Physical–chemical parameters and validation of a colorimetric method for deoxycholic and ursodeoxycholic acids: kit reagent and optical sensor

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ABSTRACT

The simple and low cost β-cyclodextrin (β-CD)–phenolphthalein (PHP) inclusion complex was used for both the study of physical–chemical parameters and validation of analytical procedures for deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) determinations in different formulations. The usefulness of this inclusion complex is proposed either in the form of kit reagent and as an original optical sensor for DCA and UDCA. The results showed that temperature had a negative effect on the equilibrium constant resulting in high negative values of enthalpy and positive values of entropy. The half-life values for DCA and UDCA measurements were 68.71 and 294.71 days, respectively. The method was validated showing limits of detection and quantification of $4.92 \times 10^{-5} \, \mathrm{mol \, L^{-1}}$ and $1.64 \times 10^{-4} \, \mathrm{mol \, L^{-1}}$ for DCA, $1.14 \times 10^{-5} \, \mathrm{mol \, L^{-1}}$ and $3.79 \times 10^{-5} \, \mathrm{mol \, L^{-1}}$ for UDCA, respectively. The developed optical sensor also showed response linearity, ease of implementation and potential application in fast screening tasks even out of the laboratory.

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1. Introduction

Besides playing an important physiological role in biological systems some bile acids such as the deoxycholic acid (DCA), are also employed to enhance oral availability of biodegradable nanoparticles (Samstein et al., 2008), as choleretic agent in liver dysfunctions (Rodriguez et al., 2000), as *N*-(2-dimethylamino)ethyl derivatives in malaria treatment (Terzic et al., 2007) and in several cosmetic preparations (Valenta et al., 1999). Injection lipolysis for subcutaneous application primarily aiming cosmetic proposes were initially used in Brazil. They were based on a mixture of phosphatidylcholine and DCA as major active components (Duncan et al., 2009; Rotunda et al., 2004), being lately replaced by formulations containing only DCA (Rotunda and Kolodney, 2006; Yagima Odo et al., 2007). However, due to the associated health risks both of them have been forbidden by national sanitary authorities like ANVISA (the Brazilian National Health Surveillance Agency)

and FDA in US. Another important bile acid, the ursodeoxycholic acid (UDCA) is also used in the treatment of primary biliary cirrhosis, primary cholangitic sclerosis (Lindor, 1997), cholelithiasis (Petroni et al., 2001), to prevent the relapse of acute pancreatitis caused by microlithiasis (Okoro et al., 2008) and to reduce alanine aminotransferase levels in hepatitis C (Ikegami and Matsuzaki, 2008).

Due to stated applications of both bile acids, a variety of methods are routinely used to measure their content in pharmaceutical formulations, such as: electrochemical (voltammetric), fluorimetric or spectrophotometric (Arias De Fuentes et al., 2000), HPLC (Scalia et al., 1989) and micellar electrokinetic chromatographic (Rodriguez et al., 2000). Nevertheless, each of one has its own disadvantage (Arias De Fuentes et al., 2000; Lin et al., 2003) which added to complexity of the samples treatment turn difficult the "in loco" control by national sanitary authorities thus usually addressing the formulation or cosmetic sample into the laboratory for further processing and analysis. The research about validated simple and low cost methods for measurement of bile acids in commercial formulations can be also useful for screening tasks during technological development replacing expensive technologies more difficult to access in developing countries. In this way, inclusion complexes formed between cyclodextrins and indicators provide the basis

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for simplified methods of measurement of hydrophobic molecules such as is the case of bile acids (Cadena et al., 2009).

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 (Yanez et al., 2004) – and their numerous derivatives is the ability to form inclusion complexes with inorganic and organic guests (Brewster and Loftsson, 2007). Concomitantly, inclusion in cyclodextrins exerts a profound effect on the physicochemical properties of guest molecules such as solubility, chemical stability, absorption and bioavailability (Zhang et al., 2009). Guests reaction with CDs by competitive complexation with indicators has been used to assess the respective equilibrium constants (Kc) and other related thermodynamic parameters due to the fails in the direct determination of the complex. For example, phenolphthalein (PHP) is a typical acid/base indicator that forms a colourless 1:1 inclusion complex with β-CD and by this used in indirect determinations of other colourless compounds by competitive complexation reaction (Afkhami et al., 2006; Glazyrin et al., 2004).

Herein, the temperature dependence of the equilibrium constants of the PHP–, DCA– and UDCA–β-cyclodextrin were used to obtain thermodynamic parameters, i.e. the free energy change (ΔG), the enthalpy change (ΔH) and the entropy change (ΔS). The usefulness of the obtained data is evidenced in the subsequent validation of analytical procedures based on the β-cyclodextrin–phenolphthalein (β-CD–PHP) inclusion complex as kit reagent or optical sensor both providing rapid, inexpensive and simple alternative method for "in loco" measurements and fast screening.

2. Experimental

2.1. Materials

Analytical grade chemicals without any further purification treatment and double deionised water were used thoroughly. β-cyclodextrin was obtained from Fluka (Steinheim, Germany). Phenolphthalein, deoxycholic acid (sodium salt) and ursodeoxycholic acid were obtained from Sigma (St. Louis, 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 Statistica software (StatSoft Inc., Tulsa, OK, USA) and the images were analysed by trial version of Adobe Photoshop CS2 software (Adobe Systems, USA).

2.2. Methods

2.2.1. Preparation of analytical reagent

The co-precipitation technique (Del Valle, 2004) enabled to prepare the inclusion complexes in batch conditions using the following solutions: 1 mL of phenolphthalein solution, 1 mL of carbonate buffer solution (pH 10.5; 150 mmol L^{-1}) (Afkhami et al., 2006) and 1 mL of β-CD solution. For having a homogeneous system the mixture was vigorously mixed after each solution addition (the order did not interferes in the result), then readily transferred into a dark flask and kept at room temperature (25 °C). Humidity and temperature effects were considered neglected because all experiments and kit storage were performed in the same acclimatized room.

2.2.2. Development of the method (kit reagent)

The competitive inclusion complexes above obtained were just homogenized with either 1 mL of water (control) or 1 mL of bile acid solution (sample). The positive control to study the absorbance of the phenolphthalein was made using 1 mL of PHP solution, 1 mL carbonate buffer (pH 10.5; 150 mmol L^{-1}) and 2 mL of water, which

was strongly mixed after each solution addition. The blank solution was composed of 1 mL the same buffer plus 3 mL of water.

Absorption spectra were obtained at pH 10.5, containing 1.55×10^{-4} mol L^{-1} phenolphthalein (PHP) and different amounts of β-cyclodextrin (β-CD), 3.88×10^{-5} up to 6.20×10^{-4} mol L^{-1}, at 25 °C. Spectra for DCA analysis were carried out using 4.38×10^{-5} up to 7.0×10^{-4} mol L^{-1} DCA solutions and β-CD–PHP complex in the fixed proportion of 6.2×10^{-4}:1.55×10^{-4} mol L^{-1}. In turn, for UDCA analysis 1.19×10^{-5} up to 1.9×10^{-4} mol L^{-1} solutions of this bile acid were assayed with the β-CD–PHP complex (3.1×10^{-4}:7.75×10^{-5} mol^{-1}).

Temperature effect was assessed in the range from 10 up to 55 °C for the 8.75×10^{-5} up to 1.40×10^{-3} mol L^{-1} DCA and 2.38×10^{-5} up to 1.9×10^{-4} mol L^{-1} UDCA concentrations, while keeping pH constant at the value of 10.5.

The effect of the ionic strength on inclusion complex formation with DCA or UDCA and β-CD was examined in the range from 0.11 up to 0.40 using β-CD–PHP – 6.2×10^{-4}:1.55×10^{-4} mol L^{-1} for DCA and 3.1×10^{-4}:7.75×10^{-5} mol L^{-1} for UDCA, according to Wang et al. (2007).

Storage stability of the kit reagent developed was evaluated by maintaining two solutions of this complex at 25 °C during 60 days in dark flasks. At defined time intervals, samples were withdrawn from each of one for testing with solutions containing 7.0×10^{-4} mol L^{-1} of DCA and 1.9×10^{-4} mol L^{-1} of UDCA, respectively.

2.2.3. Validation of the method (kit reagent)

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, May 2003).

Absorbances vs. concentration linearity was evaluated using authentic solutions of each bile acid and from them 10 samples at the range 8.3×10^{-6} up to 3.36×10^{-3} mol L^{-1} for DCA or 8.0×10^{-6} up to 4.0×10^{-4} mol L^{-1} for UDCA were measured. Assays with each solution were performed in triplicate. At the experimental conditions previously established, the linearity of the calibration graphs were validated for both bile acids by the least squares method and the one-way analysis of variance (ANOVA) for $p < 0.05$.

Standard curves were then established to ±20% over the specified range, 5.6×10^{-4} up to 8.4×10^{-4} mol L^{-1} for the DCA and from 1.52×10^{-4} up to 2.28×10^{-4} mol L^{-1} for the UDCA, respectively. Therefore, results of the triplicate assays at five different concentrations were considered.

For evaluation of the repeatability, three concentrations of the DCA (5.6×10^{-4}, 7.0×10^{-4} and 8.4×10^{-4} mol L^{-1}) and UDCA (1.52×10^{-4}, 1.9×10^{-4} and 2.28×10^{-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 was defined as $C_L = 3.3 S_B/m$, where C_L, S_B, and m are respectively the limit of detection, standard deviation and slope, also the same approach was applied for the limit of quantification defined by $C_{LQ} = 10 S_B/m$.

Accuracy was established by comparison of the concentrations found in pharmaceutical formulations: 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), water 100 mL; injectable deoxycholate formula (Yagima Odo et al., 2007) – deoxycholic acid (sodium salt) 2.5% (w/v), benzyl alcohol 1% (v/v), propylene glycol 10% (v/v), water 100 mL; Ursacol® – Ursodeoxycholic acid 300 mg and excipients (lactose, povidone, crospovidone, magnesium stearate) with the ones obtained for standardized solutions of DCA (5.6×10^{-4}, 7.0×10^{-4}

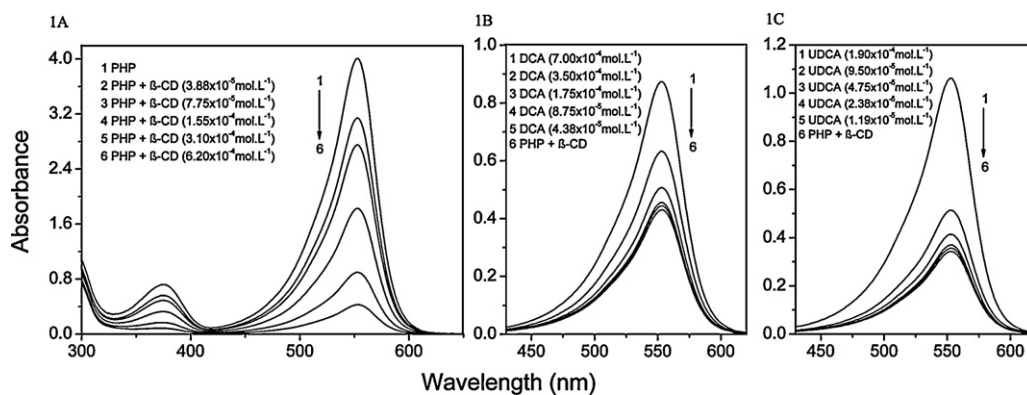**Fig. 1.** Absorption spectrum of phenolphthalein (PHP – 1A) (1.55×10^{-4} mol L^{-1}) at pH 10.5 in different β-cyclodextrin (β-CD) concentrations (3.88×10^{-5} up to 6.20×10^{-4} mol L^{-1}). Determination of (4.38×10^{-5} up to 7.0×10^{-4} mol L^{-1}) deoxycholic acid (DCA – 1B) by the inclusion complex of β-CD–PHP (6.2×10^{-4}:1.55×10^{-4} mol L^{-1}) and (1.19×10^{-5} up to 1.9×10^{-4} mol L^{-1}) of ursodeoxycholic acid concentrations (UDCA – 1C) by the inclusion complex of β-CD–PHP (3.1×10^{-4}:7.75×10^{-5} mol L^{-1}).

and 8.4×10^{-4} mol L^{-1}) and UDCA (1.52×10^{-4}, 1.9×10^{-4} and 2.28×10^{-4} mol L^{-1}) in nine determinations (3 concentrations/3 replicates).

The robustness was evaluated based on the variations in the analytical conditions, such as pH (10.3–10.7) and water (distilled, deionised, double deionised), using different settling conditions for each variation.

2.3. Optical chemical sensor for bile acids

Optical chemical sensor strips were implemented using 2 cm × 3.5 cm Bristol-paper strips afterwards soaked for 2 min in 5 mL of 1% (w/v) sodium alginate gel containing the β-CD–PHP inclusion complex (6.2×10^{-4}:1.55×10^{-4} mol L^{-1} for DCA and 3.1×10^{-4}:7.75×10^{-5} mol L^{-1} for UDCA) and finally dried for 24 h (25 °C). Later, 20 μL of DCA (1.68×10^{-3} up to 8.40×10^{-3} mol L^{-1}) or UDCA (4.56×10^{-4} up to 2.28×10^{-3} mol L^{-1}) solution was added. Colour changes were evaluated using a scanner (Model HP 5590) for obtaining images with 600dpi definition. All the images were downloaded to a computer, each image was converted to 8-bit grayscale by Adobe Photoshop software and the arithmetic mean of pixel intensity within each test zone was used to quantify the colorimetric response by the observed RGB (red, green, and blue colour system) values of digital images (Yang et al., 2007). Finally, the background-corrected response was calculated by subtraction of the each sample and control (without bile acids) values (Martinez et al., 2008a).

3. Results and discussion

3.1. Development of the method (kit reagent)

The spectral changes obtained for phenolphthalein solutions in the presence of different β-CD concentrations are shown in Fig. 1A. Phenolphthalein interacted with the β-CD in alkaline pH causing a decrease of more than 95% in the absorption band at the wavelength of 553 nm. The ionised red form of PHP was forced into β-CD cavity, forming its colourless lactone structure without protonation of the phenolic groups (Afkhami et al., 2006). Spectra of phenolphthalein solutions with increasing amount of β-CD revealed a proportional decrease of the free PHP up to concentration of 6.2×10^{-4} mol L^{-1} at pH 10.5 resulting in the ratio of 1:4 for PHP:β-CD. Higher concentrations of β-CD did not cause additional observable decrease in absorbance. However, the addition of bile acids forced the PHP leaves the cavity of β-CD molecules returning to its ionised red form in the alkaline solution (Fig. 1B and C). Thus, the extent of the

solution colour change can then be easily determined by standard curve of the free PHP and the corresponding concentration related with the amount of bile acid.

The effect of temperature on the absorbance of solutions containing the complexes with PHP, DCA and UDCA is presented in Fig. 2, where can be observed that with the increase of temperature an increase in absorbance was induced. Similar findings were previously described by Zarzycki and Lamparczyk (1998) being this effect caused by destabilization of the complex (Del Valle, 2004), 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 for the following studies was fixed at 25 °C, once it was enabled in both extended absorbance range for free PHP and β-CD–PHP inclusion complex, and there was no significant differences for robustness ($p < 0.05$ by Turkey's test) working between 20 and 30 °C.

The equilibrium constants ($Kc_{1:1}$ bile acid/cyclodextrin) for the β-CD–PHP, β-CD–DCA and β-CD–UDCA inclusion complexes were obtained by means of the Benesi–Hildbrand plot (Abdel-Shafi, 2007; Benesi and Hildebrand, 1949) according to Eqs. (1) and (2):
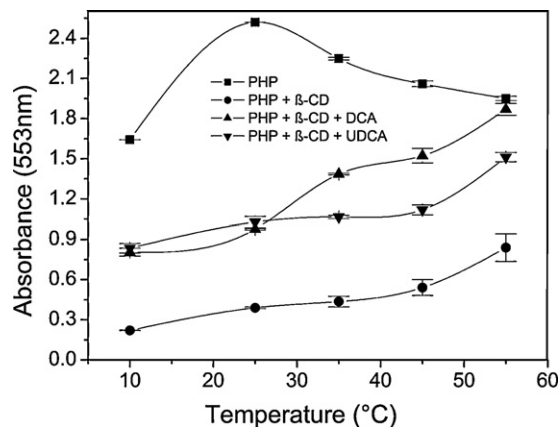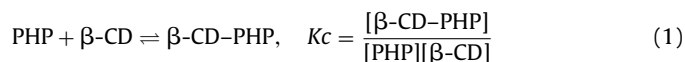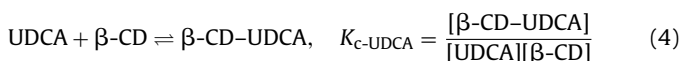
$$PHP + \beta\text{-CD} \rightleftharpoons \beta\text{-CD–PHP}, \quad Kc = \frac{[\beta\text{-CD–PHP}]}{[PHP][\beta\text{-CD}]} \tag{1}$$

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

Table 1
Equilibrium constants (Kc) of the inclusion complexes: β-cyclodextrin–phenolphthalein (β-CD–PHP) without or with the deoxycholic acid (DCA) or ursodeoxycholic acid (UDCA) at different temperatures and the thermodynamic parameters.

Inclusion complexes	T (K)	Kc ($\times 10^4$ L mol^{-1})	$\triangle G$ (kJ mol^{-1})	$\triangle H$ (kJ mol^{-1})	$\triangle S$ (J mol^{-1} K^{-1})
β-CD–PHP	298	1.7($\pm$0.1)	−2.4($\pm$0.2)	−16($\pm$1)	26($\pm$3)
	308	1.4($\pm$0.1)	−2.5($\pm$0.2)	–	–
	318	1.17($\pm$0.07)	−2.5($\pm$0.2)	–	–
	328	0.93($\pm$0.04)	−2.5($\pm$0.1)	–	–
β-CD–DCA	298	2.60($\pm$0.01)	−2.53($\pm$0.01)	−10($\pm$1)	50($\pm$5)
	308	2.39($\pm$0.01)	−2.59($\pm$0.01)	–	–
	318	2.12($\pm$0.01)	−2.64($\pm$0.01)	–	–
	328	1.77($\pm$0.01)	−2.68($\pm$0.01)	–	–
β-CD–UDCA	298	2.81($\pm$0.01)	−2.55($\pm$0.01)	−13($\pm$1)	43($\pm$3)
	308	2.43($\pm$0.01)	−2.59($\pm$0.01)	–	–
	318	2.13($\pm$0.01)	−2.64($\pm$0.01)	–	–
	328	1.76($\pm$0.01)	−2.67($\pm$0.01)	–	–

$$\frac{1}{A - A_0} = \frac{1}{a} + \frac{1}{aKc[\beta\text{-CD}_0]} \tag{2}$$

where A and A_0 are the absorbances of PHP in the presence and absence of β-CD, respectively, a is a constant related to the molar absorption coefficient changes, and $[\beta\text{-CD}_0]$ is the initial concentration of β-CD. Yuexian et al. (2005) described the competitive complexation equilibrium constant ($Kc_{1:1}$) between amino acid and methyl orange that can be applied for Kc determination between DCA or UDCA with β-CD by Eqs. (3)–(6):

$$\text{DCA} + \beta\text{-CD} \rightleftharpoons \beta\text{-CD–DCA}, \quad K_{\text{c-DCA}} = \frac{[\beta\text{-CD–DCA}]}{[\text{DCA}][\beta\text{-CD}]} \tag{3}$$

$$\text{UDCA} + \beta\text{-CD} \rightleftharpoons \beta\text{-CD–UDCA}, \quad K_{\text{c-UDCA}} = \frac{[\beta\text{-CD–UDCA}]}{[\text{UDCA}][\beta\text{-CD}]} \tag{4}$$

$$Kc_{\text{BA}} = \frac{[\beta\text{-CD}_0] - [\text{CD}]}{[\text{CD}]([\text{BA}]_0 - [\beta\text{-CD}_0] + [\text{CD}])} \tag{5}$$

where $[\text{BA}]_0$ represented the initial concentration bile acid. Kc_{BA} represented the equilibrium constant of bile acid–β-CD, [CD] was the uncomplexing β-CD concentration when bile acid, PHP and β-CD coexisted in solution and it was related to the absorbance variety of phenolphthalein in solution. [CD] could be obtained from:

$$[\text{CD}] = \frac{E_{\text{PHP}} - E}{Kc(E - E_{\beta\text{-CD–PHP}})} \tag{6}$$

where E_{PHP} and $E_{\beta\text{-CD–PHP}}$ represented the absorbance of uncomplexing phenolphthalein and β-CD–PHP inclusion complex, respectively; E was the absorbance of system in which bile acid, PHP and β-CD coexisted (Yuexian et al., 2005). 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 PHP (uncomplexed). In turn, PHP releasing causes an absorbance increase which is proportional to bile acids concentration in solution, allowing the Kc determination (Cadena et al., 2009). The values of Kc were determined at various temperatures (25 up to 55 °C) and at pH 10.5. Table 1 shows the temperature effect in the equilibrium constant ($Kc_{1:1}$ bile acid/cyclodextrin).

The thermodynamic parameters (Table 1) were calculated according to the Van't Hoff Eq. (7) which describes the temperature dependence as function of K ($Kc_{1:1}$). The ln K values were plotted as a function of the inverse temperature to give a linear relationship. Then, the apparent enthalpy ($\triangle H$) and entropy ($\triangle S$) changes were obtained from the slope and the intercept of the curve (Del Valle, 2004; Wang et al., 2007; Yuexian et al., 2005).

$$\ln K = -\frac{\triangle H}{RT} + \frac{\triangle S}{R} \tag{7}$$

Apparent free energy change ($\triangle G$) was obtained according to the Eq. (8):

$$\triangle G = -RT \ln K \tag{8}$$

The Van't Hoff plots were linear and exhibited large negative $\triangle H$ and positive $\triangle S$ for the developed complexes; these results were similar to those reported by Holm et al. (2009) for conjugated bile acids, suggesting an exothermic inclusion processes.

It was also observed an enthalpy decrease and entropy increase to the inclusion complex with the bile acids compared to β-CD–PHP inclusion complex. These results suggested that the inclusion processes of DCA and UDCA into the β-CD cavity were more favourable and spontaneous than that of PHP. In all cases $\triangle G$ was negative showing that the inclusion complexes formation can be spontaneous.

According to Jullian et al. (2008), the formation of an inclusion complex with cyclodextrin occurs due to the hydrogen bonding with the OH groups at the periphery of the cavity, Van der Waals and hydrophobic interactions. Generally, solute inclusion in the cyclodextrin cavity is associated with large negative values of $\triangle H$. Either negative or slightly positive $\triangle S$ values indicating inclusion complexation of the guest without extensive desolvation in a primarily enthalpy-driven process. The same behaviour has been reported by several authors which confirmed the great contribution from Van der Walls forces for the complexes formation (Brewster and Loftsson, 2007; Castronuovo and Niccoli, 2006).

The effect of ionic strength on inclusion of the β-CD–PHP complex at constant concentration was examined in the range from 0.11 up to 0.40. For this study, different concentrations of NaCl solution were prepared leading to systems containing H^+, Na^+, CO_3^{-2}, Cl^-, PHP, and β-CD. Due to the lower concentration of PHP and the neutral molecule β-CD, their contributions to ionic strength were negligible, so the H^+, Na^+, CO_3^{-2} and Cl^- are only responsible to ionic strength in the systems. The changes in the absorbance of inclusion complex as a function of ionic strength were carried out. The absorbance of inclusion complex gradually decreased with a rise in the ionic strength with significant difference by the Tukey test ($p < 0.05$). May be due to the system polarities were enhanced by ionic strength. Second Wang et al. (2007), the enhancement of polarity of solution is favourable to the hydrophobic interaction of β-CD molecules, which shields the interaction between β-CD molecule and PHP molecule and leads to decrease of the absorbance inclusion complex.

The study of the β-CD–PHP inclusion complex as kit reagent showed that it was stable for bile acids determination up to 12 days. A loss of about 30% for DCA and 12% regarding UDCA determinations were observed after 30 days of storage at 25 °C. The inactivation

Table 2
Validation data ($p < 0.05$).

	Linearity				
	DCA			UDCA	
Linearity range Standard curve	8.30×10^{-6}–1.68×10^{-3} mol L^{-1}			8.00×10^{-6}–2.28×10^{-3} mol L^{-1}	
	ABS $= -0.0069(\pm 0.0091) + 870.0373(\pm 12.4216)DCA_{mol/L}$			ABS$^{0.5} = 0.0699(\pm 0.011) + 4506.688(\pm 77.1179)$UDCA$_{mol/L}$	
Correlation coefficient	0.99919			0.99898	

Robustness[a]

pH	DCA	UDCA	Water	DCA	UDCA
10.3	–	97.75($\pm$1.49)	Distilled	102.38($\pm$1.52)	100.42($\pm$0.95)
10.4	97.84($\pm$3.53)	99.19($\pm$1.76)	Deionised	100.11($\pm$4.48)	98.04($\pm$1.86)
10.5	100.00($\pm$3.48)	100.0($\pm$2.15)	Double deionised	100($\pm$2.18)	100($\pm$0.66)
10.6	102.62($\pm$2.48)	99.01($\pm$1.85)			
10.7	104.50($\pm$0.46)	98.46($\pm$0.97)			

[a] %Mean $\pm$ %R.S.D.

Table 3
Accuracy of the kit reagent (β-CD–PHP) developed for different pharmaceutical formulations.

Drugs	Accuracy (%mean $\pm$ %R.S.D.)		
Deoxycholic acid standard	98.97 ($\pm$0.84)	101.36($\pm$0.36)	99.31($\pm$1.48)
Injectable Phosphatidylcholine formula (Rotunda et al., 2004)	97.11($\pm$1.43)	101.33($\pm$1.28)	100.51($\pm$0.99)
Injectable deoxycholate formula (Yagima Odo et al., 2007)	98.08($\pm$0.43)	102.77($\pm$0.63)	102.52($\pm$0.38)
Ursodeoxycholic acid standard	99.43($\pm$1.17)	100.89($\pm$0.54)	99.63($\pm$0.21)
Ursacol®	98.1($\pm$0.49)	98.7($\pm$1.86)	99.22($\pm$0.81)

constant (k_i) of the kit reagent was obtained by:

$$\ln A = \ln A_0 - k_i t \qquad (9)$$

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 ($t_{1/2}$) can be obtained by:

$$t_{1/2} = \frac{\ln 2}{k_i} \qquad (10)$$

The inactivation constants (k_i) of the kit reagent were of 1.01×10^{-2} day^{-1} for DCA and 2.35×10^{-3} day^{-1} for UDCA determinations. The half-life ($t_{1/2}$) for the kit reagent was 68.71 days for DCA and 294.71 days for the UDCA measurements. Despite the greater stability of the kit reagent for UDCA compared to that one for DCA, the last one allowed determinations in more concentrated solutions (data not shown) and pharmaceutical formulations containing high DCA concentrations (Rotunda et al., 2004; Schuller-Petrovic et al., 2008; Yagima Odo et al., 2007).

3.2. Validation of the method (kit reagent)

The spectrophotometric method using the developed kit reagent for DCA and UDCA was 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 2). The standard curves for the linearity assays were constructed with 10 concentrations and validated by the least squares method showing correlation coefficients higher than 0.998 and the one-way analysis of variance (ANOVA) showing F and p values of 4905.94 and less than 10^{-10} for DCA and 3415.11 and less than 10^{-9} for UDCA, respectively. For the range (80–120%), the standard curves were constructed with 5 concentrations showing correlation coefficients of the same order of magnitude.

The precision (repeatability) was determined by the percentage of relative standard deviation (%R.S.D.) at three levels (DCA – 5.6×10^{-4}, 7.0×10^{-4} and 8.4×10^{-4} mol L^{-1} and UDCA – 1.52×10^{-4}, 1.9×10^{-4} and 2.28×10^{-4} mol L^{-1}). The %R.S.D. values were less than 3% obtained in the concentrations studied indicating a good repeatability. The use of an experimental design is encouraged by EMEA guidelines, in this way the intermediate precision
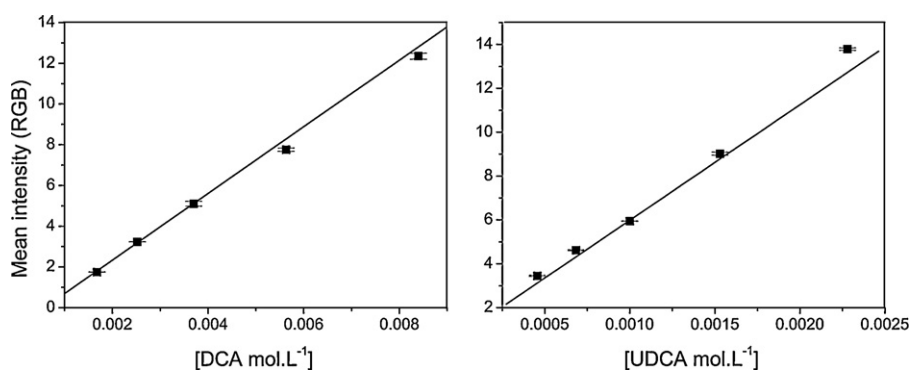

Fig. 3. Standard curve of the optical chemical sensor for deoxycholic and ursodeoxycholic acids (β-CD–PHP – 6.2×10^{-4}:1.55×10^{-4} mol L^{-1} for DCA and 3.1×10^{-4}:7.75×10^{-5} mol L^{-1} for UDCA) determinations.

was studied by a 2^2 full factorial design which did not present significant effects and interactions ($p < 0.05$). According to these results, the different analysts and equipments and your interactions did not interfere in the method.

The accuracy results demonstrated the method effectiveness for quantitative determination of DCA and UDCA in the pharmaceutical formulation. Accuracy was showed as percentage recovery of the target value, exhibited excellent results with bias lower than 2% throughout the tested range (Table 3). 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, condition that was also reported by Afkhami et al. (2006) and Glazyrin et al. (2004). Determinations performed at high pH conditions did not cause significant changes of the results and were limited by the buffering power of carbonate buffer (Table 2).

The limits of detection were of 4.92×10^{-5} mol L^{-1} for DCA and 1.14×10^{-5} mol L^{-1} for UDCA, respectively. The corresponding limits of quantification were of 1.64×10^{-4} mol L^{-1} and 3.79×10^{-5} mol L^{-1}.

3.3. Optical chemical sensor for bile acids

The results obtained concerning robustness of β-CD–PHP inclusion complex suggested its biotechnological application in the form of optical strip sensor for both qualitative and quantitative measurements of bile acids. Therefore, a mixture of alginate and β-CD–PHP inclusion complex was used to impregnate strips of paper. Alginate is largely used in biomolecule immobilization because it provides simple implementation using a biodegradable and non toxic material (Ha et al., 2009). Furthermore, it did not interfere either with the competitive complexation reaction or with the digital images obtained after colour change induced by the reaction. The quantitative analysis of digital images obtained from a scanner of the optical sensor is a recent tool to measure the amount of analytes. This new technology has been showed low cost and simplicity, very important for the developing world (Martinez et al., 2008b). The scanners are inexpensive, have high resolution, the scanned image is always in focus, the intensity of the image is not affected by lighting conditions, are portables (business card scanners or pen scanners), and they can be linked to personal digital assistants (PDAs) by wireless communication (Martinez et al., 2008a). 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 (Martinez et al., 2008a; Gaiao et al., 2006; Yang et al., 2007).

The test was only based on dropwise 20 µL of each bile acid solution and controls (buffer) in different places on the same strip, after 5 min at room temperature (25 °C) when the tip was dried, followed by immediate scanning. However, after the scanning, the tip colour was observed for 3 h without any changes. Based on collected images, it was possible to plot a standard curves (Fig. 3) for DCA obtaining the equation: RGB = 1553 ($\pm$32) DCA$_{mol/L}$ -0.8 ($\pm$0.2) with a correlation coefficient of 0.9994 and UDCA with the corresponding equation RGB = 5671 ($\pm$229) UDCA$_{mol/L}$ +0.6 ($\pm$0.3) showing the correlation coefficient of 0.998.

4. Conclusion

The development of low cost and simple method 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. The β-CD–PHP inclusion complex showed to be a good alternative method for bile acids measurement. The physical–chemical and thermodynamic parameters studies for the application of inclu-

sion complexes as kit reagent or optical sensor was carried out. The proposed method had good storage stability, 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 as kit reagent. The original optical chemical sensor developed showed good linearity suggesting that can be used for fast screening and the analyses "in loco" of bile acids in pharmaceutical formulations or raw materials.

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