nanotubes. *Carbon*, v. 46, n. 6, p. 898-906, 2008.

AL-SHERBINI, E.-S. A. M. Spectroscopic study of the inclusion complexes of 1-methyl-4-[4'-aminostyryl] quinolinium iodide with α,β,γ-cyclodextrins in the ground and excited states. *Microporous and Mesoporous Materials*, v. 85, n. 1-2, p. 25-31, 2005.

ALFAYA, A. A. S. e KUBOTA, L. T. A utilização de materiais obtidos pelo processo de sol-gel na construção de biossensores. *Química Nova*. v. 25, n. 5, pp. 835-841, 2002.

ANSELMI, C.; CENTINI, M.; MAGGIORE, M.; GAGGELLI, N.; ANDREASSI, M.; BUONOCORE, A.; BERETTA, G.; FACINO, R. M. Non-covalent inclusion of ferulic acid with α-cyclodextrin improves photo-stability and delivery: NMR and modeling studies. *Journal of Pharmaceutical and Biomedical Analysis*, v. 46, n. 4, p. 645-652, 2008.

ATKINS, P.; JONES, L. *Princípios de Química: Questionando a vida moderna e o meio ambiente*. 2. ed. Porto Alegre: Bookman, 2001.

BENESI, H. A.; HILDEBRAND, J. H. A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. *Journal of the American Chemical Society*, v. 71, n. 8, p. 2703-2707, 1949.

BREWSTER, M. E.; LOFTSSON, T. Cyclodextrins as pharmaceutical solubilizers. *Advanced Drug Delivery Reviews*, v. 59, n. 7, p. 645-666, 2007.

BRYANT, R. Controversial mesotherapy: Could it be the next Botox? *Dermatology Times*, v. 25, p. 1-2, 2004.

BUDAVARI, S.; O'NEIL, M. J.; SMITH, A.; HECKELMAN, P. E.; *The merck index: An encyclopedia of chemicals, drugs, and biologicals*. 11. ed. Whitehouse Station (NJ): Rahway, 1989.

CAI, W.; YAO, X.; SHAO, X.; PAN, Z. Bimodal complexations of steroids with cyclodextrins by a flexible docking algorithm. *Journal of Inclusion Phenomena*, v. 51, n. 1, p. 41-51, 2005.

CHALLA, R.; AHUJA, A.; ALI, J.; KHAR, R. K. Cyclodextrins in drug delivery: an updated review. *AAPS PharmSciTech [electronic resource]*. v. 6, n. 2, 2005.

CHEN, W.; CHANG, C. E.; GILSON, M. K. Calculation of cyclodextrin binding affinities: Energy, entropy, and implications for drug design. *Biophysical Journal*, v. 87, n. 5, p. 3035-3049, 2004.

CONSTANTIN, M.; FUNDUEANU, G.; BORTOLOTTI, F.; CORTESI, R.; ASCENZI, P.; MENEGATTI, E. Preparation and characterisation of poly(vinyl alcohol)/cyclodextrin microspheres as matrix for inclusion and separation of drugs. *International Journal of Pharmaceutics*, v. 285, n. 1-2, p. 87-96, 2004.

CSERNAK, O.; BUVARI-BARCZA, A.; BARCZA, L. Cyclodextrin assisted nanophase determination of alkaloid salts. *Talanta*, v. 69, n. 2, p. 425-429, 2006.

DAL GRANDE, F.; SANTOMASO, A.; CANU, P. Improving local composition measurements of binary mixtures by image analysis. *Powder Technology*, v. 187, n. 3, p. 205-213, 2008.

DE LEON-RODRIGUEZ, L. M.; BASUIL-TOBIAS, D. A. Testing the possibility of using UV-vis spectrophotometric techniques to determine non-absorbing analytes by inclusion complex competition in cyclodextrins. *Analytica Chimica Acta*, v. 543, n. 1-2, p. 282-290, 2005.

DEBRUYNE, P. R.; BRUYNEEL, E. A.; LI, X.; ZIMBER, A.; GESPACH, C.; MAREEL, M. M. The role of bile acids in carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, v. 480-481, p. 359-369, 2001.

DEL VALLE, E. M. M. Cyclodextrins and their uses: a review. *Process Biochemistry*, v. 39, n. 9, p. 1033-1046, 2004.

EMEA - Validation of Analytical Procedures: Text and Methodology CPMP/ICH/381/95. Disponível em: <http://www.emea.europa.eu/pdfs/human/ich/038195en.pdf>, Acesso em: 13 Dez. 2008.

FAN, X. D.; WHITE, I. M.; SHOPOUA, S. I.; ZHU, H. Y.; SUTER, J. D.; SUN, Y. Z. Sensitive optical biosensors for unlabeled targets: A review. *Analytica Chimica Acta*, v. 620, n. 1-2, p. 8-26, 2008.

FERNANDEZ-LEYES, M. D.; MESSINA, P. V.; SCHULZ, P. C. Aqueous sodium dehydrocholate-sodium deoxycholate mixtures at low concentration. *Journal of Colloid and Interface Science*, v. 314, n. 2, p. 659-664, 2007.

GAIAO, E. D. N.; MARTINS, V. L.; LYRA, W. D. S.; ALMEIDA, L. F. D.; SILVA, E. C. D.; ARAÚJO, M. C. U. Digital image-based titrations. *Analytica Chimica Acta*, v. 570, n. 2, p. 283-290, 2006.

GEORGIOU, M. E.; GEORGIOU, C. A.; KOUPPARIS, M. A. Flow injection gradient technique in spectrophotometric determination of formation constants of micromolecule-cyclodextrin complexes. *Analytical Chemistry*, v. 67, n. 1, p. 114-123, 1995.

GHOREISHI, S. M.; BEHPOUR, M.; GOLESTANEH, M. Study of inclusion complex formation between a cationic surfactant, two cyclodextrins and a drug. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, v. 62, n. 3-4, p. 279-284, 2008.

GLAZYRIN, A. E.; GRACHEV, M. K.; KUROCHKINA, G. I.; NIFANT'EV, E. E. Inclusion compounds of some water-soluble β-cyclodextrin derivatives with phenolphthalein. *Russian Journal of General Chemistry*, v. 74, n. 12, p. 1922-1925, 2004.

GODINHO, M. D. S.; PEREIRA, R. O.; RIBEIRO, K. D. O.; SCHIMIDT, F.; OLIVEIRA, A. E. D.; OLIVEIRA, S. B. D. Classificação de refrigerantes através de análise de imagens e análise de componentes principais (PCA). *Química Nova*, v. 31, p. 1485-1489, 2008.

GUNARATNE, A.; CORKE, H. Effect of hydroxypropyl β-cyclodextrin on physical properties and transition parameters of amylose-lipid complexes of native and acetylated starches. *Food Chemistry*, v. 108, n. 1, p. 14-22, 2008.

HERNÁNDEZ BALBOA, M. A.; ARÉVALO, V. V.; ABREGO REYES, V. H.; VELÁZQUEZ, A. M.; GANEM-QUINTANAR, A.; QUINTANAR, D.; CAMACHO, B.; ARZALUZ, G. N.; ROSALES-HOZ, M.; LEYVA, M. A.; ANGELES, E. Study of chlorambucil and chlorambucil-trimethyl-β-cyclodextrin inclusion complex by CE. *Chromatographia*, v. 67, n. 1-2, p. 193-196, 2008.

HIRLEKAR, R.; KADAM, V. Preparation and characterization of inclusion complexes of carvedilol with methyl-β-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, p. 1-6, 2008.

HOFMANN, A. F.; HAGEY, L. R. Bile acids: Chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cellular and Molecular Life Sciences*, v. 65, n. 16, p. 2461-2483, 2008.

IKEGAMI, T.; MATSUZAKI, Y. Ursodeoxycholic acid: Mechanism of action and novel clinical applications. *Hepatology Research*, v. 38, n. 2, p. 123-131, 2008.

JADHAV, G. S.; VAVIA, P. R. Physicochemical, in silico and in vivo evaluation of a danazol-β-cyclodextrin complex. *International Journal of Pharmaceutics*, v. 352, n. 1-2, p. 5-16, 2008.

KADRI, M.; DJEMIL, R.; ABDAOUI, M.; WINUM, J.-Y.; COUTROT, F.; MONTERO, J.-L. Inclusion complexes of N-sulfamoyloxazolidinones with β-cyclodextrin. *Bioorganic & Medicinal Chemistry Letters*, v. 15, n. 4, p. 889-894, 2005.

KANG, S.-Z.; CUI, Z.; MU, J. Electrochemical behavior of sodium cholate and deoxycholate on an electrode modified with multi-walled carbon nanotubes (MWNTs) linked up with cyclodextrin. *Diamond and Related Materials*, v. 16, n. 1, p. 12-15, 2007.

KUWABARA, T.; NAKAMURA, A.; UENO, A.; TODA, F. Inclusion complexes and guest-induced color changes of pH-indicator-modified β-cyclodextrins. *Journal of Physical Chemistry*, v. 98, n. 25, p. 6297-6303, 1994.

LEI, Y.; CHEN, W.; MULCHANDANI, A. Microbial biosensors. *Analytica Chimica Acta*, v. 568, n. 1-2, p. 200-210, 2006.

LI, J.; ZHAO, C.; CHAO, J. Investigation on the inclusion behavior of Norfloxacin with 2-methyl-β-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, v. 62, n. 3-4, p. 325-331, 2008.

LIN, M.-C.; WU, H.-L.; KOU, H.-S.; WU, S.-M.; CHEN, S.-H. Simple and sensitive fluorimetric liquid chromatography for simultaneous analysis of chenodiol and ursodiol in pharmaceutical formulations. *Analytica Chimica Acta*, v. 493, n. 2, p. 159-166, 2003.

LINDOR, K. D. Ursodiol for primary sclerosing cholangitis. *New England Journal of Medicine*, v. 336, n. 10, p. 691-695, 1997.

LIU, Y.; SHI, J.; GUO, D. S. Novel permethylated β-cyclodextrin derivatives appended with chromophores as efficient fluorescent sensors for the molecular recognition of bile salts. *Journal of Organic Chemistry*, v. 72, n. 22, p. 8227-8234, 2007.

LOFTSSON, T.; DUCHËNE, D. Cyclodextrins and their pharmaceutical applications. *International Journal of Pharmaceutics*, v. 329, n. 1-2, p. 1-11, 2007.

MARPLES, B. A.; STRETTON, R. J. Antifungal utility of bile acids. *UNITED STATES PATENT AND TRADEMARK OFFICE GRANTED PATENT*, 1987.

MEADOWS, D. Recent developments with biosensing technology and applications in the pharmaceutical industry. *Advanced Drug Delivery Reviews*, v. 21, n. 3, p. 179-189, 1996.

MOMOSE, T.; MURE, M.; IIDA, T.; GOTO, J.; NAMBARA, T. Method for the separation of the unconjugates and conjugates of chenodeoxycholic acid and deoxycholic acid by two-dimensional reversed-phase thin-layer chromatography with methyl [beta]-cyclodextrin. *Journal of Chromatography A*, v. 811, n. 1-2, p. 171-180, 1998.

MUKHOPADHYAY, S.; MAITRA, U. Chemistry and biology of bile acids. *Current Science*, v. 87, n. 12, p. 1666-1683, 2004.

MURA, P.; FAUCCI, M. T.; MANDERIOLI, A.; BRAMANTI, G. Influence of the preparation method on the physicochemical properties of binary systems of econazole with cyclodextrins. *International Journal of Pharmaceutics*, v. 193, n. 1, p. 85-95, 1999.

NAKASHIMA, T.; ANNO, T.; KANDA, H.; SATO, Y.; KUROI, T.; FUJII, H.; NAGADOME, S.; SUGIHARA, G. Potentiometric study on critical micellization concentrations (CMC) of sodium salts of bile acids and their amino acid derivatives. *Colloids and Surfaces B: Biointerfaces*, v. 24, n. 2, p. 103-110, 2002.

NELSON, D.; LEHNINGER, A.; COX, M. *Lehninger princípios de bioquímica*. 3. ed. São Paulo: Sarvier, 2002.

OKORO, N.; PATEL, A.; GOLDSTEIN, M.; NARAHARI, N.; CAI, Q. Ursodeoxycholic acid treatment for patients with postcholecystectomy pain and bile microlithiasis. *Gastrointestinal Endoscopy*, v. 68, n. 1, p. 69-74, 2008.

ORIENTI, I.; CERCHIARA, T.; ZECCHI, V.; ARIAS BLANCO, M. J.; GINES, J. M.; MOYANO, J. R.; RABASCO ALVAREZ, A. M. Complexation of ursodeoxycholic acid with β-cyclodextrin-choline dichloride coprecipitate. *International Journal of Pharmaceutics*, v. 190, n. 2, p. 139-153, 1999.

PETRONI, M. L.; JAZRAWI, R. P.; PAZZI, P.; LANZINI, A.; ZUIN, M.; PIGOZZI, M. G.; FRACCHIA, M.; GALATOLA, G.; ALVISI, V.; HEATON, K. W.; PODDA, M.; NORTHFIELD, T. C. Ursodeoxycholic acid alone or with chenodeoxycholic acid for dissolution of cholesterol gallstones: A randomized multicentre trial. *Alimentary Pharmacology and Therapeutics*, v. 15, n. 1, p. 123-128, 2001.

RICCARDI, C. D. S.; COSTA, P. I. D.; YAMANAKA, H. Imunossensor amperométrico. *Química Nova*, v. 25, n. 2, p. 316-320, 2002.

RODRIGUEZ, V. G.; LUCANGIOLI, S. E.; FERNANDEZ OTERO, G. C.; CARDUCCI, C. N. Determination of bile acids in pharmaceutical formulations using micellar electrokinetic chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, v. 23, n. 2-3, p. 375-381, 2000.

ROTUNDA, A. M.; KOLODNEY, M. S. Mesotherapy and phosphatidylcholine injections: Historical clarification and review. *Dermatologic Surgery*, v. 32, n. 4, p. 465-480, 2006.

ROTUNDA, A. M.; SUZUKI, H.; MOY, R. L.; KOLODNEY, M. S. Detergent effects of sodium deoxycholate are a major feature of an injectable phosphatidylcholine formulation used for localized fat dissolution. *Dermatologic Surgery*, v. 30, n. 7, p. 1001-1008, 2004.

RUBINSTEIN, F. ANVISA, RESOLUÇÃO - RE Nº 2682, DE 20 DE OUTUBRO DE 2005. Disponível em: <http://e-legis.anvisa.gov.br/leisref/public/showAct.php?id=19261&word=>, Acesso em: 13 Dez. 2008.

RUMEL, D. ANVISA, Resolução - RE nº 899, de 29 de maio de 2003. Disponível em: <http://www.anvisa.gov.br/legis/resol/2003/re/899_03re.htm>, Acesso em: 13 Dez. 2008.

SAMSTEIN, R. M.; PERICA, K.; BALDERRAMA, F.; LOOK, M.; FAHMY, T. M. The use of deoxycholic acid to enhance the oral bioavailability of biodegradable nanoparticles. *Biomaterials*, v. 29, n. 6, p. 703-708, 2008.

SCALIA, S. Bile acid separation. *Journal of Chromatography B: Biomedical Sciences and Applications*, v. 671, n. 1-2, p. 299-317, 1995.

SETCHELL, K. D. R.; GALZIGNA, L.; O'CONNELL, N.; BRUNETTI, G.; TAUSCHEL, H. D. Bioequivalence of a new liquid formulation of ursodeoxycholic acid (Ursofalk suspension) and Ursofalk capsules measured by plasma pharmacokinetics and biliary enrichment. *Alimentary Pharmacology and Therapeutics*, v. 21, n. 6, p. 709-721, 2005.

SHANMUGAM, M.; RAMESH, D.; NAGALAKSHMI, V.; KAVITHA, R.; RAJAMOHAN, R.; STALIN, T. Host-guest interaction of l-tyrosine with β-cyclodextrin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, v. 71, n. 1, p. 125-132, 2008.

SILVA, F. R. D. O. *Desenvolvimento de um biossensor de peróxido de hidrogênio de baixo custo baseado na emissão de európio III*. (Mestrado) - Departamento de Enegenharia de Sistemas Eletrônicos, Universidade de São Paulo, 2008.

SIVANANTHA RAJA, A.; SANKARANARAYANAN, K. Performance analysis of a colorimeter designed with RGB color sensor. In: 2007 International Conference on Intelligent and Advanced Systems, ICIAS 2007. 2007. p.305-310. Disponível em:<http://www.scopus.com/scopus/inward/record.url?eid=2-s2.0-57949083878&partnerID=40 >. Acesso em 21 fev. 2009.

SOLA, S.; GARSHELIS, D. L.; AMARAL, J. D.; NOYCE, K. V.; COY, P. L.; STEER, C. J.; IAIZZO, P. A.; RODRIGUES, C. M. P. Plasma levels of ursodeoxycholic acid in black bears, Ursus americanus: Seasonal changes. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, v. 143, n. 2, p. 204-208, 2006.

SZEJTLI, J. Introduction and general overview of cyclodextrin chemistry. *Chemical Reviews*, v. 98, n. 5, p. 1743-1753, 1998.

TAWARAH, K. M.; KHOURI, S. I. J. Determination of the stability and stoichiometry of p-methyl red inclusion complexes with γ-cyclodextrin. *Dyes and Pigments*, v. 45, n. 3, p. 229-233, 2000.

TAY, J.; TINMOUTH, A.; FERGUSSON, D.; HUEBSCH, L.; ALLAN, D. S. Systematic Review of Controlled Clinical Trials on the Use of Ursodeoxycholic Acid for the Prevention of Hepatic Veno-occlusive Disease in Hematopoietic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation*, v. 13, n. 2, p. 206-217, 2007.

TERCI, D. B. L.; ROSSI, A. V. Indicadores naturais de pH: usar papel ou solução? *Química Nova*, v. 25, p. 684-688, 2002.

TERZIC, N.; OPSENICA, D.; MILIC, D.; TINANT, B.; SMITH, K. S.; MILHOUS, W. K.; SÔLAJA, B. A. Deoxycholic acid-derived tetraoxane antimalarials and antiproliferatives. *Journal of Medicinal Chemistry*, v. 50, n. 21, p. 5118-5127, 2007.

THÉVENOT, D. R.; TOTH, K.; DURST, R. A.; WILSON, G. S. Electrochemical biosensors: Recommended definitions and classification. *Biosensors and Bioelectronics*, v. 16, n. 1-2, p. 121-131, 2001.

VALENTA, C.; NOWACK, E.; BERNKOP-SCHNURCH, A. Deoxycholate-hydrogels: novel drug carrier systems for topical use. *International Journal of Pharmaceutics*, v. 185, n. 1, p. 103-111, 1999.

VAN DER VEEN, B. A.; UITDEHAAG, J. C. M.; DIJKSTRA, B. W.; DIJKHUIZEN, L. Engineering of cyclodextrin glycosyltransferase reaction and product specificity. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, v. 1543, n. 2, p. 336-360, 2001.

VEIGA, F.; PECORELLI, C.; RIBEIRO, L. *As ciclodextrinas em tecnologia farmacêutica*. Coimbra: Minerva Coimbra, 2006.

VISCARRA ROSSEL, R. A.; FOUAD, Y.; WALTER, C. Using a digital camera to measure soil organic carbon and iron contents. *Biosystems Engineering*, v. 100, n. 2, p. 149-159, 2008.

VLAHCEVIC, Z. R.; EGGERTSEN, G.; BJÖRKHEM, I.; HYLEMON, P. B.; REDFORD, K.; PANDAK, W. M. Regulation of sterol 12α-hydroxylase and cholic acid biosynthesis in the rat. *Gastroenterology*, v. 118, n. 3, p. 599-607, 2000.

YANEZ, C.; SALAZAR, R.; NUNEZ-VERGARA, L. J.; SQUELLA, J. A. Spectrophotometric and electrochemical study of the inclusion complex between β-cyclodextrin and furnidipine. *Journal of Pharmaceutical and Biomedical Analysis*, v. 35, n. 1, p. 51-56, 2004.

YOO, S. H. Preparation of aqueous clear solution dosage forms with bile acids. *UNITED STATES PATENT AND TRADEMARK OFFICE GRANTED PATENT*, 2007.

YUAN, C.; JIN, Z.; LI, X. Evaluation of complex forming ability of hydroxypropyl-β-cyclodextrins. *Food Chemistry*, v. 106, n. 1, p. 50-55, 2008.

YUEXIAN, F.; YU, Y.; SHAOMIN, S.; CHUAN, D. Molecular recognition of α-cyclodextrin (CD) to choral amino acids based on methyl orange as a molecular probe. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, v. 61, n. 5, p. 953-959, 2005.

ZAITSEV, V. N.; KHALAF, V. A.; ZAITSEVA, G. N. Organosilica composite for preconcentration of phenolic compounds from aqueous solutions. *Analytical and Bioanalytical Chemistry*, v. 391, n. 4, p. 1335-1342, 2008.

ZARZYCKI, P. K.; LAMPARCZYK, H. The equilibrium constant of β-cyclodextrin-phenolphtalein complex; influence of temperature and tetrahydrofuran addition. *Journal of Pharmaceutical and Biomedical Analysis*, v. 18, n. 1-2, p. 165-170, 1998.

ZHANG, M.; LI, J.; ZHANG, L.; CHAO, J. Preparation and spectral investigation of inclusion complex of caffeic acid with hydroxypropyl-β-cyclodextrin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, v. 71, n. 5, p. 1891-1895, 2009.

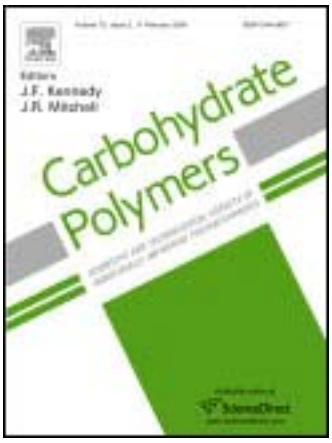
ZHENG, L.; XIONG, L.; LI, J.; LI, X.; SUN, J.; YANG, S.; XIA, J. Synthesis of a novel β-cyclodextrin derivative with high solubility and the electrochemical properties of ferrocene-carbonyl-β-cyclodextrin inclusion complex as an electron transfer mediator. *Electrochemistry Communications*, v. 10, n. 2, p. 340-345, 2008.

ZHU, M.; HAN, S.; YUAN, Z. β-Cyclodextrin polymer as the immobilization matrix for peroxidase and mediator in the fabrication of a sensor for hydrogen peroxide. *Journal of Electroanalytical Chemistry*, v. 480, n. 1-2, p. 255-261, 2000.

ZHU, X.; SUN, J.; WU, J. Study on the inclusion interactions of β-cyclodextrin and its derivative with dyes by spectrofluorimetry and its analytical application. *Talanta*, v. 72, n. 1, p. 237-242, 2007.

CAPÍTULO 1

Artigo submetido à revista Carbohydrate Polymers



Spectrophotometric determination of deoxycholic and ursodeoxycholic acids by competitive complexation with phenolphthalein-β-cyclodextrin

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Abstract

An expeditious colorimetric methodology for the determination of the deoxycholic acid (DCA) and of the ursodeoxycholic acid (UDCA) in pharmaceutical formulations is reported. The method is based on their competitive complexation reaction with a colour indicator to form β-cyclodextrin-inclusion complexes. Several pH colour indicators were tested, but phenolphthalein (PHP) showed the best interaction with the β-cyclodextrin (β-CD) with an inclusion yield higher than 95%. The best concentrations of β-cyclodextrin to form inclusion complexes were $1.24x10^{-3}mol{\cdot}L^{-1}$ and $6.2x10^{-4}mol{\cdot}L^{-1}$ at the pH of 9.5 and 10.5. Statistical analysis of the results showed that pH had a significant effect on the DCA determination and that high β-CD-PHP inclusion complex concentrations had a negative significant effect on the UDCA determination ($p<0.05$). The limit of detection and limit of quantification were $3.94x10^{-5}mol{\cdot}L^{-1}$ and $1.31x10^{-4}mol{\cdot}L^{-1}$ for DCA (range: $6.1x10^{-6}$-$3.13x10^{-3}mol{\cdot}L^{-1}$), $4.08x10^{-5}mol{\cdot}L^{-1}$ and $1.36x10^{-4}mol{\cdot}L^{-1}$ for UDCA (range: $6.05x10^{-6}$-$3.88x10^{-4}mol{\cdot}L^{-1}$). This simple and cheap method showed high stability and feasible instrumentation.

Keywords: β-Cyclodextrin; Phenolphthalein; Inclusion complex, Deoxycholic acid; Ursodeoxycholic acid

1. Introduction

Bile acids are organic metabolites of cholesterol with natural ionic detergent proprieties playing a pivotal role in the absorption, transport, and secretion of lipids or as therapeutic agents (Yoo, 2007). The deoxycholic acid, (3α, 5β, 12α)-3,12-dihydroxy-5-cholan-24-oic acid (DCA), is a secondary bile acid formed from conjugated forms of the colic acid (Fernandez-Leyes et al., 2007). It is of common use as choleretic agent in therapy of liver dysfunctions, or as additive in cosmetic preparations and antibiotics (Rodriguez et al., 2000). Furthermore, it constitutes the major active ingredient of "phosphatidylcholine injection" which is a controversial popular technique to treat localized fat accumulation, now prohibited by ANVISA (The Brazilian National Health Surveillance Agency) and FDA agencies (Rotunda & Kolodney, 2006). Ursodeoxycholic acid, (3α, 5β, 7β)-3,7-dihydroxycholan-24-oic acid (UDCA), is a minor constituent in human bile (Sola et al., 2006) used as a drug to dissolve cholesterol gallstones, to treat biliary cirrhosis, bile reflux gastritis, treatment of colorectal cancer and a range of other adult cholestatic conditions (Tay et al., 2007; Setchell et al., 2005). Several instrumental approaches were described for the analysis of bile acids in bulk or in pharmaceutical preparations, including potentiometry, voltammetry, micellar electrokinetic chromatography (MEKC), supercritical fluid chromatography (SFC), gas chromatography and HPLC. However, the procedures are generally quite onerous and involve difficult or time-consuming extraction procedures (Lin et al., 2003; Momose et al., 1998). The extensive use of bile acids shows the needs of the biotechnological studies to develop alternative methods for fast analysis of pharmaceutical formulations.

In this context, cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides with six (α-), seven (β-) or eight (γ-) glucose residues linked by α-(1–

4) glycosidic bonds (Wang & Cai, 2008b; Teranishi & Nishiguchi, 2004). In aqueous solution, they possess a truncated cone shaped structure with a hydrophilic exterior and a hydrophobic interior (Wang & Cai, 2008a) capable to form inclusion complexes with a wide variety of substrates such as dyes, drugs, small anions, carboxylic acids and alcohols (Gunaratne & Corke, 2008). The inclusion complexes formed are often able to promote enhancements or perturbations of the photophysical and photochemical properties of included guest molecules (Yuexian et al., 2005). Hence, investigation of the inclusion mechanisms can be accomplished by a great variety of methods including molecular absorption spectrophotometry and fluorometry, HPLC, surface tension, electrochemistry and calorimetry (Zhu et al., 2007). However, reports of the usage of CDs as analytical reagents in UV–Vis spectrophotometry are scarce. Afkhami et al. (2006) used the inclusion complex β-CD-PHP in a competitive assay to determine fluoxetine. Xie et al. 2005 used the absorption spectra of the dibenzoyl peroxide-β-cyclodextin complex in the analysis of benzoic acid concentrations. Yanez et al. (2004) proposed the use of β-CD to directly quantify the furnidipine through of the inclusion complex formed and Yuexian et al. (2005) resorted to the methyl orange-α-cyclodextrin inclusion complex to determine aromatic amino acids. More commonly, CDs have been used in UV–Vis spectrophotometry mainly to improve the solubility and stability of coloured compounds and to increase the sensitivity and selectivity of colorimetric reactions (De Leon-Rodriguez & Basuil-Tobias, 2005).

Phenolphthalein (PHP) is a typical acid/base indicator that forms a colourless 1:1 inclusion complex with β-CD and can be used for the indirect determination of colourless compounds through competitive complexation reaction (Afkhami et al., 2006; Glazyrin et al., 2004). In this context, the objective of this work is the proposal of the colorimetric determination of deoxycholic and ursodeoxycholic acid based on

competitive complexation reaction with phenolphthalein-β-cyclodextrin inclusion complexes. Two factorial designs were developed to identify the constraints and study the experimental conditions to accomplish optimized determination procedures. Figures of merit of the proposed procedures enable the envisagement of low cost and simple application in large scale monitoring and control tasks.

2. Experimental

2.1. Materials

Absorption spectra and data were collected by means of a Pharmacia Ultraspec 3000pro UV/Vis spectrophotometer using 1-cm path length quartz cells. Statistical evaluations were carried out by means of the Statistica software (StatSoft Inc., Tulsa, OK, USA).

Double deionised water and analytical grade chemicals were used without further purification. β-cyclodextrin was obtained from Fluka (Steinheim, Germany). Phenolphthalein, deoxycholic (sodium salt) and ursodeoxycholic acids were obtained from Sigma (St. Luiz, MO, USA). At acidic conditions, the measurements were performed using a 150 $mmol \cdot L^{-1}$ KCl-HCl buffer solution, pH 2.0. A 150 $mmol \cdot L^{-1}$ carbonate buffer solution was prepared to provide measurements at basic conditions (Afkhami et al., 2006).

2.2. Methods

The co-precipitation technique (Del Valle, 2004) was used to prepare the inclusion complexes. Therefore, solutions were mixed in the following order: 1.00 mL of the indicator solution, 1.00 mL of buffer, 1.00 mL of β-CD solution and 1.00 mL bile acid sample (The order did not interfere in the results). The mixture was strongly stirred after the addition of each solution except for the one containing the bile acid who was just homogenized. The blank solution was composed of 1.00 mL of buffer and 3.00 mL of water.

A 1:6 ratio between the concentrations of indicator and β-CD was kept to compare the best indicator inclusion complex to accomplish the bile acids determinations. Thus, the following concentrations of indicators were considered: bromophenol blue ($4x10^{-5}mol{\cdot}L^{-1}$), bromocresol blue ($1.85x10^{-5}$ $mol{\cdot}L^{-1}$), methyl orange ($5.1x10^{-5}mol{\cdot}L^{-1}$ at pH 2.0 and $4.0x10^{-4}mol{\cdot}L^{-1}$ at pH 9.5), methyl red ($3.1x10^{-4}$ $mol{\cdot}L^{-1}$), phenol red ($7.5x10^{-5}mol{\cdot}L^{-1}$), phenolphthalein ($3.1x10^{-4}mol{\cdot}L^{-1}$, only in basic conditions) and thymol blue ($1.3x10^{-4}mol{\cdot}L^{-1}$). All the concentrations referred were in the linear range of spectrophotometric response for the respective indicator.

To access the formation constants of the β-CD-PHP, solutions of $3.1x10^{-4}mol{\cdot}L^{-1}$ phenolphthalein in different amounts of β-CD ($5.17x10^{-5}$ to $1.86x10^{-3}mol{\cdot}L^{-1}$) at pH 9.5 or of $1.55x10^{-4}mol{\cdot}L^{-1}$ phenolphthalein in the $2.58x10^{-5}$ to $9.3x10^{-4}mol{\cdot}L^{-1}$ β-CD range at pH 10.5, were respectively prepared. To evaluate the formation constants of the β-CD-DCA, DCA was added to final concentrations in between $1.56x10^{-5}$ to $6.25x10^{-3}mol{\cdot}L^{-1}$ to the β-CD-PHP ($1.24x10^{-3}$:$3.1x10^{-4}mol{\cdot}L^{-1}$) solution. A similarly study was performed for assessing the β-CD-UDCA constant, for which solutions with UDCA in the interval

of $4.84x10^{-5}$ to $3.1x10^{-3}mol{\cdot}L^{-1}$ were prepared with a constant ratio of β-CD-PHP ($6.2x10^{-4}$:$1.55x10^{-4}mol{\cdot}L^{-1}$) concentrations.

2.3. Applications

The study was performed by the addition of known amounts of the DCA and UDCA standards to a sample with a known amount of pharmaceutical formulations such as, Injectable Phosphatidylcholine formula (Rotunda et al., 2004) (phosphatidylcholine 5% w/v, deoxycholic acid sodium salt 4.75% w/v, benzyl alcohol 0.9% v/v, and water up to the volume of 100 mL) for the DCA and Ursacol (labeled with 300mg of ursodeoxycholic acid per pill) for the UDCA.

3. Results and discussion

3.1. Evaluation of the inclusion complexes between ß-cyclodextrin and different indicators

The Fig. 1 shows the spectral changes of different common pH indicators after addition of β-CD up to the concentrations ratio of 6:1 in pH 2.0 and 9.5 media. For bromocresol blue, bromophenol blue, thymol blue and phenol red, no significant changes were observed in the respective spectra when β-CD was added. However, the phenolphthalein (PHP) showed a strong interaction with the β-CD in alkaline pH since there was a decrease in the absorption peak (at the wavelength of 553nm) (Fig. 1A) of more than 95%. At this pH condition the ionized red form of PHP becomes enclosed in the β-CD cage, where it is forced into its colourless lactone structure without, however,

protonating the phenolic groups (Afkhami et al., 2006). Inclusion complexes between methyl orange and the β-CD were observed on both pH conditions tested (Fig. 1B). At pH 2.0 the absorption peak at 506nm decreased slightly, whereas at pH 9.5 the formation of two isobestics points at 400 and 456nm and a blue shift of the absorption band of about 6 nm were registered. The decrease in absorbance values is due to the lack of coplanarity of methyl orange caused by the constrained conformation of methyl orange in the cyclodextrin cavity (Zhang et al., 2006; Yuexian et al., 2005). The same authors reported a similar behavior between the methyl orange and α-CD. In the basic pH, the formation of isosbestic point was similarly noticed by Sueishi & Miyakawa (1999). The methyl red also formed inclusion complexes with β-CD at the two pH (Fig. 1C) media, evidenced by the appearance of two isosbestic points in acid (484nm and 579nm), basic pH (335m and 433nm) and a blue shift of 8nm in the wavelength corresponding to the absorption maxima. At pH 2.0, the absorbance decrease and the isosbestic points appearance characterizes the formation of the azonium tautomer inclusion complex, one of the cationic protonated forms of methyl red. Similar findings were previously described by Tawarah & Khouri (2000) and Kuwabara et al. (1994) concerning the inclusion complexes with γ-CD and β-CD, respectively.

[FIGURE 1 here]

Based on the previous observations it is clear that inclusion complexes are formed with particular forms of the indicators, but the spectral changes are more pronounced in the case of the β-CD-PHP complex. When the PHP is forced to leave the cavity of β-CD by a competitive colourless host it will transmit again the red colour to the solution. Thus, the extent of the solution colour change can then be easily

determined against a calibration curve of the free indicator and the corresponding amount related with the amount of the substance hosted. The absorption spectra of phenolphthalein (PHP) solutions with concentrations of β-CD ranging from $5.17x10^{-5}$ to $1.86x10^{-3}$ mol.L^{-1} revealed a proportional decrease of the free PHP up to a concentration of $1.24x10^{-3}$mol·L^{-1} in pH 9.5 medium and up to $6.2x10^{-4}$mol·L^{-1} at pH 10.5 medium (concentrations ratio of 1:4 PHP:β-CD). Higher concentrations of β-CD did not cause additional observable decrease in absorbency at both pH conditions. By establishing the premise that in the assayed conditions phenolphthalein is only capable of forming 1:1 inclusion complexes with β-cyclodextrin (Brewster & Loftsson, 2007; Afkhami et al., 2006; Del Valle, 2004), the corresponding equilibrium constant (*Kc*) could be derived as in the relationships of Eq. (1). After mathematical treatment it can be shown that the value for this constant can be easily graphically found using the linear double reciprocal plot obtained by Eq. (2) (Abdel-Shafi, 2007; Benesi & Hildebrand, 1949):

$$PHP + \beta - CD \rightleftharpoons \beta - CD - PHP,\ K_c = \frac{[\beta - CD - PHP]}{[PHP][\beta - CD]} (1)$$

$$\frac{1}{A - A_0} = \frac{1}{a} + \frac{1}{aK_c[\beta - CD_0]} (2)$$

where A and A_0 are the absorbance of PHP in the presence and absence of β-CD, respectively, K_c is the equilibrium constant for the formation of 1:1 inclusion complex, a is a constant related to the molar absorption coefficients changes, and $[\beta\text{-}CD_0]$ is the initial concentration of β-CD. The equilibrium constants (*Kc*) were then determined both at pH 9.5 and pH 10.5 from the linear plots experimentally obtained. The respective values of $1.65(\pm0.98)x10^4$ and $5.10(\pm0.37)x10^4$ L.mol^{-1} were enabled

showing that the smaller amount of free PHP found at the higher pH is congruent with the higher value of the complex formation constant.

3.2. Determination of deoxicholic acid and ursodeoxicholic acid by their competitive complexation reaction with β-cyclodextrin-phenolphthalein inclusion complex

The Fig. 2 shows the absorption spectra of the β-CD-PHP inclusion complex under various concentrations of DCA (Fig. 2A) and the UDCA (Fig. 2B). It is clear that with the addition of DCA or UDCA an increase in the monitored absorbance occurred. This behaviour indicates competition of the DCA or of the UDCA with PHP to form the inclusion complex with the β-CD. However, at pH 9.5 the spectral changes of the solution with the increasing amounts of UDCA were absent. Aiming both to study the conditions for the determinations of both acids and simultaneously evaluate the robustness of the experimental conditions, two independent factorial designs were developed considering the pH changes, the temperature, the concentration of the indicator inclusion complex and the buffer concentration (Table 1). The central points of both designs were also assayed in quadruplicate for determination of the pure error.

[TABLE 1 here]

[FIGURE 2 here]

The results obtained through a 2^3 full factorial design for the study of the variables that influence in the determination of DCA pointed out that the pH had the main statistically significant positive effect ($p<0.05$). In fact, there was an increase of

about 73.2% in the answer for DCA ($1.88x10^{-3}L{\cdot}mol^{-1}$) among the pH 9.2 and 10.7 (Fig. 3), and only of 2.85% between the pH 10.5 and 10.7 (increase not significant by the Tukey test ($p<0.05$)). Thus, the adoption of the higher pH conditions provided the larger variation in the absorbance of the sample in the studied conditions. The second order interactions between the pH and β-CD-PHP concentration and between temperature and β-CD-PHP concentration also presented positive effects, meaning that the pH main effect is slightly enhanced by raising the levels of the other two variables. The other main effects and third order interactions were not statistically significant ($p<0.05$). To calculate the formation constant of the β-CD-DCA inclusion complexes with a 1:1 stoichiometry, the modified Benesi–Hildbrand (Abdel-Shafi, 2007; Benesi & Hildebrand, 1949) equation Eq. (3) can be resorted to, since the β-CD-PHP complex is colourless at the monitoring wavelength and any absorbance of the solution is due to the free PHP_0 displaced from the complex and thus proportional to bile acid concentration.

$$\frac{1}{A-A_0}=\frac{1}{a}+\frac{1}{aK_c[PHP_0]} \quad (3)$$

The values of K_c of $8.65x10^3$ and $2.58x10^4$ $L.mol^{-1}$ were respectively found at the pH of 9.5 and 10.5, respectively.

[FIGURE 3 here]

The results obtained through the 2^4 full factorial design for the study of the variables with significant influence in the determination of UDCA are presented in the Table 1. The concentration of the β-CD-PHP complex has a negative and highly significant effect ($p<0.05$), indicating that smaller inclusion complex concentrations

lead to larger absorbance values, in the studied conditions. The pH (positive effect) and buffer concentration (negative effect) variables used in the solution of UDCA presented low statistically significant effects. Only a second-order interaction was significant, namely the interaction between temperature and β-CD-PHP concentration, indicating a positive effect of those variables on the absorbance. As can be seen on Fig. 4, and settling a constant 1:4 ratio between β-CD and PHP concentrations, the absorbance of the complex remains constant above $1.94x10^{-5}$:$7.75x10^{-5}$ $mol{\cdot}L^{-1}$, but after addition of $3.88x10^{-4}mol{\cdot}L^{-1}$ of UDCA, the amount of PHP displaced from the complex was greater. The univariant optimization of the amount of β-CD for that amount of PHP revealed an increase of the absorbance value of 43.2% (difference statistically significant by Tukey test, $p<0.05$) as response to the same concentration of UDCA previously used. In those conditions the straight line obtained from the Benesi–Hildbrand plot enabled to obtain the value of $2.22x10^4$ $L.mol^{-1}$ for K_c.

[FIGURE 4 here]

3.3. Analytical Parameters

From the measurements performed under the optimum conditions described above, the calibration graph was linear in the range $6.1x10^{-6}$-$3.13x10^{-3}mol{\cdot}L^{-1}$ of DCA allowing the establishment of a regression line of equation: $A = 698(\pm10)\ DCA_{mol/L} - 0.005(\pm0.012)$ with a correlation coefficient of 0.9995. For UDCA a quadratic relationship was found in the range $6.05x10^{-6}$-$3.88x10^{-4}mol{\cdot}L^{-1}$, but translated in a linear regression line if this is established in the form of $A^{0.5} = 3814(\pm133)\ UDCA_{mol/L} + 0.03\ (\pm0.01)$ with a correlation coefficient of 0.9991. The limits of detection were of

$3.94x10^{-5}mol{\cdot}L^{-1}$ for DCA and of $4.08x10^{-5}mol{\cdot}L^{-1}$ for UDCA. The corresponding limits of quantification were respectively of $1.31x10^{-4}mol{\cdot}L^{-1}$ and $1.36x10^{-4}mol{\cdot}L^{-1}$.

Based on Turkey's test there were no significant difference between the standards and the pharmaceutical formulations (3 replicates, $p<0.05$), according to the method described in the experimental section (variation: 4% for DCA and 1% for UDCA). Table 2 shows a summary of the analytical parameters for the determination of DCA and UDCA.

[TABLE 2 here]

The advantages of the procedure proposed herein are the short time for the analysis completion, the use of low cost reagents and the low concentrations determination enabled. The best pH conditions founded to determinate DCA was 10.5 and similar to the proposed by Afkhami et al. (2006) to accomplish fluoxetine determinations. Zarzycki & Lamparczyk (1998) also adopted a pH 10.5 to assess the inclusion complex between β-CD-PHP and tetrahydrofuran. The development of low cost methods for determining the DCA are important in quality control of pharmaceutical formulations, for example, in deoxycholate-hydrogels (Valenta et al., 1999) to prevent misuses when considering aesthetic usage purposes. The limit of detection of $3.94x10^{-5}mol{\cdot}L^{-1}$ and a linearity varying the order of $6.1x10^{-6}$-$3.13x10^{-3}mol{\cdot}L^{-1}$ enable the envisagement of the determination of the its content in samples of phosphatidylcholine injection (50mg/mL) indiscriminately used in mesotherapy (Rotunda & Kolodney, 2006a, b, 2004). The limit of detection for the UDCA determinations is more than ten times lower of the concentrations found in liver 5-

$6x10^{-4}mol{\cdot}L^{-1}$ (Poupon & Poupon, 1995) and the typical pattern provided by the European Pharmacopoeia for the pills is 60mg.

4. Conclusion

DCA and UDCA were satisfactorily determined in the pharmaceutical formulations (error: 4% for DCA and 1% for UDCA) by competitive complexation reaction with a colour indicator to form β-cyclodextrin-inclusion complexes. The best pH conditions to determinate DCA and UDCA concentrations were 10.5. The limits of detection were $3.94x10^{-5}mol{\cdot}L^{-1}$ and $4.08x10^{-5}mol{\cdot}L^{-1}$ for DCA and UDCA, respectively. The linearity ranges were $6.1x10^{-6}$-$3.13x10^{-3}mol{\cdot}L^{-1}$ for DCA and $6.05x10^{-6}$-$3.88x10^{-4}mol{\cdot}L^{-1}$ for UDCA. This method showed high stability at large temperature range, instantaneous reaction, simple and low cost for reagents and instrumentation.

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References

Abdel-Shafi, A. A. (2007). Spectroscopic studies on the inclusion complex of 2-naphthol-6-sulfonate with β-cyclodextrin. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy, 66*(3), 732-738.

Afkhami, A., Madrakian, T., & Khalafi, L. (2006). Spectrophotometric determination of fluoxetine by batch and flow injection methods. *Chemical and Pharmaceutical Bulletin, 54*(12), 1642-1646.

Benesi, H. A., & Hildebrand, J. H. (1949). A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. *Journal of the American Chemical Society, 71*(8), 2703-2707.

Brewster, M. E., & Loftsson, T. (2007). Cyclodextrins as pharmaceutical solubilizers. *Advanced Drug Delivery Reviews, 59*(7), 645-666.

De Leon-Rodriguez, L. M., & Basuil-Tobias, D. A. (2005). Testing the possibility of using UV-vis spectrophotometric techniques to determine non-absorbing analytes by inclusion complex competition in cyclodextrins. *Analytica Chimica Acta, 543*(1-2), 282-290.

Del Valle, E. M. M. (2004). Cyclodextrins and their uses: a review. *Process Biochemistry, 39*(9), 1033-1046.

Fernandez-Leyes, M. D., Messina, P. V., & Schulz, P. C. (2007). Aqueous sodium dehydrocholate-sodium deoxycholate mixtures at low concentration. *Journal of Colloid and Interface Science, 314*(2), 659-664.

Glazyrin, A. E., Grachev, M. K., Kurochkina, G. I., & Nifant'ev, E. E. (2004). Inclusion compounds of some water-soluble β-cyclodextrin derivatives with phenolphthalein. *Russian Journal of General Chemistry, 74*(12), 1922-1925.

Gunaratne, A., & Corke, H. (2008). Effect of hydroxypropyl β-cyclodextrin on physical properties and transition parameters of amylose-lipid complexes of native and acetylated starches. *Food Chemistry, 108*(1), 14-22.

Kuwabara, T., Nakamura, A., Ueno, A., & Toda, F. (1994). Inclusion complexes and guest-induced color changes of pH-indicator-modified β-cyclodextrins. *Journal of Physical Chemistry, 98*(25), 6297-6303.

Lin, M.-C., Wu, H.-L., Kou, H.-S., Wu, S.-M., & Chen, S.-H. (2003). Simple and sensitive fluorimetric liquid chromatography for simultaneous analysis of chenodiol and ursodiol in pharmaceutical formulations. *Analytica Chimica Acta, 493*(2), 159-166.

Momose, T., Mure, M., Iida, T., Goto, J., & Nambara, T. (1998). Method for the separation of the unconjugates and conjugates of chenodeoxycholic acid and deoxycholic acid by two-dimensional reversed-phase thin-layer chromatography with methyl [beta]-cyclodextrin. *Journal of Chromatography A, 811*(1-2), 171-180.

Poupon, R., & Poupon, R. E. (1995). Ursodeoxycholic acid therapy of chronic cholestatic conditions in adults and children. *Pharmacology and Therapeutics, 66*(1), 1-15.

Rodriguez, V. G., Lucangioli, S. E., Fernandez Otero, G. C., & Carducci, C. N. (2000). Determination of bile acids in pharmaceutical formulations using micellar electrokinetic chromatography. *Journal of Pharmaceutical and Biomedical Analysis, 23*(2-3), 375-381.

Rotunda, A. M., & Kolodney, M. S. (2006a). Mesotherapy and phosphatidylcholine injections: Historical clarification and review. *Dermatologic Surgery, 32*(4), 465-480.

Rotunda, A. M., Suzuki, H., Moy, R. L., & Kolodney, M. S. (2004b). Detergent effects of sodium deoxycholate are a major feature of an injectable phosphatidylcholine formulation used for localized fat dissolution. *Dermatologic Surgery, 30*(7), 1001-1008.

Setchell, K. D. R., Galzigna, L., O'Connell, N., Brunetti, G., & Tauschel, H. D. (2005). Bioequivalence of a new liquid formulation of ursodeoxycholic acid (Ursofalk suspension) and Ursofalk capsules measured by plasma pharmacokinetics and biliary enrichment. *Alimentary Pharmacology and Therapeutics, 21*(6), 709-721.

Sola, S., Garshelis, D. L., Amaral, J. D., Noyce, K. V., Coy, P. L., Steer, C. J., Iaizzo, P. A., & Rodrigues, C. M. P. (2006). Plasma levels of ursodeoxycholic acid in black bears, Ursus americanus: Seasonal changes. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143*(2), 204-208.

Sueishi, Y., & Miyakawa, T. (1999). Complexation of phenols with β- and γ-cyclodextrins: Determination of the association constants by using the isomerization of spiropyran. *Journal of Physical Organic Chemistry, 12*(7), 541-546.

Tawarah, K. M., & Khouri, S. i. J. (2000). Determination of the stability and stoichiometry of p-methyl red inclusion complexes with γ-cyclodextrin. *Dyes and Pigments, 45*(3), 229-233.

Tay, J., Tinmouth, A., Fergusson, D., Huebsch, L., & Allan, D. S. (2007). Systematic Review of Controlled Clinical Trials on the Use of Ursodeoxycholic Acid for the Prevention of Hepatic Veno-occlusive Disease in Hematopoietic Stem Cell Transplantation. *Biology of Blood and Marrow Transplantation, 13*(2), 206-217.

Teranishi, K., & Nishiguchi, T. (2004). Cyclodextrin-bound 6-(4-methoxyphenyl)imidazo[1,2-α]pyrazin-3(7H)- ones with fluorescein as green chemiluminescent probes for superoxide anions. *Analytical Biochemistry, 325*(2), 185-195.

Valenta, C., Nowack, E., & Bernkop-Schnurch, A. (1999). Deoxycholate-hydrogels: novel drug carrier systems for topical use. *International Journal of Pharmaceutics, 185*(1), 103-111.

Wang, J. h., & Cai, Z. (2008a). Incorporation of the antibacterial agent, miconazole nitrate into a cellulosic fabric grafted with β-cyclodextrin. *Carbohydrate Polymers, 72*(4), 695-700.

Wang, J. h., & Cai, Z. (2008b). Investigation of inclusion complex of miconazole nitrate with β-cyclodextrin. *Carbohydrate Polymers, 72*(2), 255-260.

Xie, H., Wang, H. Y., Ma, L. Y., Xiao, Y., & Han, J. (2005). Spectrophotometric study of the inclusion complex between β-cyclodextrin and dibenzoyl peroxide and its analytical application. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 62*(1-3), 197-202.

Yanez, C., Salazar, R., Nunez-Vergara, L. J., & Squella, J. A. (2004). Spectrophotometric and electrochemical study of the inclusion complex between β-cyclodextrin and furnidipine. *Journal of Pharmaceutical and Biomedical Analysis, 35*(1), 51-56.

Yoo, S. H. (2007). Preparation of aqueous clear solution dosage forms with bile acids. *UNITED STATES PATENT AND TRADEMARK OFFICE GRANTED PATENT*.

Yuexian, F., Yu, Y., Shaomin, S., & Chuan, D. (2005). Molecular recognition of [alpha]-cyclodextrin (CD) to choral amino acids based on methyl orange as a molecular probe. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 61*(5), 953-959.

Zarzycki, P. K., & Lamparczyk, H. (1998). The equilibrium constant of β-cyclodextrin-phenolphtalein complex; influence of temperature and tetrahydrofuran addition. *Journal of Pharmaceutical and Biomedical Analysis, 18*(1-2), 165-170.

Zhang, H., Chen, G., Wang, L., Ding, L., Tian, Y., Jin, W., & Zhang, H. (2006). Study on the inclusion complexes of cyclodextrin and sulphonated azo dyes by electrospray ionization mass spectrometry. *International Journal of Mass Spectrometry, 252*(1), 1-10.

Zhu, X., Sun, J., & Wu, J. (2007). Study on the inclusion interactions of β-cyclodextrin and its derivative with dyes by spectrofluorimetry and its analytical application. *Talanta, 72*(1), 237-242.

Spectrophotometric determination of deoxycholic and ursodeoxycholic acids by competitive complexation with phenolphthalein-β-cyclodextrin

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Captions for figures

Fig. 1. Absorption spectrum at acid (2.0) and alkaline pH (9.5) of A: phenolphthalein ($3.1x10^{-4}mol{\cdot}L^{-1}$, only in basic solution); B: methyl orange ($5.1x10^{-5}mol{\cdot}L^{-1}$ at pH 2.0 and $4.0x10^{-4}mol{\cdot}L^{-1}$ at pH 9.5); C: methyl red ($3.1x10^{-4}mol{\cdot}L^{-1}$), submitted to proportion 1:6 with β-CD.

Fig. 2. Determination of various concentrations of DCA (A) by the inclusion complex of β-CD-PHP at pH 9.5 (β-CD-PHP: $1.24x10^{-3}$:$3.1x10^{-4}mol{\cdot}L^{-1}$) and various concentrations of UDCA at pH 10.5 (β-CD-PHP: $6.2x10^{-4}$:$1.55x10^{-4}mol{\cdot}L^{-1}$).

Fig. 3. pH effect in the inclusion complex β-CD-PHP ($6.2x10^{-4}$:$1.55x10^{-4}mol{\cdot}L^{-1}$) formation and your interaction with DCA ($1.88x10^{-3}mol{\cdot}L^{-1}$).

Fig. 4. Concentration effect in the PHP, β-CD-PHP inclusion complex and determination of UDCA ($3.88x10^{-4}mol{\cdot}L^{-1}$) by β-CD-PHP inclusion complex (same concentration).

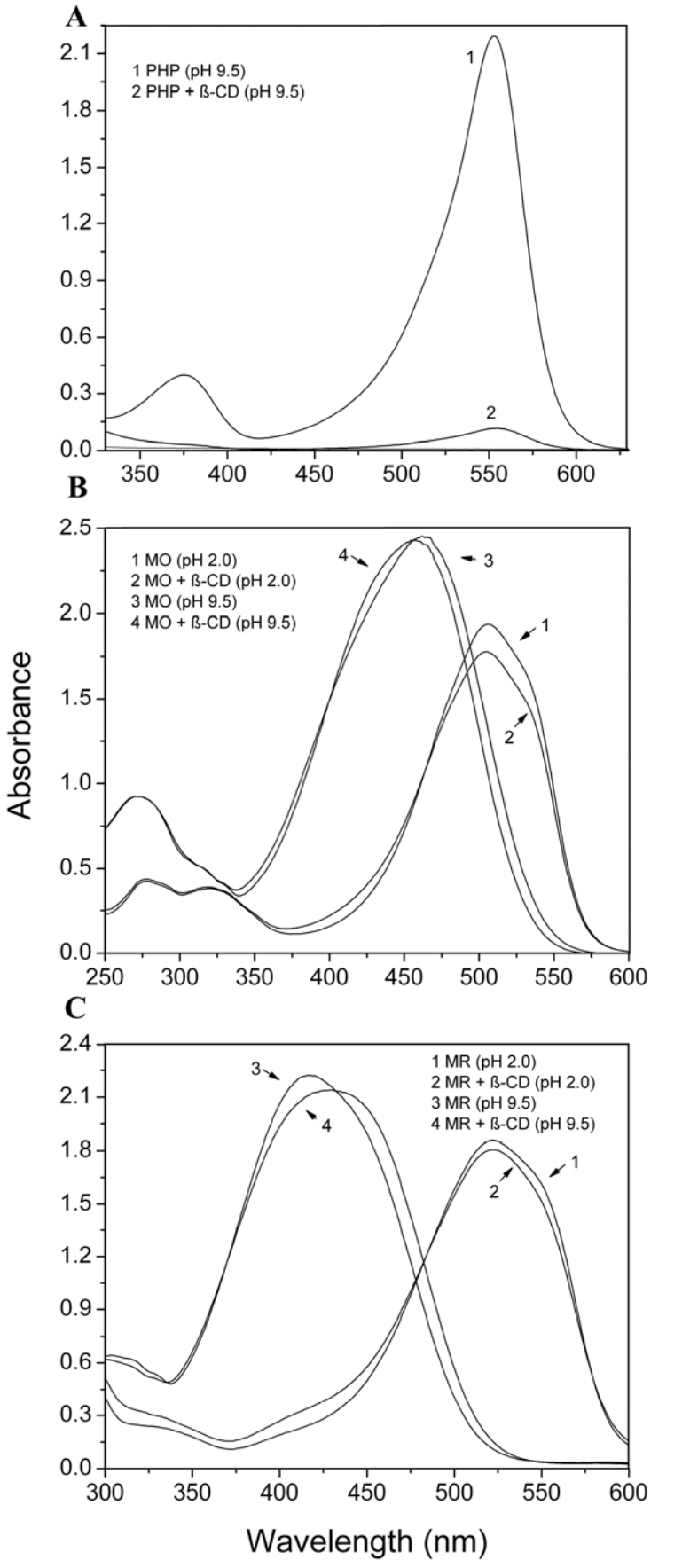
A
1 PHP (pH 9.5)
2 PHP + ß-CD (pH 9.5)
B
1 MO (pH 2.0)
2 MO + ß-CD (pH 2.0)
3 MO (pH 9.5)
4 MO + ß-CD (pH 9.5)
C
1 MR (pH 2.0)
2 MR + ß-CD (pH 2.0)
3 MR (pH 9.5)
4 MR + ß-CD (pH 9.5)
Absorbance
Wavelength (nm)

Figure 1

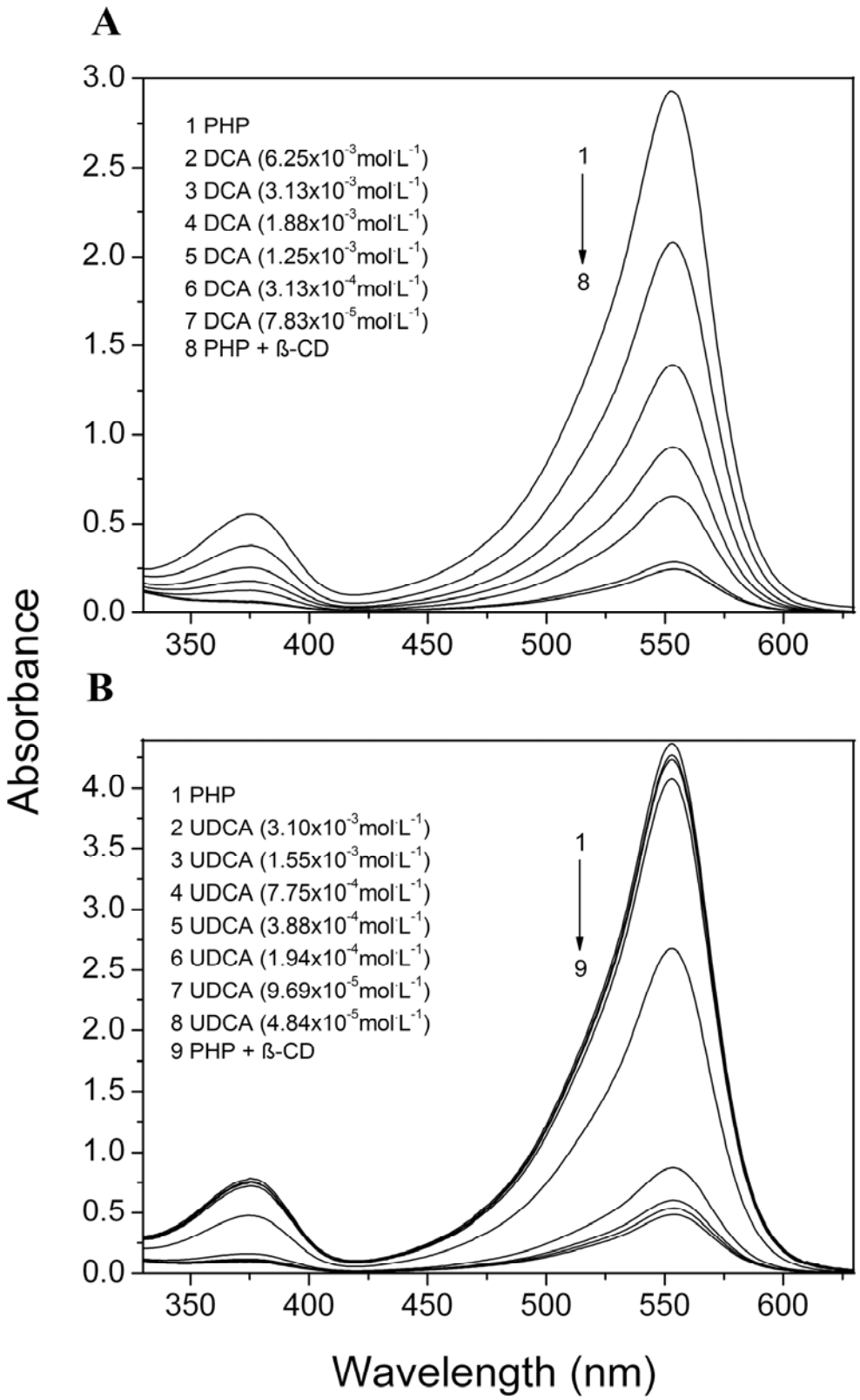
A
1 PHP
2 DCA (6.25x10-3mol·L-1)
3 DCA (3.13x10-3mol·L-1)
4 DCA (1.88x10-3mol·L-1)
5 DCA (1.25x10-3mol·L-1)
6 DCA (3.13x10-4mol·L-1)
7 DCA (7.83x10-5mol·L-1)
8 PHP + ß-CD
1
8
B
1 PHP
2 UDCA (3.10x10-3mol·L-1)
3 UDCA (1.55x10-3mol·L-1)
4 UDCA (7.75x10-4mol·L-1)
5 UDCA (3.88x10-4mol·L-1)
6 UDCA (1.94x10-4mol·L-1)
7 UDCA (9.69x10-5mol·L-1)
8 UDCA (4.84x10-5mol·L-1)
9 PHP + ß-CD
1
9
Absorbance
Wavelength (nm)

Figure 2

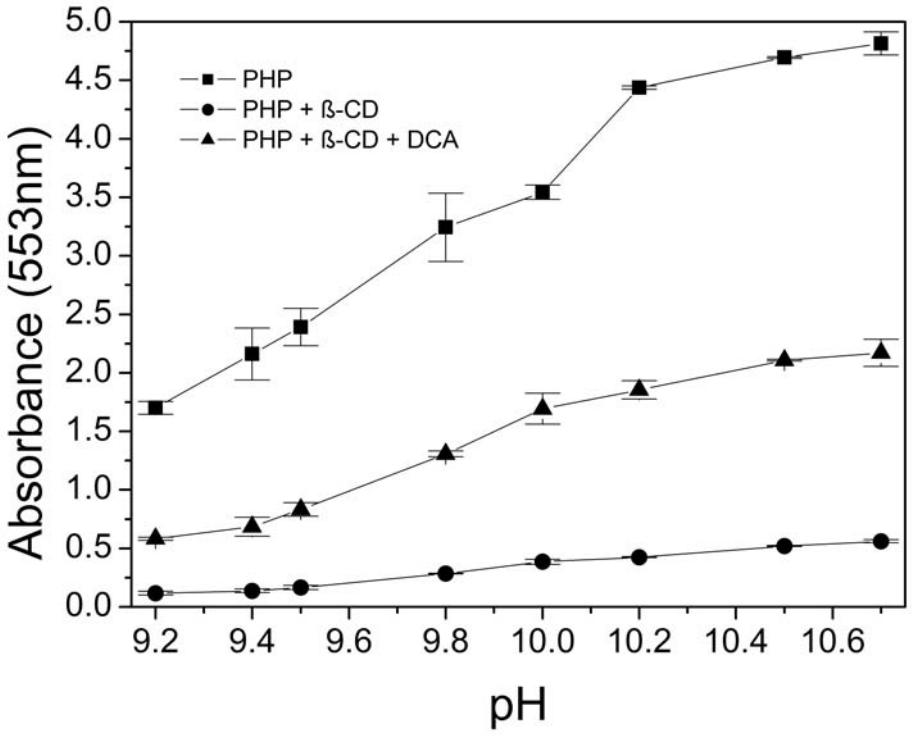
PHP
PHP + ß-CD
PHP + ß-CD + DCA
Absorbance (553nm)
pH
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6

Figure 3

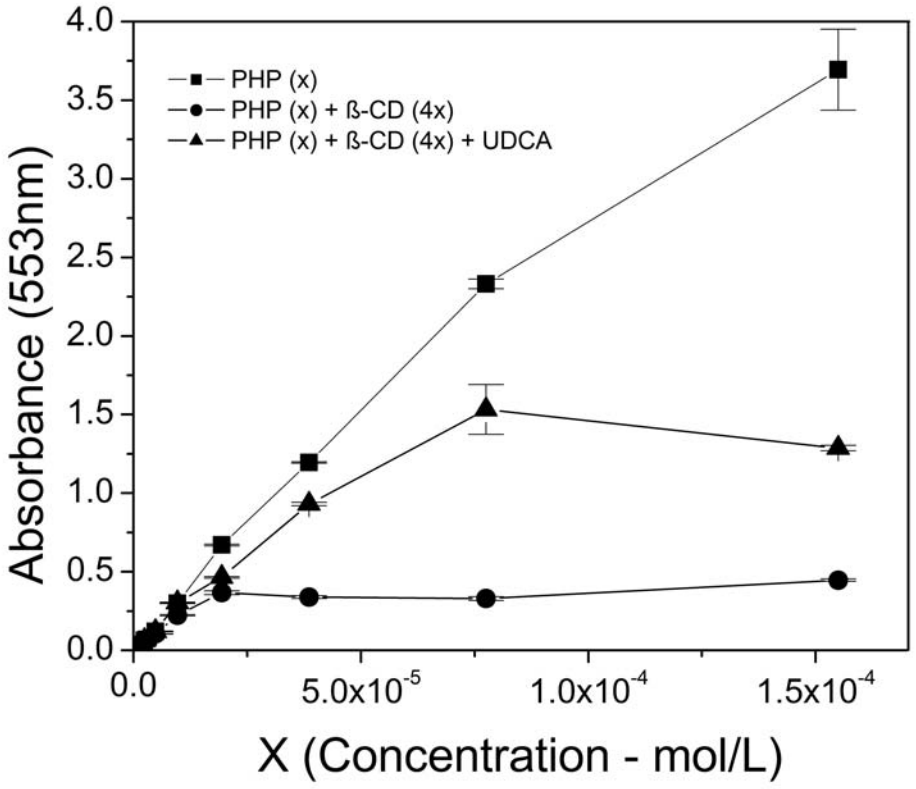
PHP (x)
PHP (x) + ß-CD (4x)
PHP (x) + ß-CD (4x) + UDCA
Absorbance (553nm)
X (Concentration - mol/L)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0.0
5.0x10-5
1.0x10-4
1.5x10-4

Figure 4

Spectrophotometric determination of deoxycholic and ursodeoxycholic acids by competitive complexation with phenolphthalein-β-cyclodextrin

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Tables

Table 1 -Experimental parameters using a full factorial design to determinate DCA[(a)] (2^3 factorial design) and UDCA[(b)] (2^4 factorial design). Factors in bold were statistically significant ($p<0.05$) and pure error **p** was of $1.017x10^{-4}$ and of $1.25x10^{-3}$ for DCA and UDCA, respectively.

Factors	*-1*	*Central point*	**+1**
pH	9.2[(a)]; 10.3[(b)]	9.5[(a)]; 10.5[(b)]	9.8[(a)]; 10.7[(b)]
Temperature (°C)	20	25	30
Concentration – β-CD-PHP ($mol \cdot L^{-1}$)	A[(a)]: $6.2x10^{-4}$:$1.55x10^{-4}$	B[(a)]: $1.24x10^{-3}$:$3.1x10^{-4}$	C[(a)]: $1.9x10^{-3}$:$4.65x10^{-4}$
	A[(b)]: $3.1x10^{-4}$:$7.75x10^{-5}$	B[(b)]: $6.2x10^{-4}$:$1.55x10^{-4}$	C[(b)]: $9.3x10^{-4}$:$2.33x10^{-4}$
Concentration - Buffer (mM)	50[(b)]	200[(b)]	350[(b)]

Results of the 2^3 full factorial design:

Factors	*effect*	**p**
Mean/interaction	**0.638500**	**0.00000021**
(1) pH	**0.430500**	**0.00001001**
(2) Temperature	0.013500	0.15462335
(3) [B-CD:PHP]	0.003000	0.70224584
1 by 2	-0.011000	0.22055080
1 by 3	**0.050500**	**0.00578764**
2 by 3	**0.028500**	**0.02805735**
1*2*3	0.003000	0.70224584

Results of the 2^4 factorial design

Factors	*effect*	**p**
Mean/Interaction	**1.26055**	**0.000001**
(1) pH	**0.11525**	**0.007339**
(2) Temperature	-0.03975	0.110165
(3) [BCD-PHP]	**-1.01550**	**0.000012**
(4) Buffer	**-0.05900**	**0.044509**
1 by 2	0.03175	0.170465
1 by 3	0.05150	0.061885
1 by 4	0.02700	0.224251
2 by 3	**0.10700**	**0.009056**
2 by 4	-0.01350	0.500771
3 by 4	-0.01075	0.586191
1*2*3	0.04150	0.100604
1*2*4	0.00000	1.000000
1*3*4	0.03125	0.175374

Table 2 - Summary of the best analytical parameters for the detrmination of deoxycholic and ursodeoxycholic acids using inclusion complex.

Analytical Parameters	*Deoxycholic acid*	*Ursodeoxycholic acid*
β-CD-PHP inclusion complex ($mol \cdot L^{-1}$)	$6.2x10^{-4}$:$1.55x10^{-4}$	$3.1x10^{-4}$:$7.75x10^{-5}$
Temperature (°C)	20-30	20-30
pH	10.5	10.5
Solubility	Water	Carbonate buffer 50mM
Reaction time	Instantaneous	Instantaneous
Wavelength (nm)	553	553
Linearity range ($mol \cdot L^{-1}$)	$6.1x10^{-6}$-$3.13x10^{-3}$	$6.05x10^{-6}$-$3.88x10^{-4}$
Limit of detection ($mol \cdot L^{-1}$)	$3.94x10^{-5}$	$4.08x10^{-5}$
Limit of quantification ($mol \cdot L^{-1}$)	$1.31x10^{-4}$	$1.36x10^{-4}$

Supplementary Material

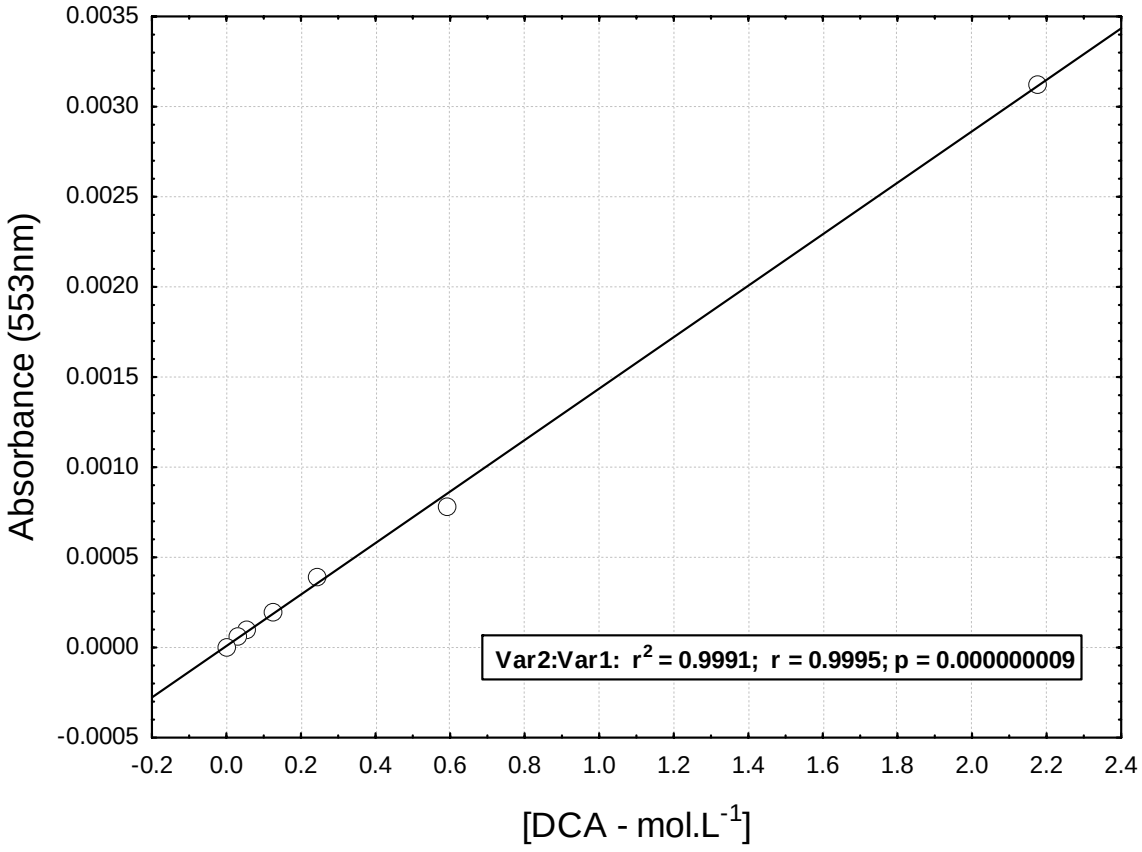

Fig. 1. Deoxycholic acid calibration graph at pH 10.5.

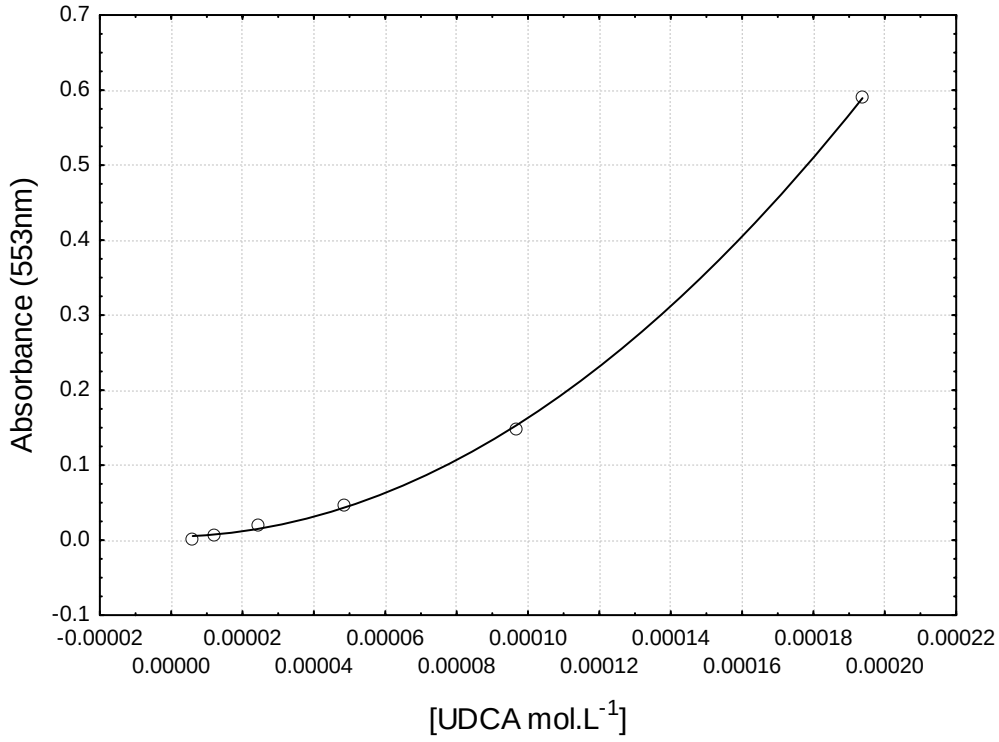

Fig. 2. Ursodeoxycholic acid quadratic calibration graph at pH 10.5.

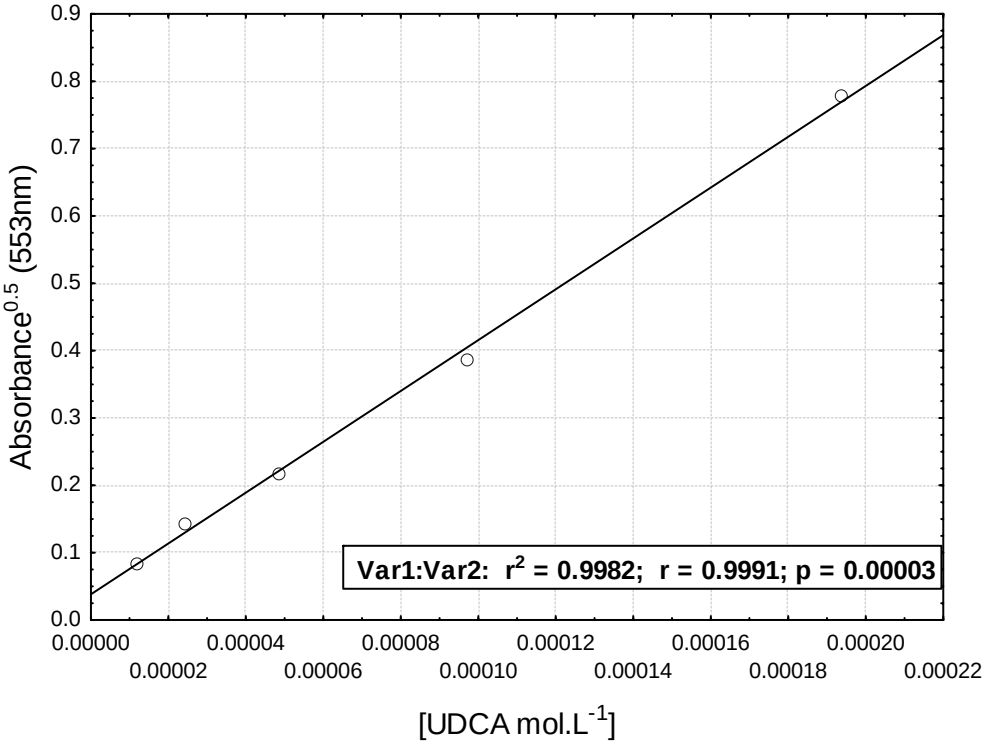

Fig. 3. Ursodeoxycholic acid calibration graph at pH 10.5.

CAPÍTULO 2

Artigo submetido à revista Biophysical Chemistry

Physical-chemical parameters and validation of a spectrophotometric method for deoxycholic and ursodeoxycholic acids determination in pharmaceuticals

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Abstract

This work reports the study of physical-chemical parameters and methodology validation for deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) assessment in different formulations. Moreover a simple dry chemistry approach is proposed. The method is based on competitive complexation reaction with phenolphthalein (PHP) to form β-cyclodextrin inclusion complexes. Temperature has a negative effect on the equilibrium constant resulting in high negative values of enthalpy and positive values of entropy. The inclusion complexes were stable for 12 days with a half-life of 68.71 days to DCA and 294.71 days for the UDCA determination. The methods were validated showing respectively limits of detection and limits of quantification of $4.92x10^{-5}mol{\cdot}L^{-1}$ and $1.64x10^{-4}mol{\cdot}L^{-1}$ for the DCA, $1.14x10^{-5}mol{\cdot}L^{-1}$ and $3.79x10^{-5}mol{\cdot}L^{-1}$ for UDCA. The method exhibits high stability, instantaneous reaction and affordability for optical chemical sensor implementation.

Keywords: Deoxycholic acid; Ursodeoxycolic acid; β-Cyclodextrin; Inclusion complex; Phenolphthalein; Thermodynamic parameters.

1. Introduction

Mesotherapy uses cutaneous injections of a mixture of compounds to treat localised pathologic conditions and with cosmetic proposes. It is of common use in Brazil by resorting to phosphatidylcholine injections [1,2], although they have been forbidden by national sanitary authorities like ANVISA (the Brazilian National Health Surveillance Agency) and FDA at US. The phosphatidylcholine injection possesses deoxycholic acid (DCA) as its major active component [3]. This is also used to enhance oral availability of biodegradable nanoparticles [4], as choleretic agent in liver dysfunctions [5], as *N*-(2-dimethylamino)ethyl derivatives in malaria [6] and in several cosmetic preparations [7]. Another important bile acid, the ursodeoxycholic acid (UDCA) is also widely used in the treatment of primary biliary cirrhosis, primary cholangitic sclerosis [8], cholelithiasis [9], to prevent the relapse of acute pancreatitis caused by microlithiasis [10] and to reduce alanine aminotransferase levels in hepatitis C [11]. Due to large application of both bile acids, a variety of methods are used to accomplish their content in pharmaceutical formulations, namely electrochemical (voltammetric), fluorimetric or spectrophtometric [12], HPLC [13] and micellar electrokinetic chromatographic [5]. In this work, a colorimetric approach based on the use of biodegradable cyclodextrins is proposed, once it enables quicker and inexpensive alternative for routine analysis.

The important property of cyclodextrins (CDs) - cyclic oligosaccharides with six (α-), seven (β-) or eight (γ-) glucose residues linked by α-(1–4) glycosidic bonds [14] - and their numerous derivatives is the ability to form inclusion complexes with inorganic and organic guests [15]. Concomitantly, inclusion in cyclodextrins exerts a profound effect on the physicochemical properties of guest molecules such as solubility, chemical

stability, absorption and bioavailability [16]. Guests reaction with CDs through competitive complexation with indicators has been used to assess the respective equilibrium constants (*Kc*) and other related thermodynamic parameters when direct determination of the complex fails. For example, phenolphthalein (PHP) is a typical acid/base indicator that forms a colorless 1:1 inclusion complex with β-CD and by this used in indirect determinations of colorless compounds by competitive complexation reaction [17,18]. Herein, the temperature dependence of the equilibrium constants of the PHP-, DCA- and UDCA-β-cyclodextrin was used to obtain the respective thermodynamic parameters, i.e. the standard free energy change ($\Delta G°$), the standard enthalpy change ($\Delta H°$) and the standard entropy change ($\Delta S°$), for subsequent validation of an alternative methodology for DCA and UDCA determinations and optical sensing approach.

2. Experimental

2.1. Materials

Analytical grade chemicals without any further purification treatment and double deionised water were thoroughly used. β-cyclodextrin was obtained from Fluka (Steinheim, Germany). Phenolphthalein, deoxycholic acid (sodium salt) and ursodeoxycholic acid were obtained from Sigma (St. Luiz, MO, USA). Absorption spectra were collected from a Pharmacia Ultrospec 3000pro UV/Vis spectrophotometer using 1-cm path length quartz cells. Statistical evaluations were carried out by means of the Statistica software (StatSoft Inc., Tulsa, OK, USA) and analysed images by means of a trial version of Adobe Photoshop CS2 software (Adobe systems, USA).

2.2. Methods

2.2.1. Methods development

The co-precipitation technique [19] enabled to prepare the inclusion complexes in batch conditions using the following order to mixture solutions: 1mL phenolphthalein solution, 1mL carbonate buffer (pH 10.5; 150mmol/L) [17], 1mL β-CD (1mL water in the control) and 1mL bile acid (1mL water in the control). The mixture was strongly mixed after each solution addition except for the one corresponding to the bile acid addition where the obtained mixture was just homogenized. The blank solution was composed of 1mL the same buffer plus 3mL of water.

Diverse absorption spectra were collected for pH 10.5 buffer solutions containing $1.55x10^{-4}mol{\cdot}L^{-1}$ phenolphthalein (PHP) and different amounts of β-cyclodextrin (β-CD), $3.88x10^{-5}$ to $6.20x10^{-4}mol{\cdot}L^{-1}$, at 25°C. For the spectra corresponding to DCA analysis, concentrations of this between $4.38x10^{-5}$ and $7.0x10^{-4}$ $mol{\cdot}L^{-1}$ and β-CD-PHP in the proportions $6.2x10^{-4}$:$1.55x10^{-4}mol{\cdot}L^{-1}$ was settled. Concerning analysis of UDCA, solutions with concentrations in between $1.19x10^{-5}$ and $1.9x10^{-4}mol{\cdot}L^{-1}$ of the bile acid plus β-CD-PHP – $3.1x10^{-4}$:$7.75x10^{-5}mol{\cdot}L^{-1}$ were sampled.

Optimum temperature conditions were assessed in the interval of 10 to 55°C for the concentrations of DCA ($8.75x10^{-5}$ to $1.40x10^{-3}mol{\cdot}L^{-1}$) and UDCA ($2.38x10^{-5}$ to $1.9x10^{-4}$ $mol{\cdot}L^{-1}$) while keeping pH constant at the value of 10.5.

Storage stability of β-cyclodextrin-phenolphthalein inclusion complex was evaluated by means of maintaining two solutions of the β-cyclodextrin-phenolphthalein complex at 25°C during 60 days, and using them to determine DCA ($7.0x10^{-4}mol{\cdot}L^{-1}$) and UDCA ($1.9x10^{-4}mol{\cdot}L^{-1}$) during this period.

2.2.2. Validation of methods

The methodology used to validate the determination of DCA and UDCA followed the procedures presented by the EMEA (European Medicines Agency – CPMP/ICH/381/95) and ANVISA (Brazilian National Health Surveillance Agency – RE 899, 29/05/03). Absorbance vs. concentration linearity was evaluated by triplicate calibration assays using ten solutions of either DCA with concentrations ranging from $8.3x10^{-6}$ to $3.36x10^{-3}$ mol·L^{-1} or from $8.0x10^{-6}$ to $4.0x10^{-4}$ mol·L^{-1} in UDCA. The assays were conducted following the experimental conditions previously established and linearity of the calibration graphs validated by both the least squares method and the one-way analysis of variance (ANOVA) for $p<0.05$. Calibrations were then established to ±20% of the test concentration using five different concentrations in triplicate: $5.6x10^{-4}$ to $8.4x10^{-4}$ mol·L^{-1} for the DCA and from $1.52x10^{-4}$ to $2.28x10^{-4}$ mol·L^{-1} for the UDCA, respectively. For evaluation of the repeatability, three concentrations of the DCA ($5.6x10^{-4}$, $7.0x10^{-4}$ and $8.4x10^{-4}$ mol·L^{-1}) and UDCA ($1.52x10^{-4}$, $1.9x10^{-4}$ and $2.28x10^{-4}$ mol·L^{-1}) were assayed in nine determinations (3 concentrations/3 replicates). Intermediate precision was assessed with solutions of the same concentration in a 2^2 full factorial design considering different analysts and equipments (Pharmacia Ultrospec 3000pro and Micronal B582). The limit of detection defined as $C_L = 3.3S_B/m$, where C_L, S_B, and m are respectively the limit of detection, standard deviation and slope, was calculated. Also the same approach was for the limit of quantification defined by $C_{LQ} = 10S_B/m$. Accuracy was established by comparison of the concentrations found in pharmaceutical formulations (Table 1) with the ones obtained for standardized solutions of DCA ($5.6x10^{-4}$, $7.0x10^{-4}$ and $8.4x10^{-4}$ mol·L^{-1}) and UDCA ($1.52x10^{-4}$, $1.9x10^{-4}$ and $2.28x10^{-4}$ mol·L^{-1}) in nine determinations (3 concentrations/ 3 replicates).

[TABLE 1 here]

The procedures robustness was evaluated through variations in its defining parameters (pH and water) using different settling conditions for each variation.

3.3. Optical chemical sensor approach

Optical chemical sensor strips were implemented using 2x3.5cm Bristol-paper strips afterwards soaked for 2 minutes in 5mL of 1% (w/v) sodium alginate gel containing the β-CD-PHP inclusion complex ($6.2x10^{-4}$:$1.55x10^{-4}$ $mol{\cdot}L^{-1}$ for DCA and $3.1x10^{-4}$:$7.75x10^{-5}$ $mol{\cdot}L^{-1}$ for UDCA) and finally dried for 24h (25°C). Later, 20µL of DCA ($1.68x10^{-3}$ to $8.40x10^{-3}$ $mol{\cdot}L^{-1}$) or UDCA ($4.56x10^{-4}$ to $2.28x10^{-3}$ $mol{\cdot}L^{-1}$) solution was added. Colour changes were evaluated using a scanner (Model HP 5590) in which the images of high definition 600dpi were produced. The monitored signals of the DCA and UDCA concentrations were obtained by the observed RGB (red, green, and blue colour system) values of digital images [20].

3. Results and Discussion

3.1. Methods development

The spectral changes obtained for phenolphthalein solutions in the presence of different β-CD concentrations are shown in Fig. 1A. Phenolphthalein interacts strongly with the β-CD in alkaline pH since a more than 95% decrease in the absorption band at the wavelength of 553nm is observed. Whilst enclosed in the β-CD cage, ionized red

form of PHP is forced into its colourless lactone structure, however without protonation of the phenolic groups [17]. Spectra of phenolphthalein solutions with increasing concentrations of β-CD revealed a proportional decrease of the free PHP up to a concentration of $6.2x10^{-4}mol{\cdot}L^{-1}$ at pH 10.5 (concentrations ratio of 1:4 PHP:β-CD). Higher concentrations of β-CD did not cause additional observable decrease in absorbency. However, following the addition of bile acids PHP is forced to leave the cavity of β-CD molecules transmitting again red colour to the solution (Fig. 1B and 1C). Thus, the extent of the solution colour change can then be easily determined by calibration curve of the free PHP and the corresponding concentration related with the amount of bile acid.

[FIGURE 1 Here]

Influence of temperature on the absorbance of solutions containing the complexes with PHP, DCA and UDCA is revealed in Fig. 2. It is noticed that with the increase of temperature a corresponding increase in absorbance is induced. Similar findings were previously described by Zarzycki and Lamparczyk [21] being this effect caused by destabilization of the complex [19], with consequent phenolphthalein release. Hence, it becomes difficult to distinguish between the absorbance increase caused by an increase in the bile acid affinity by the β-CD cavity or the simple destabilization of the inclusion complex. For this reason the temperature in the remaining studies was fixed at 25°C, once it enabled both an extended absorbance range for free PHP and β-CD-PHP inclusion complex, and robustness since no significant differences ($p < 0.05$ by Turkey's test) of results are obtained working between 20-30°C.

[FIGURE 2 Here]

The equilibrium constant ($Kc_{1:1}$ bile acid/cyclodextrin) for the β-CD-PHP, β-CD-DCA and β-CD-UDCA inclusion complexes can be obtained by the Benesi–Hildbrand plot [22,23] according to Eq. (1-2):

$$PHP + \beta - CD \rightleftharpoons \beta - CD - PHP\,, K_c = \frac{[\beta - CD - PHP]}{[PHP][\beta - CD]} \quad (1)$$

$$\frac{1}{A - A_0} = \frac{1}{a} + \frac{1}{aK_c[\beta - CD_0]} \quad (2)$$

where A and A_0 are respectively the absorbance of PHP in the presence and absence of β-CD, a is a constant related to the molar absorption coefficients changes, and $[\beta\text{-}CD]_0$ is the initial concentration of β-CD. The competitive complexation equilibrium among DCA or UDCA with β-CD is described by Eqs. (3 - 5):

$$DCA + \beta - CD \rightleftharpoons \beta - CD - DCA\,,\ K_{c-DCA} = \frac{[\beta - CD - DCA]}{[DCA][\beta - CD]} \quad (3)$$

$$UDCA + \beta - CD \rightleftharpoons \beta - CD - UDCA\,, K_{c-UDCA} = \frac{[\beta - CD - UDCA]}{[UDCA][\beta - CD]} \quad (4)$$

$$\frac{1}{A - A_0} = \frac{1}{a} + \frac{1}{aK_c[PHP_0]} \quad (5)$$

For the selected wavelength of 553 nm the corresponding molar absorptivity β-CD-PHP complex is negligible. The absorbance of the solution is mainly due to free (uncomplexed) PHP and indicative of the respective concentration. In turn, PHP releasing determines an absorbance increase that is proportional to bile acids concentration in solution, so allowing calculating *Kc*. The $[PHP_0]$ is the PHP concentration released (uncomplexed) for β-CD. *Kc* was determined in various temperatures (25 to 55°C) at pH 10.5. The Table 2 shows the temperature influence in the equilibrium constant ($Kc_{1:1}$ bile acid/cyclodextrin).

The thermodynamic parameters (Table 2) were calculated according to the Van´t Hoff Eq. (5) which describes the temperature dependence in function of *K*. The *lnK* values were plotted as a function of the inverse temperature to give a linear relationship. Then, the enthalpy (*ΔH*°) and entropy (*ΔS*°) changes were obtained from the slope and the intercept of the curve [19,24,25].

$$\ln K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (6)$$

Standard free energy change (*ΔG*°) was obtained according to the Eq. (7):

$$\Delta G^\circ = -RT \ln K \quad (7)$$

The Van't Hoff plots for the studied complexes were linear and exhibited largely negative *ΔH*°, thus indicating exothermic inclusion processes and positive *ΔS*°. It was also observed an enthalpy decrease and entropy increase with the bile acids inclusion complexes formation in respect to the β-CD-PHP inclusion complex. The inclusion processes of DCA and UDCA in β-CD cage is more favourable and spontaneous than that of PHP. In all cases *ΔG*° was negative indicating that the inclusion complexes formation was spontaneous [19].

[TABLE 2 Here]

The formation of an inclusion complex with cyclodextrin is caused by interactions such as hydrogen bonding with the OH groups at the periphery of the cavity, Van der Waals interactions and hydrophobic effects. Generally, solute inclusion in the cyclodextrin cavity is associated with large negative values of *ΔH*°. Either negative or slightly positive *ΔS*° values indicate inclusion complexation of the guest without extensive desolvation in a primarily enthalpy-driven process [26]. The negative enthalpy values strength the contribution from Van der Waals forces in the formation of

the inclusion complex while the positive entropy explained the relaxation of water molecules from the cavity and from the hydration shell of the including guest [15,27].

In general, the β-CD-PHP inclusion complex was stable for 12 days. A loss of about 30% in DCA determinations and of 12% regarding UDCA determinations were observed after 30 days of storage at 25°C (Fig. 3). The inactivation constant (k_i) of the inclusion complex was enabled by:

$$\ln A = \ln A_0 - k_i t \quad (7)$$

where A_0 is the initial absorbance of the bile acids and A is the final absorbance of the bile acids after 60 days. Furthermore, the half-life ($t_{½}$) for the inclusion complex can be obtained by:

$$t_{1/2} = \ln 2 / k_i \quad (8)$$

[FIGURE 3 Here]

The inactivation constant (*ki*) of the inclusion complex was calculated at 25°C with value of $1.01 \times 10^{-2} days^{-1}$ for DCA determination and $2.35 \times 10^{-3} days^{-1}$ for UDCA determination. The half-life (t½) for the inclusion complex was 68.71 days for DCA determination and 294.71 days for the UDCA determination. Despite the greater stability of the β-CD-PHP inclusion complex for UDCA relatively to β-CD-PHP complex for DCA determination, the latter allows determinations in more concentrated solutions (data not shown) and pharmaceuticals formulations containing high DCA concentrations [2,3,28].

3.2. Validation of methods

The proposed spectrophotometric methods for DCA and UDCA determinations were validated according to the EMEA and ANVISA guidelines. Thus the validation characteristics addressed were linearity, accuracy, precision, specificity, limits of detection and quantification and robustness (Table 3). The calibration graphs for the linearity assays were constructed with 10 concentrations showing correlation coefficients higher than 0.998. For the range (80-120%), the calibration graph was constructed with 5 concentrations showing a correlation coefficient of the same order of magnitude. The methods showed to be precise, since coefficients of variation less than 5% were obtained. Furthermore, the results obtained through a 2^2 full factorial design for the study of the intermediate precision showed no significant effects ($p<0.05$). These results showed that different analysts and equipments do not interfere in the methods. The accuracy was assessed through comparison of the concentrations found in pharmaceutical formulations with standardised solutions for which recovery data corroborates the assumption. In the concentrations studied, the excipients used in the formulations and water type did not interfere in the results. The optimum pH was fixed at 10.5, similar results were obtained by Afkhami et al. [17] and Glazyrin et al. [18] Determinations performed at higher pH conditions did not cause significant changes of results and are limited by buffering power of carbonate buffer.

[TABLE 3 Here]

The limits of detection were of $4.92x10^{-5}mol{\cdot}L^{-1}$ for DCA and of $1.14x10^{-5}mol{\cdot}L^{-1}$ for UDCA determinations, respectively. The corresponding limits of quantification were of $1.64x10^{-4}mol{\cdot}L^{-1}$ and $3.79x10^{-5}mol{\cdot}L^{-1}$.

3.3. 3.3. Optical chemical sensor approach

Alginates are largely used for biomolecule immobilisation because they provide simple implementation using a biodegradable and non toxic material [29]. In the present study, the alginate did not interfere in the competitive complexation reaction because only the last one caused colour changes in the digital images created. The exploitation of digital images obtained from a scanner is a recent instrumental detection technique for optical sensing applications. In this technique the analytical signal corresponds to the RGB-based value that was calculated from each digital image, using the proposed procedure based on the red, green, and blue colour system [20,30]. The test was simply based on drop wise 20μL of bile acid solution and immediate scan of the strip, which it was possible to attain a calibration graph for DCA obeying to the equation: RGB = 1553.89 (±31.79) $DCA_{mol/L}$ - 0.778(±0.16) with a correlation coefficient of 0.99937. For UDCA the corresponding equation was of RGB = 5671.43(±228.78) $UDCA_{mol/L}$ + 0.611(±0.31) with the correlation coefficient of 0.99757.

4. Conclusion

The development of low cost methods for determining DCA and UDCA are important in quality control of raw materials and pharmaceutical formulations to prevent misuses and accidents when used for aesthetic purposes, specifically for the

DCA. On the basis of this work, the competitive complexation reaction was used for the study of physical-chemical parameters. The temperature had a negative effect on the equilibrium constant and should be settled between 20-30°C for optimised bile acids determination. Thermodynamics parameters were evaluated showing in all cases, that spontaneous competitive complexation reactions took place. The inclusion complexes were stable for 12 days with a half-life of 68.71 days to DCA and 294.71 days for the UDCA determination. The proposed methods offer good linearity and precision and can be applied to the analysis of a wide concentration range of DCA and UDCA in real samples with satisfactory results. The optical chemical sensor method offers good linearity and can be used as an alternative to the spectrophotometric method.

Acknowledgements

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References

[1]A.M. Rotunda, and M.S. Kolodney, Mesotherapy and phosphatidylcholine injections: Historical clarification and review. Dermatol. Surg. 32 (2006) 465-480.

[2]M.E. Yagima Odo, L.C. Cuce, L.M. Odo, and A. Natrielli, Action of sodium deoxycholate on subcutaneous human tissue: Local and systemic effects. Dermatol. Surg. 33 (2007) 178-188.

[3]A.M. Rotunda, H. Suzuki, R.L. Moy, and M.S. Kolodney, Detergent effects of sodium deoxycholate are a major feature of an injectable phosphatidylcholine formulation used for localized fat dissolution. Dermatol. Surg. 30 (2004) 1001-1008.

[4]R.M. Samstein, K. Perica, F. Balderrama, M. Look, and T.M. Fahmy, The use of deoxycholic acid to enhance the oral bioavailability of biodegradable nanoparticles. Biomaterials 29 (2008) 703-708.

[5]V.G. Rodriguez, S.E. Lucangioli, G.C. Fernandez Otero, and C.N. Carducci, Determination of bile acids in pharmaceutical formulations using micellar electrokinetic chromatography. J. Pharm. Biomed. Anal. 23 (2000) 375-381.
[6]N. Terzic, D. Opsenica, D. Milic, B. Tinant, K.S. Smith, W.K. Milhous, and B.A. Ŝolaja, Deoxycholic acid-derived tetraoxane antimalarials and antiproliferatives. J. Med. Chem. 50 (2007) 5118-5127.
[7]C. Valenta, E. Nowack, and A. Bernkop-Schnurch, Deoxycholate-hydrogels: novel drug carrier systems for topical use. Int. J. Pharm. 185 (1999) 103-111.
[8]K.D. Lindor, Ursodiol for primary sclerosing cholangitis. N. Engl. J. Med. 336 (1997) 691-695.
[9]M.L. Petroni, R.P. Jazrawi, P. Pazzi, A. Lanzini, M. Zuin, M.G. Pigozzi, M. Fracchia, G. Galatola, V. Alvisi, K.W. Heaton, M. Podda, and T.C. Northfield, Ursodeoxycholic acid alone or with chenodeoxycholic acid for dissolution of cholesterol gallstones: A randomized multicentre trial. Aliment. Pharmacol. Ther. 15 (2001) 123-128.
[10]N. Okoro, A. Patel, M. Goldstein, N. Narahari, and Q. Cai, Ursodeoxycholic acid treatment for patients with postcholecystectomy pain and bile microlithiasis. Gastrointest. Endosc. 68 (2008) 69-74.
[11]T. Ikegami, and Y. Matsuzaki, Ursodeoxycholic acid: Mechanism of action and novel clinical applications. Hepatol. Res. 38 (2008) 123-131.
[12]O. Arias De Fuentes, L. Campanella, G. Crescentini, A. Falcioni, M.P. Sammartino, and M. Tomassetti, Flow injection analysis of cholic acids in pharmaceutical preparations using a polymeric membrane ISE as detector. J. Pharm. Biomed. Anal. 23 (2000) 89-98.
[13]S. Scalia, P. Pazzi, and M. Guarneri, Determination of bile acids in pharmaceutical dosage forms by HPLC. Anal. Lett. 22 (1989) 915-927.
[14]C. Yanez, R. Salazar, L.J. Nunez-Vergara, and J.A. Squella, Spectrophotometric and electrochemical study of the inclusion complex between β-cyclodextrin and furnidipine. J. Pharm. Biomed. Anal. 35 (2004) 51-56.
[15]M.E. Brewster, and T. Loftsson, Cyclodextrins as pharmaceutical solubilizers. Adv. Drug Deliver. Rev. 59 (2007) 645-666.
[16]M. Zhang, J. Li, L. Zhang, and J. Chao, Preparation and spectral investigation of inclusion complex of caffeic acid with hydroxypropyl-β-cyclodextrin. Spectrochim. Acta A 71 (2009) 1891-1895.
[17]A. Afkhami, T. Madrakian, and L. Khalafi, Spectrophotometric determination of fluoxetine by batch and flow injection methods. Chem. Pharm. Bull. 54 (2006) 1642-1646.
[18]A.E. Glazyrin, M.K. Grachev, G.I. Kurochkina, and E.E. Nifant'ev, Inclusion compounds of some water-soluble β-cyclodextrin derivatives with phenolphthalein. Russ. J. Gen. Chem. 74 (2004) 1922-1925.
[19]E.M.M. Del Valle, Cyclodextrins and their uses: a review. Process Biochem. 39 (2004) 1033-1046.
[20]C.X. Yang, X.Y. Sun, B. Liu, and H.T. Lian, Determination of Total Phosphorus in Water Sample by Digital Imaging Colorimetry. Chinese J. Anal. Chem. 35 (2007) 850-853.
[21]P.K. Zarzycki, and H. Lamparczyk, The equilibrium constant of β-cyclodextrin-phenolphtalein complex; influence of temperature and tetrahydrofuran addition. J. Pharm. Biomed. Anal. 18 (1998) 165-170.
[22]A.A. Abdel-Shafi, Spectroscopic studies on the inclusion complex of 2-naphthol-6-sulfonate with β-cyclodextrin. Spectrochim. Acta A 66 (2007) 732-738.

[23]H.A. Benesi, and J.H. Hildebrand, A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. J. Am. Chem. Soc. 71 (1949) 2703-2707.
[24]H.Y. Wang, J. Han, and X.G. Feng, Spectroscopic study of orange G-β-cyclodextrin complex and its analytical application. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 66 (2007) 578-585.
[25]F. Yuexian, Y. Yu, S. Shaomin, and D. Chuan, Molecular recognition of α-cyclodextrin (CD) to choral amino acids based on methyl orange as a molecular probe. Spectrochim. Acta A 61 (2005) 953-959.
[26]C. Jullian, T. Orosteguis, F. Pérez-Cruz, P. Sánchez, F. Mendizabal, and C. Olea-Azar, Complexation of morin with three kinds of cyclodextrin. A thermodynamic and reactivity study. Spectrochim. Acta A 71 (2008) 269-275.
[27]G. Castronuovo, and M. Niccoli, Thermodynamics of inclusion complexes of natural and modified cyclodextrins with propranolol in aqueous solution at 298 K. Bioorg. Med. Chem. 14 (2006) 3883-3887.
[28]S. Schuller-Petrovic, G. Wölkart, G. Höfler, N. Neuhold, F. Freisinger, and F. Brunner, Tissue-toxic effects of phosphatidylcholine/deoxycholate after subcutaneous injection for fat dissolution in rats and a human volunteer. Dermatol. Surg. 34 (2008) 529-542.
[29]J. Ha, C.R. Engler, and J.R. Wild, Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads. Bioresour. Technol. 100 (2009) 1138-1142.
[30]E.d.N. Gaiao, V.L. Martins, W.d.S. Lyra, L.F.d. Almeida, E.C.d. Silva, and M.C.U. Araújo, Digital image-based titrations. Anal. Chim. Acta 570 (2006) 283-290.

Physical-chemical parameters and validation of a spectrophotometric method for deoxycholic and ursodeoxycholic acid determinations in pharmaceuticals

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Captions for figures

Fig. 1. Absorption spectrum of phenolphthalein (PHP - 1A) ($1.55x10^{-4}$ mol·L^{-1}) at pH 10.5 in different β-cyclodextrin (β-CD) concentrations ($3.88x10^{-5}$ to $6.20x10^{-4}$ mol·L^{-1}). Determination of ($4.38x10^{-5}$ to $7.0x10^{-4}$ mol·L^{-1}) deoxycholic acid concentrations (DCA - 1B) by the inclusion complex of β-CD-PHP ($6.2x10^{-4}$:$1.55x10^{-4}$ mol·L^{-1}) and ($1.19x10^{-5}$ to $1.9x10^{-4}$ mol·L^{-1}) of ursodeoxycholic acid concentrations (UDCA - 1C) by the inclusion complex of β-CD-PHP ($3.1x10^{-4}$:$7.75x10^{-5}$ mol·L^{-1}).

Fig. 2. Temperature effect (10-55°C) on the phenolphthalein (PHP): β-CD-PHP inclusion complex ($6.2x10^{-4}$:$1.55x10^{-4}$ mol·L^{-1}) formation and complex interaction with deoxycholic (DCA - $7.0x10^{-4}$ mol·L^{-1}) and ursodeoxycholic acids (UDCA - $1.9x10^{-4}$ mol·L^{-1}).

Fig 3. Storage stability of inclusion complex (β-CD-PHP – $6.2x10^{-4}$:$1.55x10^{-4}$ mol·L^{-1} for DCA and $3.1x10^{-4}$:$7.75x10^{-5}$ mol·L^{-1} for UDCA) for bile acids determination.

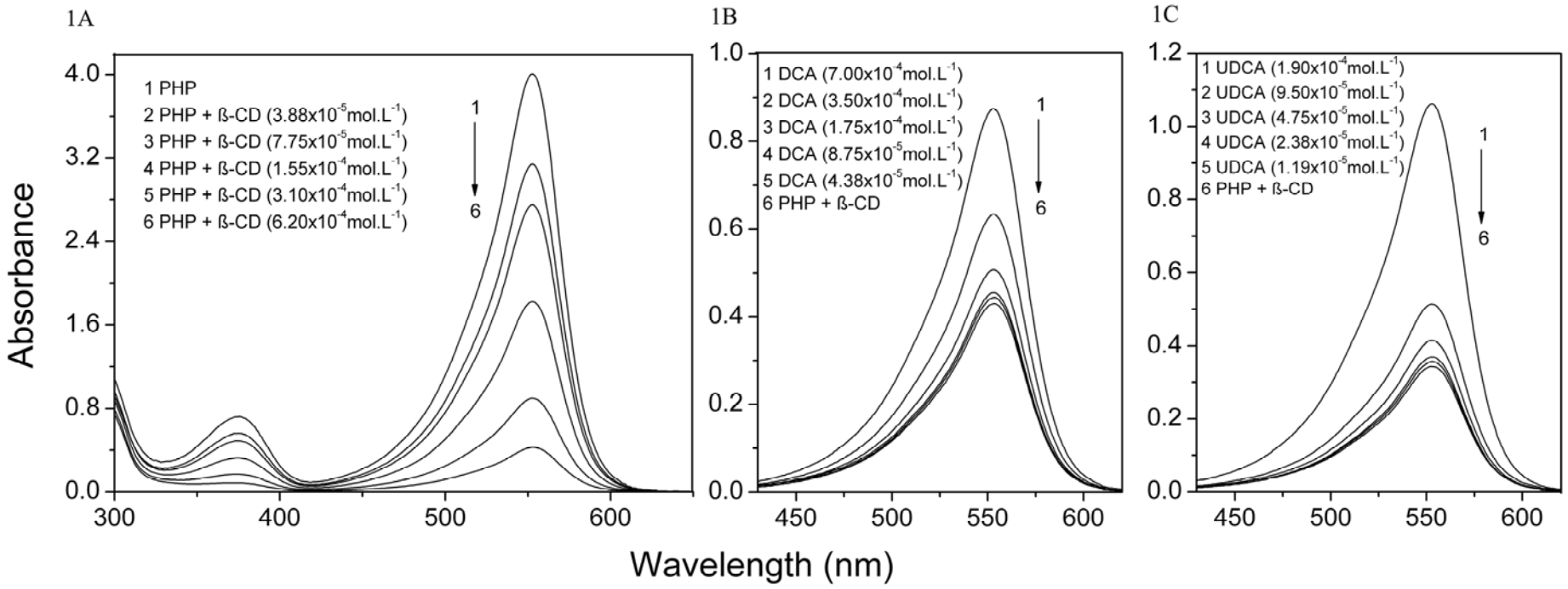
1A
1 PHP
2 PHP + ß-CD (3.88x10-5mol.L-1)
3 PHP + ß-CD (7.75x10-5mol.L-1)
4 PHP + ß-CD (1.55x10-4mol.L-1)
5 PHP + ß-CD (3.10x10-4mol.L-1)
6 PHP + ß-CD (6.20x10-4mol.L-1)
1
6
1B
1 DCA (7.00x10-4mol.L-1)
2 DCA (3.50x10-4mol.L-1)
3 DCA (1.75x10-4mol.L-1)
4 DCA (8.75x10-5mol.L-1)
5 DCA (4.38x10-5mol.L-1)
6 PHP + ß-CD
1C
1 UDCA (1.90x10-4mol.L-1)
2 UDCA (9.50x10-5mol.L-1)
3 UDCA (4.75x10-5mol.L-1)
4 UDCA (2.38x10-5mol.L-1)
5 UDCA (1.19x10-5mol.L-1)
6 PHP + ß-CD
Absorbance
Wavelength (nm)

Figure 1

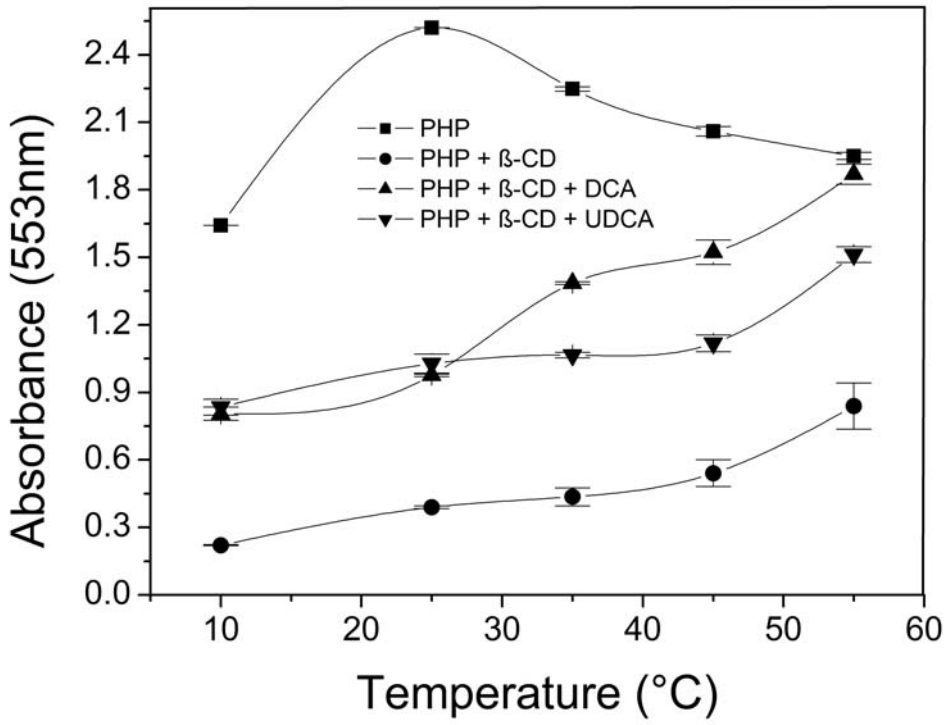
Absorbance (553nm)
Temperature (°C)
PHP
PHP + ß-CD
PHP + ß-CD + DCA
PHP + ß-CD + UDCA
0.0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
10
20
30
40
50
60

Figure 2

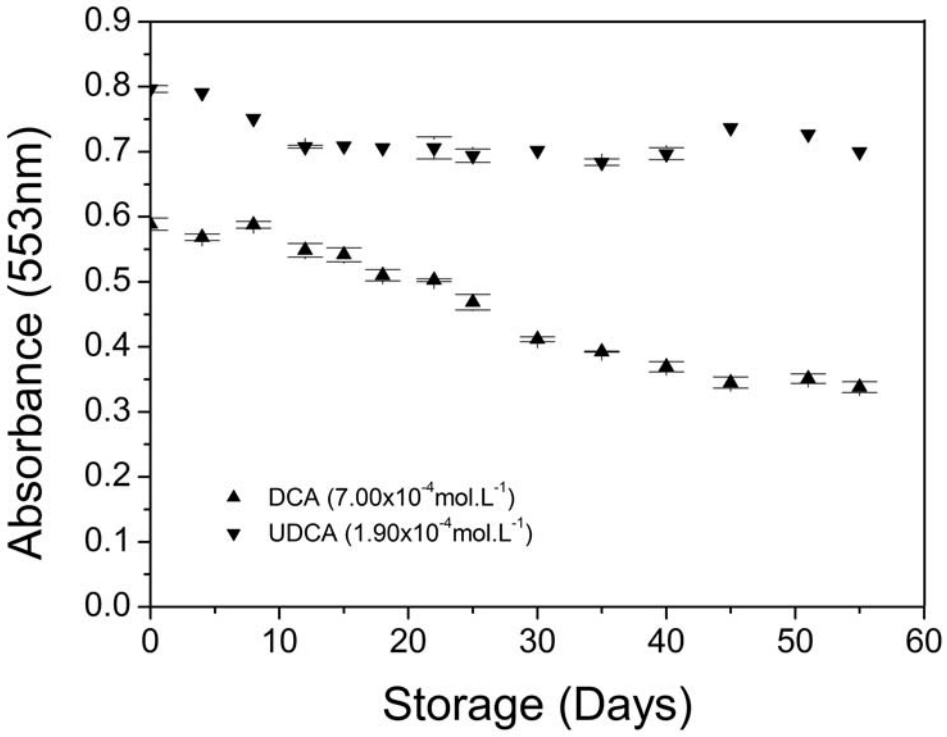
Absorbance (553nm)
Storage (Days)
DCA (7.00x10^{-4}mol.L^{-1})
UDCA (1.90x10^{-4}mol.L^{-1})
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
10
20
30
40
50
60

Figure 3

Physical-chemical parameters and validation of a spectrophotometric method for deoxycholic and ursodeoxycholic acids determination in pharmaceuticals

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Tables

Table 1

Pharmaceutical formulations used in the study of the accuracy evaluation.

Drugs	*Formulations*
Injectable Phosphatidylcholine formula [3]	Phosphatidylcholine 5% (w/v), deoxycholic acid (sodium salt) 4.75% (w/v), Benzyl alcohol 0.9% (v/v), Water 100 mL.
Injectable deoxycholate formula [2]	Deoxycholic acid (sodium salt) 2.5% (w/v), Benzyl alcohol 1% (v/v), propylene glycol 10% (v/v), Water 100mL.
Ursacol	Ursodeoxycholic acid 300mg and excipients: lactose, povidone, crospovidone, Magnesium stearate

Table 2

The values of the equilibrium constant (*Kc*) of β-cyclodextrin-phenolphtalein (β-CD-PHP) complex without and with the addition of deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) calculated at different temperatures and the thermodynamic parameters of inclusion complexes. Some values are shown without signalled precision once this was better than 0.005.

T (K)	*298*	*308*	*318*	*328*
β-CD-PHP				
Kc (x10^4L·mol^{-1})	1.67(±0.11)	1.40(±0.11)	1.17(±0.07)	0.93(±0.04)
ΔG° (kJ·mol^{-1})	-2.42(±0.19)	-2.45(±0.16)	-2,49(±0.19)	-2.50(±0.13)
ΔH° (kJ·mol^{-1})	-15.62(±1.05)			
ΔS° (J·mol^{-1}°K^{-1})	25.56(±3.35)			
β-CD-DCA				
Kc (x10^4L·mol^{-1})	2.60	2.39	2.12	1.77
ΔG° (kJ·mol^{-1})	-2.53	-2.59	-2.64	-2.68
ΔH° (kJ·mol^{-1})	-10.25(±1.48)			
ΔS° (J·mol^{-1}°K^{-1})	50.31(±4.74)			
β-CD-UDCA				
Kc (x10^4L·mol^{-1})	2.81	2.43	2.13	1.76
ΔG° (kJ·mol^{-1})	-2.55	-2.59	-2.64	-2.67
ΔH° (kJ·mol^{-1})	-12.47(±0.96)			
ΔS° (J·mol^{-1}°K^{-1})	43.42(±3.12)			

Table 3

Validation data ($p<0.05$)

Linearity							
Linearity range		DCA $8.30x10^{-6}$-$1.68x10^{-3}$ mol·L^{-1}		UDCA $8.00x10^{-6}$-$2.28x10^{-3}$ mol·L^{-1}			
Calibration curve		ABS = -0.0069(±0.0091) + 870.0373(±12.4216)$DCA_{mol/L}$		$ABS^{0.5}$=0.0699(±0.011) + 4506.688(±77.1179)$UDCA_{mol/L}$			
Correlation coefficient		0.99919		0.99898			
ANOVA[1]							
		Sums of squares	d.f.	Mean squares	F		
DCA	Regression	**1.802878**	**1**	1.802878	**4905.942**		
	Residual	0.002940	8	0.000367			
	Total	1.805818					
UDCA	Regression	**1.208491**	**1**	1.208491	**3415.105**		
	Residual	0.002477	7	0.000354			
	Total	1.210968					
Range							
Correlation coefficient		0.99898		0.99989			
Precision							
Repeatability							
	[2]	ABS	%R.S.D.	ABS	%R.S.D.	ABS	%R.S.D.
DCA	$8.4x10^{-4}$ mol·L^{-1}	0.680	0.96	0.640	0.72	0.648	1.49
	$7.0x10^{-4}$ mol·L^{-1}	0.555	1.10	0.544	0.18	0.567	0.35
	$5.6x10^{-4}$ mol·L^{-1}	0.421	0.99	0.433	2.91	0.442	0.85
UDCA[3]	$2.28x10^{-4}$ mol·L^{-1}	1.123	0.21	1.129	0.51	1.118	0.56
	$1.9x10^{-4}$ mol·L^{-1}	0.936	0.54	0.928	1.08	0.920	1.36
	$1.52x10^{-4}$ mol·L^{-1}	0.724	1.17	0.728	1.87	0.725	2.11
Intermediate precision (Results of the 2^3 full factorial design)[1]							
		DCA		UDCA			
Factors		effect	p	effect	p		
Mean/Interaction		0.557250	0	0.751500	0		
Analysts (1)		-0.006500	0.460437	-0.019333	0.348727		
Equipments (2)		0	1.000000	-0.019667	0.340665		
1 by 2		0.010667	0.231109	-0.010333	0.613656		
Accuracy[4]							
		Pure sample	Injectable Phosphatidylcholine		Injectable deoxycholate		
DCA	$5.6x10^{-4}$ mol·L^{-1}	98.97(±0.84)	97.11(±1.43)		98.08(±0.43)		
	$7.0x10^{-4}$ mol·L^{-1}	101.36(±0.36)	101.33(±1.28)		102.77(±0.63)		
	$8.4x10^{-4}$ mol·L^{-1}	99.31(±1.48)	100.51(±0.99)		102.52(±0.38)		
		Pure sample	Ursacol				
UDCA[3]	$1.52x10^{-4}$ mol·L^{-1}	99.43(±1.17)	98.1(±0.49)				
	$1.9x10^{-4}$ mol·L^{-1}	100.89(±0.54)	98.7(±1.86)				
	$2.28x10^{-4}$ mol·L^{-1}	99.63(±0.21)	99.22(±0.81)				
Robustness[4]							
pH	DCA	UDCA	Water	DCA	UDCA		
10.3	-	97.75(±1.49)	Distilled	102.38(±1.52)	100.42(±0.95)		
10.4	97.84(±3.53)	99.19(±1.76)	Deionised	100.11(±4.48)	98.04(±1.86)		
10.5	100.00(±3.48)	100.0(±2.15)	Double	100(±2.18)	100(±0.66)		
10.6	102.62(±2.48)	99.01(±1.85)	deionised				
10.7	104.50(±0.46)	98.46(±0.97)					
Limit of detection		DCA - $4.92x10^{-5}$ mol·L^{-1}		UDCA - $1.14x10^{-5}$ mol·L^{-1}			
Limit of quantification		DCA - $1.64x10^{-4}$ mol·L^{-1}		UDCA - $3.79x10^{-5}$ mol·L^{-1}			

[1] Factors in bold were statistically significant ($p<0.05$).
[2] ABS – Absorbance; %R.S.D. – Relative standard deviation (%).
[3] $(Absorbance)^{0.5}$.
[4] %mean ± %R.S.D..

Supplementary Material

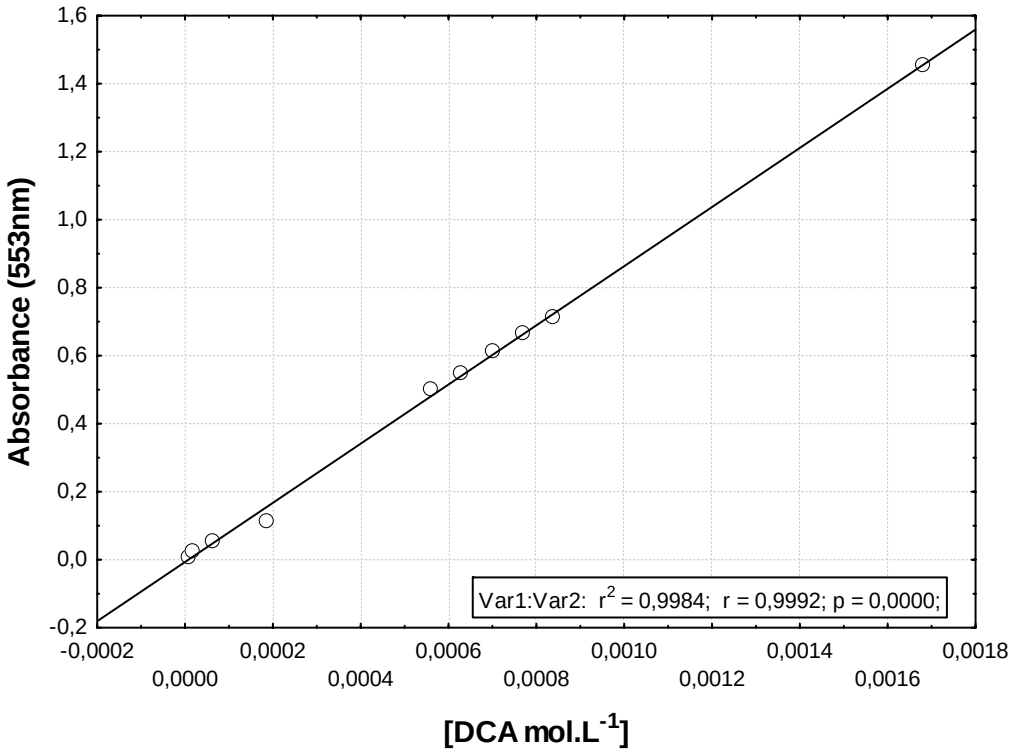

Fig. 1. Deoxycholic acid calibration graph at pH 10.5.

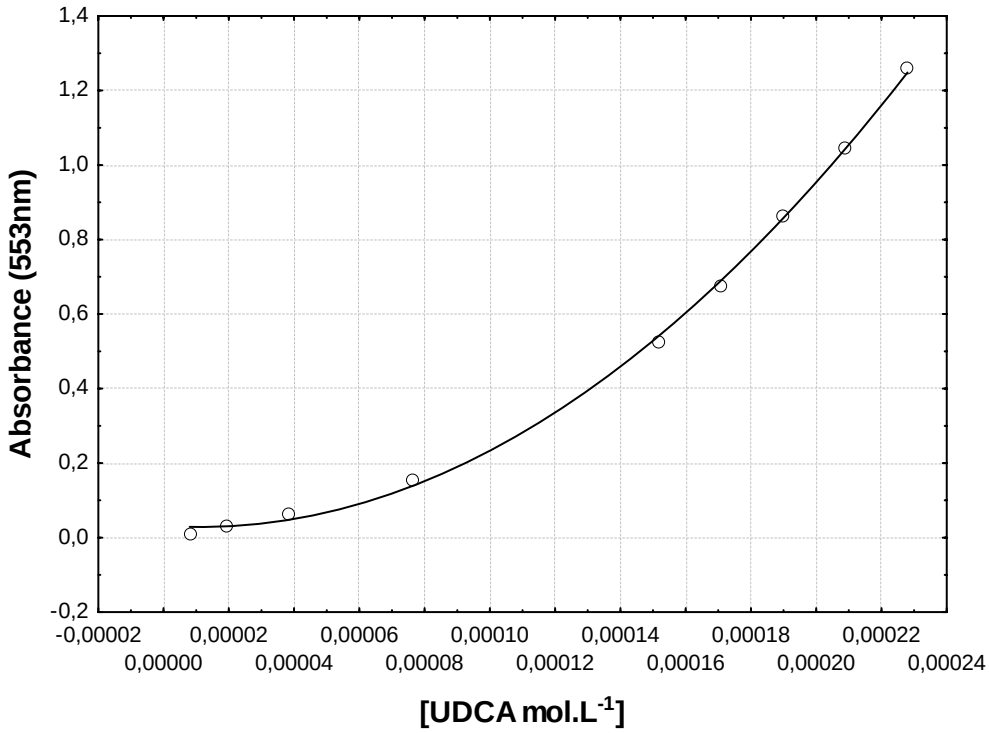

Fig. 2. Ursodeoxycholic acid quadratic calibration graph at pH 10.5.

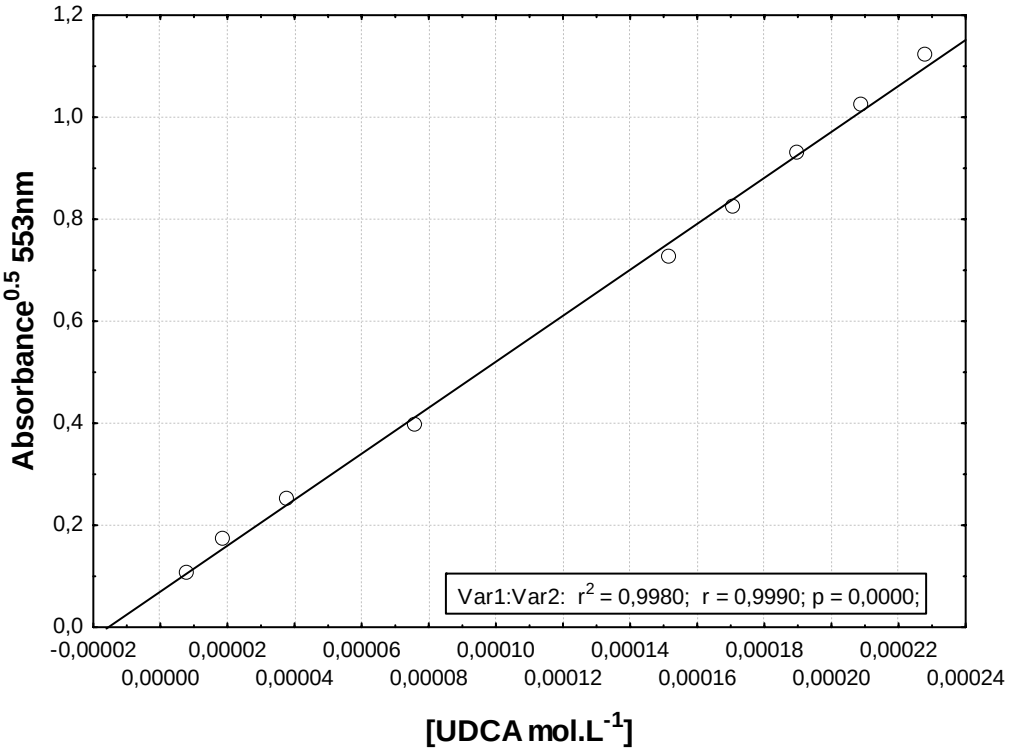

Fig. 3. Ursodeoxycholic acid calibration graph at pH 10.5.

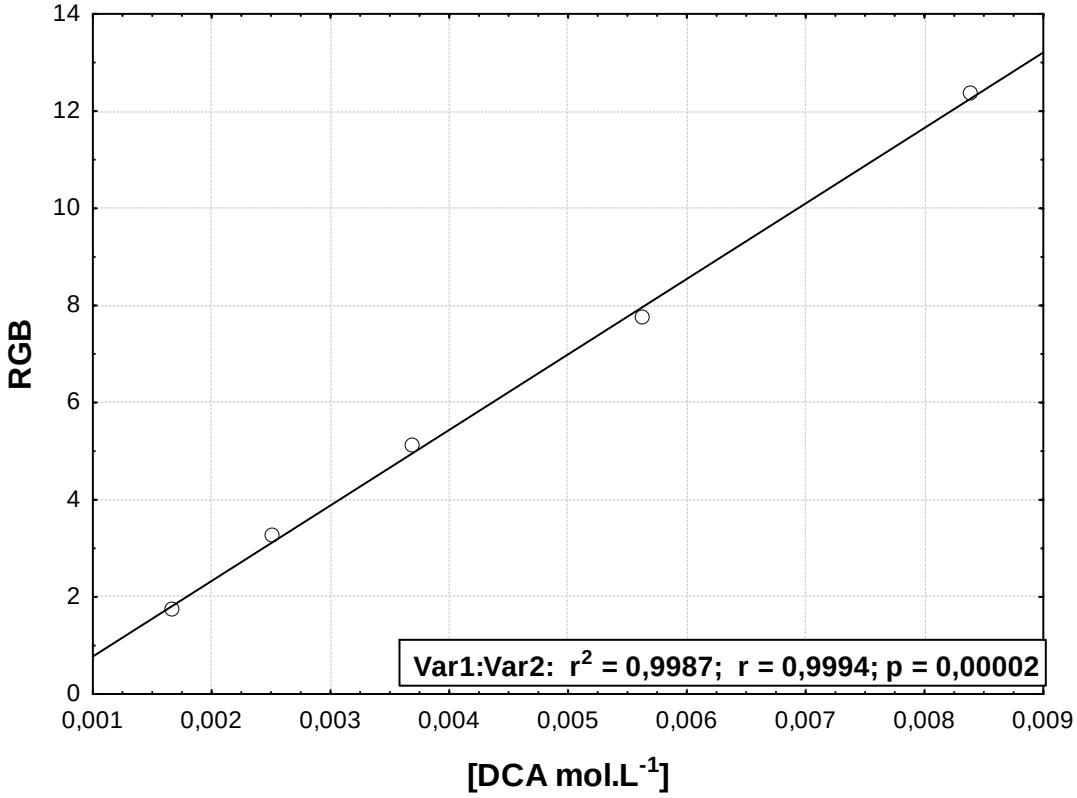

Fig. 4. Optical sensor calibration graph for deoxycholic acid determination.

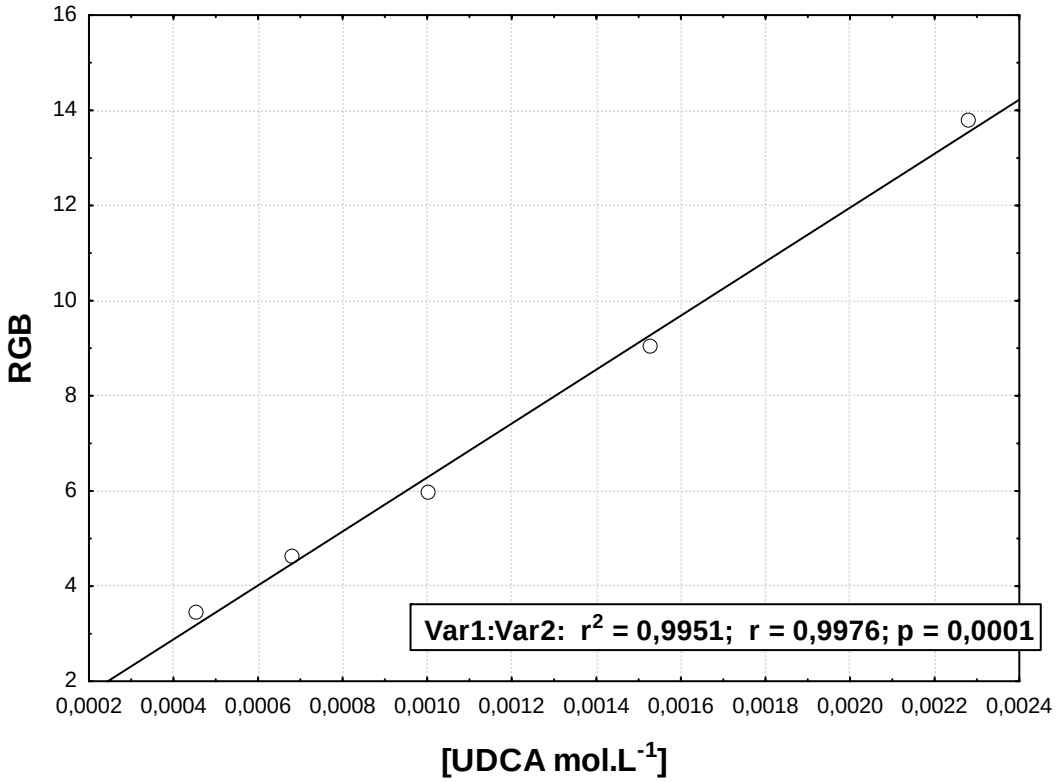

Fig. 5. Optical sensor calibration graph for ursodeoxycholic acid determination.

CONCLUSÃO

Os estudos realizados mostraram que:

- O melhor indicador para formar o complexo de inclusão com a β-ciclodextrina foi à fenolftaleína com mais de 95% de complexação. Através da formação deste complexo, foi possível a determinação dos ácidos desoxicólico e ursodesoxicólico em formulações farmacêuticas;
- O pH teve o efeito positivo mais estatisticamente significativo ($p<0,05$) na determinação do ácido desoxicólico havendo um aumento na eficiência de determinação de 73,2% entre os pH 9,2 a 10,7. O pH 10,5 foi considerado como ótimo. Os outros fatores (Concentração e Temperatura) não apresentaram efeitos significativos indicando boa resistência à temperatura;
- A concentração teve o efeito negativo mais estatisticamente significativo ($p<0,05$) na determinação do ácido ursodesoxicólico havendo um aumento na eficiência de determinação de 43,2% com redução à metade da concentração do complexo de inclusão. Os outros fatores (pH, Temperatura e concentração do tampão carbonato) apresentaram baixo ou nenhum efeito significativo indicando boa resistência as variações de temperatura e pH;
- Os limites de detecção e quantificação para a determinação dos ácidos desoxicólico e ursodesoxicólico foram $3,94x10^{-5} mol \cdot L^{-1}$ e $4,08x10^{-5}$ $mol \cdot L^{-1}$, respectivamente. Os correspondentes limites de quantificação foram de $1,31x10^{-4} mol \cdot L^{-1}$ para o ácido desoxicólico e $1,36x10^{-4} mol \cdot L^{-1}$ para o ácido ursodesoxicólico;
- O aumento do pH favoreceu o aumento das constantes de equilíbrio indicando o favorecimento da formação dos produtos – ácido biliar-β-ciclodextrina. Os complexos mantiveram-se estáveis nos 12 primeiros dias apresentando os tempos de meia-vida (t½) de 68,71 dias para a determinação do ácido desoxicólico e de 294,71 dias para a determinação do ácido ursodesoxicólico;

- Os parâmetros termodinâmicos mostraram altos valores negativos de $\Delta H°$ indicando que o processo de inclusão é exotérmico e com contribuição das forças de van der Waals e $\Delta S°$ foi positivo originado do relaxamento da camada de solvatação que envolvia os ácidos biliares e a cavidade da CD. Em todos os casos $\Delta G°$ foi negativo indicando que a formação dos complexos de inclusão foi espontânea;
- O método espectrofotométrico para a determinação dos ácidos desoxicólico e ursodesoxicólico foi validado segundo metodologia proposta pela ANVISA e EMEA. O mesmo apresentou boa linearidade e precisão podendo ser aplicado para análise de matérias primas e de formulações farmacêuticas que contenham os ácidos biliares propostos em sua composição;
- A reação de complexação competitiva também foi aplicada na construção de sensores químicos ópticos. Baseado nestes resultados, este método mostrou alta estabilidade, bom intervalo de temperatura, reação instantânea, baixo custo de reagentes e não exige instrumentação sofisticada atendendo a realidade do Sistema Único de Saúde - SUS.

ANEXOS

1. Trabalhos apresentados em Congressos:

Detecção espectrofotométrica do ácido desoxicólico e ursodesoxicólico através do complexo de inclusão β-ciclodextrina-indicador, 2008. **CADENA, P.G.**; ARAÚJO, A.N.; PIMENTEL, M.C.B.; LIMA FILHO, J.L.; SILVA, V.L. *XVII Congresso Brasileiro de Engenharia Química (COBEQ)*, Recife-PE. Trabalho 987.

β-Ciclodextrin complexation with phenolphthalein: Spectrophotometric determination of deoxycholic acid (DCA), 2008. **CADENA, P.G.**; OLIVEIRA, E.C.; SILVA, R.A.; ARAÚJO, A.N.; MONTENEGRO, M.C.B.S.M.; PIMENTEL, M.C.B.; LIMA FILHO, J.L.; SILVA, V.L. *XXXVII Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq) and XI Congress of the Panamerican Association for Biochemistry and Molecular Biology (PABMB)*, Águas de Lindóia-SP. Registration Number 8007. Online.

Purificação da bromelina cisteína peptidase do Ananas comosus (Abacaxi-Bromeliaceae), 2008. SILVA, R.A.; **CADENA, P.G.**; PIMENTEL, M.C.B.; LIMA FILHO, J.L. I Workshop internacional em biotecnologia III Encontro Alfa-Valnatura III Jornada Científica do Lika, Recife-PE. Anais: v. I, IND-73. CD-ROM.

Spectrophotometric determination of caffeine based on competitive reaction with dyes-cyclodextrin inclusion complexes, 2008. OLIVEIRA, E.C.; **CADENA, P.G.**; ARAÚJO, A.N.; MONTENEGRO, M.C.B.S.M.; SILVA, V.L.; LIMA FILHO, J.L.; PIMENTEL, M.C.B. I Workshop internacional em biotecnologia III Encontro Alfa-Valnatura III Jornada Científica do Lika, Recife-PE. Anais: v. I, IND-78. CD-ROM.

2. Guide for Authors (Carbohydrate Polymers)

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Closs, C. B., Roberts, I. D., Conde-Petit, B., & Eschler, F. (1997). Phase separation and rheology of aqueous amylopectin/ galactomannan systems. In E. J. Windhab, & B. Wolf. *Proceedings of the 1st international symposium on food rheology and structure* (pp. 233-237). Hannover: Vincentz Verlag.

Stephen, A. M. (1995). *Food polysaccharides and their applications*. New York: Marcel Dekker.

Norton, I. T., & Foster, T. J.. (2002). Hydrocolloids in real food systems. In P. A. Williams & G. O. Phillips (Eds.). *Gums and stabilisers for the food industry* (Vol. 11, pp. 187-200). Cambridge, UK: The Royal Society of Chemistry.

Babtsov, V., Shapiro, Y., & Kvitnitsky, E. (2005). Method of microencapsulation. *US Patent Office*, Pat. No. 6 932 984.

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