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Effect of cucurbitacins on bilirubin–albumin binding in human plasma

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Abstract

The aim of this study is to investigate the effect of three cucurbitacins (Cuc) E, D and I on the bilirubin–albumin binding, both in human serum albumin (HSA) and in plasma. Bilirubin–HSA solution and plasma free of cucurbitacins were prepared as well as others containing serial concentrations of cucurbitacins. The concentration of unbound bilirubin was determined in bilirubin–HSA solution and the direct and total bilirubin concentrations were measured in plasma (with normal or elevated bilirubinemia) by Jendrassik and Grof method. In the conditions we adopted Cuc E and D (to a lesser extent), decreased the levels of unbound bilirubin in bilirubin–HSA solution and decreased direct bilirubin concentration and total bilirubin concentration in plasma in a dose-dependent manner while Cuc I had no effect. The effect of Cuc is related to the presence of native HSA. Thus, when albumin was absent or has been denatured by heating or by urea, Cuc E did not modify bilirubin levels, suggesting that the native structure of albumin is essential for such activity. The interaction of HSA with Cuc E was investigated by fluorescence spectroscopy. Cuc E increased the intrinsic fluorescence of the protein and the magnitude of fluorescence intensity of bilirubin–albumin complex. We concluded that Cuc E and D produced a rearrangement in the structure of albumin, particularly in the domain-II, resulting in an increase in the binding of bilirubin to albumin regardless to whether it's conjugated to glucuronic acid or unconjugated.

Keywords: Cucurbitacin; Albumin; Bilirubin; Human plasma

Introduction

Cucurbitacins are tetracyclic triterpenes. These highly oxygenated compounds which are mainly present in Cucurbitaceae, are also found in other plants (Chen et al., 2005). 19 types of cucurbitacins have been extracted and characterized from plants. They differ from each other by hydroxylation at C-2, -3, -19, -24, the presence of ketone function at C-3, double bond between C-1 and C-2, double bond between C-23 and C-24 and by the acetylation of the C-26 hydroxy group. Cucurbitacin glycosides, diacetate cucurbitacins and hexanorcucurbitacins are part of cucurbitacins derivatives (Chen et al., 2005).

A large number of biological activities have been attributed to cucurbitacins and to their glycosylderivatives (Chen et al., 2005; Panosian et al., 1987, 1989; Huang et al., 1998). Thus, Cuc E (Duncan and Duncan, 1997; Duncan et al., 1996; Musza et al., 1994), Cuc I (Blaskovich et al., 2003), Cuc Q (Sun et al., 2005), Cuc B (Arisawa et al., 1984) and Cuc D (Rodriguez et al., 2003) exhibit anti-proliferative activity on various cancer cell lines. Besides, Cuc R (Recio et al., 2004) and Cuc B (Peters et al., 1999; Yesilada et al., 1988) produced an anti-inflammatory activity; in addition to a hepatocurative and hepatoprotective effects for Cuc B (Agil et al., 1999).

As cytotoxic agents, nanoparticles containing polylactic acid were developed in order to specifically target cancer cells (Yang et al., 2003).

Numerous studies reported that the activity of Cuc is related to its structure (Sun et al., 2005; Dinan et al., 1997). In this context, Bartalis and Halaweish (2005) demonstrated that cytotoxicity increased linearly with hydrophobicity.

To study whether cucurbitacin can affect the binding of endogenous compounds to plasma proteins, the interaction of bilirubin with albumin has been evaluated in the presence and absence of the substance.

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Bilirubin produced from the degradation of haemoproteins in mammals, is transported to the liver for conjugation by glucuronidation and subsequent excretion from the body (Ostrow et al., 1994). Human serum albumin (HSA), the major plasma protein, binds bilirubin reversibly and acts as a buffer preventing its transfer from blood to the tissues. Plasma contains two forms of bilirubin: the first one is conjugated to glucuronic acid and is mainly unbound to proteins (direct bilirubin), and the unconjugated form mainly bound to albumin (indirect bilirubin). Total bilirubin concentration is determined after addition of accelerator agents that destroy the interaction between bilirubin and albumin and allow determination of both forms of bilirubin (Mullon and Langer, 1987; Doumas et al., 1985). A small proportion of unconjugated form is unbound and is called unbound unconjugated "free" bilirubin; it increases in case of hyperbilirubinemia and is responsible for bilirubin toxicity (Ostrow et al., 1994).

In this study, the effect of cucurbitacins E, D and I on the bilirubin levels was evaluated in HSA solution and in human plasma. The Jendrassic–Grof procedure based on the reactivity of bilirubin with diazotized sulfanilic acid (Mullon and Langer, 1987; Doumas et al., 1985) was used to determine unbound bilirubin concentration in Bilirubin–HSA solution, direct bilirubin and total bilirubin concentrations in human plasma (with normal and hyperbilirubinemia) in the presence and absence of cucurbitacins. Additional experiments were performed to identify the mechanism by which the cucurbitacins could produce variation in the bilirubin levels. Fluorescence spectroscopy is a powerful tool for the study of the interaction of chemicals with proteins. Thus, the intrinsic fluorescence of the protein and the fluorescence intensity of bilirubin–albumin complex were evaluated in the presence and absence of cucurbitacin.

Materials and methods

Cucurbitacins D, E and I were purchased from Extrasynthese SA, Genay, France; Human serum albumin (fraction V, purity ≤ 98.0%, fatty acid free) and caffein from Fluka, Biochemica, USA; bilirubin, sodium potassium tartrate, ascorbic acid, hydrated sodium acetate, EDTA, and NaOH from Himedia Laboratories, India; disodium hydrogen orthophosphate $2H_2O$, potassium dihydrogen phosphate, urea and sulfanilic acid from Needham Market Suffolk, England; albumin-kit from bioMerieux, France; hydrochloric acid 37 % from Scharlau, Swiss; dimethylsulfoxid from Chemie, Switzerland; sodium benzoate from Scharlau Chemie, Spain; sodium nitrite from BDH laboratory, England; methanol from Labscan-analytical sciences, Ireland.

Plasma samples from healthy subjects ($n=6$) and from patient with hyperbilirubinemia were obtained from Notre Dame de Secours hospital, Jbeil, Lebanon. A spectrophotometer (Heλiosá; Thermelectron, U.S.A.; bandpass: 2 nm) was used for absorbance measurements at 607 nm.

Human serum albumin denaturation

HSA (580 μM), prepared in 67 mM phosphate buffer pH 7.4, was denatured by heating at 100 °C for 2 h. HSA denatured by urea was prepared by dissolving HSA 580 μM in 67 mM phosphate buffer pH 7.4 containing 10 M urea. The final solution was incubated for 15 h at room temperature (Ahmad et al., 2004).

Preparation of solutions

HSA was diluted just before experiments in 67 mM phosphate buffer, pH 7.4. Bilirubin stock solution (stock A) (171 μM) was prepared by dissolving 10 mg of bilirubin in 2 mL of dimethylsulfoxide and 0.5 mL of NaOH 0.4 M and completed to 100 mL with HSA 580 μM. Bilirubin stock solution (stock B) (171 μM) was prepared by dissolving 10 mg of bilirubin in 2 mL of dimethylsulfoxide and 0.5 mL of NaOH 0.4 M and completed to 100 mL with phosphate buffer (pH 7.4). Bilirubin stock solutions were stored in the dark at 4 °C. Stock solutions of cucurbitacins E, D and I were prepared in methanol (2 mg/mL).

Experimental protocol

Bilirubin–HSA solutions were prepared by diluting bilirubin, taken from stock solution (A), in HSA solution. Different studies were carried out on HSA solution with a physiological concentration of bilirubin (17 μM). Bilirubin–HSA solution or human plasma was added to tubes containing Cuc (which were dried by evaporating methanol, the solvent used in the preparation of stock solutions of Cuc). The final concentrations of Cuc varied from 5 to 250 μM. After 10 min incubation at 37 °C, aliquots were taken from bilirubin–HSA solution for unbound bilirubin analysis and from human plasma for direct and total bilirubin analysis according to Jendrassik and Grof method (Doumas et al., 1985).

In order to identify the mechanism by which Cuc E (100 μM) could modify the bilirubin levels, a series of experiments were realized:

- Bilirubin, taken from stock solution (B), was diluted in 67 mM phosphate buffer pH 7.4 and the mixture was then added to tubes containing Cuc E.
- Bilirubin from stock (B) was diluted in HSA (denatured previously by heating or by urea) and the mixture was then added to tubes containing Cuc E.

Solutions containing bilirubin and the reactions were protected from light by wrapping the containers with aluminum foil.

Fluorescence and UV spectroscopic measurements

The fluorescence emission spectra were carried out on a spectrofluorimeter (Thermo Spectronic, Aminco. Bowman Series 2, USA) equipped with a data recorder at 25±0.1 °C. The slits were set at 3 and 5 nm for excitation and emission respectively. The pathlength of the sample cuvette was 1 cm.

The cucurbitacin E solution (2 mg/mL) was dissolved in dimethylsulfoxid and the HSA (1×10^{-5} M) solution was prepared in phosphate buffer 67 mM (pH 7.4). To a fixed volume (5.0 mL) of protein solution, increased volumes of

cucurbitacin solution or of solvent (dimethylsulfoxid) were added and the fluorescence was measured after 20 min. The molar ratios of cucurbitacin to HSA were 0.5; 1; 2.5; 5 and 10.

The fluorescence was recorded in the wavelength range 290–500 nm after exciting the protein solution either at 295 nm exclusively for tryptophan fluorescence or at 280 nm for total protein fluorescence.

Bilirubin binding

Bilirubin solution was prepared by dissolving 5 mg of bilirubin in 0.5 N NaOH containing 1 mM EDTA and immediately diluting it to the desired volume with 0.067 M sodium phosphate, pH 7.4. To a fixed volume of HSA solution, increasing volumes of stock bilirubin solution were added to achieve different bilirubin/albumin molar ratios. The Cuc E was then added at a molar ratio cucurbitacin/HSA equal to 1. The fluorescence was measured after 20 min of the addition of cucurbitacin or dimethylsulfoxid at 530 nm after exciting the bilirubin–albumin complex at 466 nm in dark. The data were plotted as relative fluorescence against bilirubin/albumin molar ratio.

Statistical analysis

All values are presented as the mean ± SEM of n independent experiments. Data were analyzed by use of Mann–Whitney test. $P<0.05$ was considered significant.

Results

As shown in Fig. 1, Cuc E and D decreased the concentration of unbound bilirubin in Bilirubin–HSA solution while Cuc I was non-effective even at 100 μM. This decrease was more important with Cuc E than with Cuc D, whatever the concentration of Cuc i.e. at 100 μM Cuc, the ratio UBC_{CucE}/UBC_0 (unbound bilirubin concentration in the presence of Cuc E/unbound bilirubin concentration of the control) and UBC_{CucD}/UBC_0 (unbound bilirubin concentration in the presence of Cuc D/unbound bilirubin concentration of the control) were respectively 0.48 ± 0.01 and 0.66 ± 0.01. Hence, both cucurbitacins acted in a dose-dependent manner.

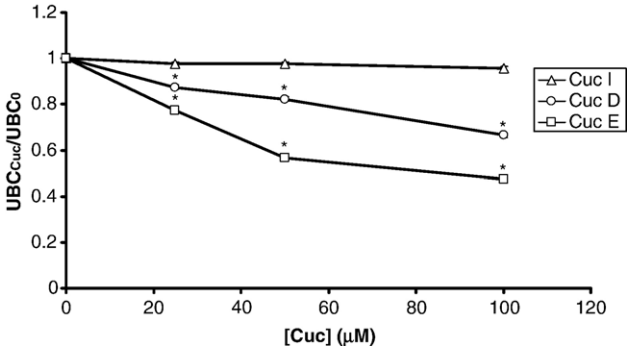


Fig. 1. Effect of different concentrations of Cuc I, D and E on the ratio: unbound bilirubin concentration in the presence of Cuc (UBC_{cuc})/unbound bilirubin concentration in the absence of Cuc (UBC_0) in human serum albumin solution (580 μM). [B]: 17 μM; pH 7.4; 37 °C; 10 min. Values are the means ± SEM of quadruplicate experiments. The SEM are presented but they are too little to be seen. (*) indicates a significant difference from values for control ($P<0.05$).

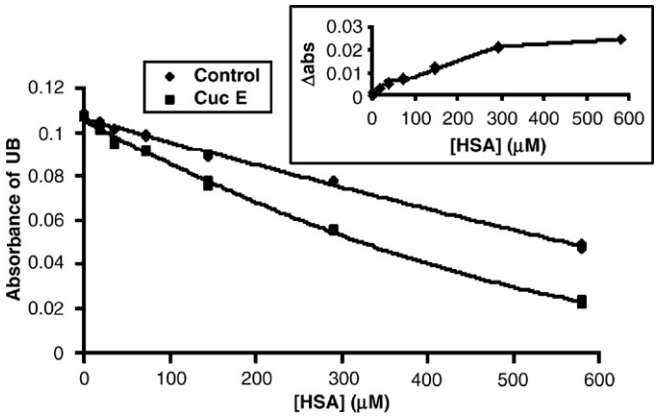


Fig. 2. Values of absorbance of unbound bilirubin at various concentrations of human serum albumin (HSA) solutions in the presence and absence of Cuc E. The extrapolation of the difference of absorbance values obtained in the presence and absence of Cuc E ($\Delta Abs = Abs_0 - Abs_{CucE}$) at varying concentrations of HSA is represented in the inset. [Cuc E]: 100 μM; [B]: 17 μM; 37 °C; pH 7.4; 10 min. Values are the means ± SEM of quadruplicate experiments. The SEM are presented but they are too little to be seen. (*) indicates a significant difference from values of control ($P<0.05$).

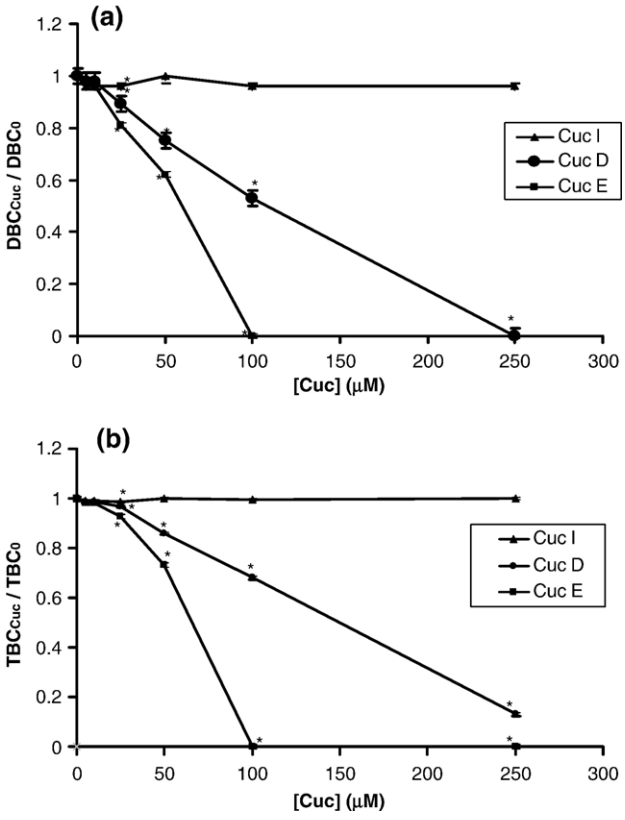


Fig. 3. Effect of different concentrations of cucurbitacins I, D and E on the ratios (a) DBC_{Cuc}/DBC_0 and (b) TBC_{Cuc}/TBC_0 in the presence (DBC_{Cuc} and TBC_{Cuc}) and absence (DBC_0 and TBC_0) of cucurbitacin in plasma. 37 °C, 10 min. Values are the means ± SEM of quadruplicate experiments. The SEM are presented but they are too little to be seen. (*) indicates a significant difference from values of control ($P<0.05$).

Since Cuc E induced the strongest effects, it was chosen for the subsequent experiments. In the absence of Cuc E, the levels of unbound bilirubin decreased with the increase of HSA concentration (Fig. 2). In the presence of Cuc E, whatever the concentration of HSA, these values were lower than those obtained in the controls without Cuc. The difference ($Abs_0 - Abs_{CucE}$) increased with HSA concentration (Fig. 2).

In the absence of Cuc E, the absorbance values of unbound bilirubin were 0.108±0.001 in the buffer and in HSA which has been denatured by heating or by urea. The presence of Cuc E (100 μM) decreased the absorbance values of unbound bilirubin in a medium containing native HSA (580 μM, pH 7.4, 37 °C). These values were respectively 0.048±0.001 and 0.023±0.001 in the absence and presence of Cuc E. However, in buffer and in HSA denatured solutions, Cuc E produced no effect (data not shown).

Results obtained with human plasma were similar to those obtained in Bilirubin–HSA solution (Fig. 3). In fact, Cuc E and D decreased bilirubin levels in a dose-dependent manner, and no variation of bilirubin levels was obtained with Cuc I. With Cuc E 100 μM, the ratios DBC_{CucE}/DBC_0 (direct bilirubin concentration in the presence of Cuc E/direct bilirubin concentration of the control) (Fig. 3a) and TBC_{CucE}/TBC_0 (total bilirubin concentration in the presence of Cuc E/total bilirubin concentration of the control) (Fig. 3b) were equal to zero, whereas, for the same concentration of Cuc D, they were 0.53±0.01 (Fig. 3a) and 0.68±0.02 (Fig. 3b), respectively.

The denaturation of plasma proteins by urea produced a complete release of bilirubin from the albumin and thus the values of direct bilirubin concentration and total bilirubin concentration were similar (data not shown). Cuc E was inactive on plasma with denatured proteins (data not shown).

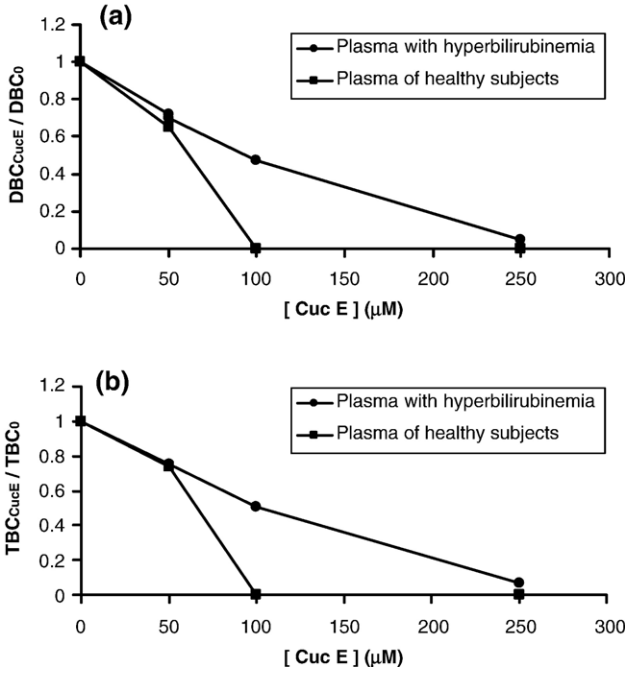


Fig. 4. Comparison of the effect of Cuc E (100 μM) on the ratios (a) DBC_{Cuc}/DBC_0 and (b) TBC_{Cuc}/TBC_0 in the presence (DBC_{Cuc} and TBC_{Cuc}) and absence (DBC_0 and TBC_0) of Cuc E in plasma obtained from healthy subjects ($n=6$) and plasma from patient with hyperbilirubinemia. Plasma, 37 °C, 10 min. Values are the means of duplicate experiments in plasma from healthy subjects.

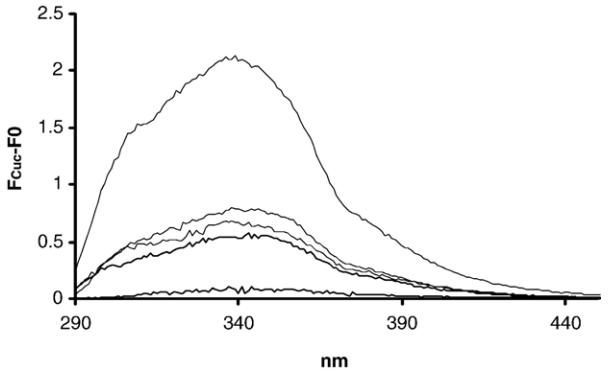


Fig. 5. Effect of cucurbitacin on the intrinsic fluorescence of HSA (10 μM) at pH 7.4. The protein was excited at 280 nm. The cucurbitacin concentration from bottom to top was varied as 5; 10; 25; 50 and 100 μM. Each data point was the average of three independent determinations.

The results obtained with plasma presenting a hyperbilirubinemia (DBC_0 and TBC_0 were respectively 40 and 200 μM), showed that Cuc E decreased markedly the bilirubin levels (Fig. 4). In fact, at 100 μM Cuc E, the ratio DBC_{CucE}/DBC_0 and TBC_{CucE}/TBC_0 reached approximately 0.5.

Cuc E had no intrinsic fluorescence at the excitation wavelengths of 280, 295 or 466 nm. Fig. 5 shows the difference of fluorescence emission spectra of HSA determined in the presence and absence of cucurbitacin E after excitation at 280 nm. We can see that the fluorescence intensity of HSA increased with the cucurbitacin E concentration. Likewise, increase in the fluorescence of the protein was obtained after excitation of the protein at 295 nm (data not shown).

When a mixture of bilirubin and albumin was excited at 466 nm, it resulted the appearance of a fluorescence band in the wavelength region 500–600 nm with an emission maximum at 530 nm. This magnitude of fluorescence intensity at 530 nm increased with bilirubin/albumin molar ratio as shown in Fig. 6.

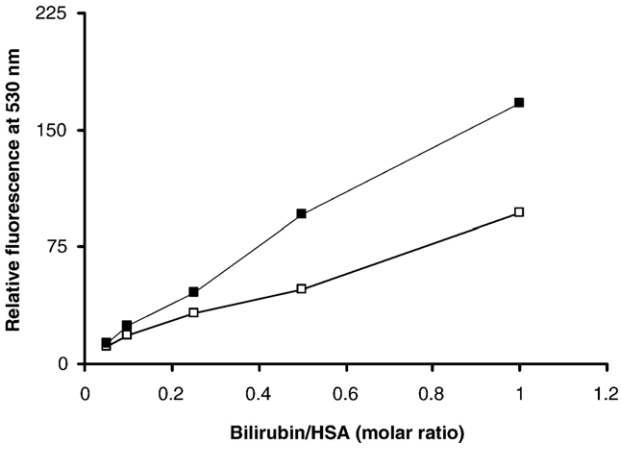


Fig. 6. Relative fluorescence of bilirubin bound to HSA in the presence (solid squares) and absence (open squares) of Cuc E (10 μM). The fluorescence was recorded at 530 nm after exciting the bilirubin–albumin complex at 466 nm.

Whatever the bilirubin/albumin molar ratio, the addition of Cuc E to HSA at a molar ratio 1:1 produced an increase in the magnitude of fluorescence intensity at 530 nm.

Discussion

Our results suggest that cucurbitacins E and D produced modifications in the levels of bilirubin in a medium containing HSA. Elayan et al. (1989) have shown that the administration of *Ecballium elaterium* juice to male rats with surgically induced jaundice produced a decrease in serum bilirubin concentration. However the mechanism by which the juice affects this parameter was unclear. The fresh crude juice obtained from the fruit of *E. elaterium* contains cucurbitacins B, D, E, I, L, R (Attard and Sciluna-Spiteri, 2001; Rao et al., 1974; Chen et al., 2005; Toker et al., 2003; Seger et al., 2005) and their derivatives glycosylcucurbitacins and hexanorcucurbitacins (Rao et al., 1974; Seifert and Elgamal, 1997). The activity of the juice may be at least in part induced by cucurbitacins E and D.

Because Cuc E was more efficient in decreasing bilirubin levels, we have been interested in determining its mechanism of action. When albumin was absent, Cuc E did not affect the bilirubin levels. This result has been found in several experiments and at various concentrations of Cuc E, varying from 50 to 500 μM (data not shown). In HSA solutions (pH 7.4), the binding of bilirubin increased with HSA concentration according to results published by Athar et al. (1999). The intensity of the effect of Cuc was related to the level of albumin molecules, suggesting that albumin is involved in the mechanism of action of cucurbitacin.

The denaturation of HSA by heating at 100 °C suppressed its activity. In the same way, urea (10 M) produced a complete denaturation of HSA at pH 7.4 (Ahmad et al., 2004). When HSA was denatured, Cuc E was inactive. The results suggest that native structure of albumin is required so that Cuc E could produce a decrease in the bilirubin levels, and suppose that cucurbitacin produces a modification in the structure of albumin in such a way that allows bilirubin to better bind the protein.

The fluorescence of HSA results from the presence of the tryptophan, tyrosine and phenylalanine residues. Actually, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group, or a tryptophan. This view-point was well supported by the experimental observations of Sulkowska (2002). The change of intrinsic fluorescence intensity of HSA is that of tryptophan residue when molecules are bound to HSA. Cuc E increased the fluorescence of albumin either after excitation of the tryptophan alone or of the protein. Since HSA contains only one tryptophan residue (Trp-214), which resides in domain-II, decrease in fluorescence intensity observed after exciting the protein at 295 nm may be ascribed to the conformational changes in domain-II. It can be inferred that no structural change occurred in domain-II in the cucurbitacin concentration range 5–100 μM. The microenvironment of the tryptophan residue in a hydrophobic pocket was not affected by cucurbitacin binding.

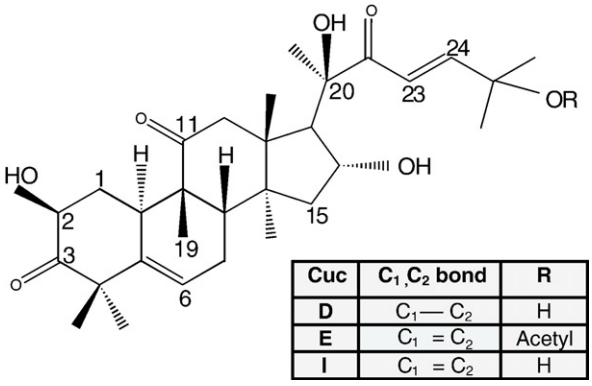
Bilirubin binds to albumin with high affinity at a site located at or near loop 4 in domain II-A. Increase in bilirubin binding to HSA caused by cucurbitacin cannot be due to non-specific interaction of cucurbitacin with bilirubin as no fluorescence was observed with free bilirubin mixed with cucurbitacin. Since the occurrence of bilirubin fluorescence is due to acquisition of helicity in the pigment upon binding to albumin (Chen, 1974), a significant increase in bilirubin fluorescence due to cucurbitacin suggests that domain II, implied in the binding of bilirubin, undergoes rearrangement as to allow the bound pigment to acquire a tight helical twisting.

Several compounds are capable of modifying the interaction of bilirubin with albumin by competing with bilirubin to its binding site on albumin, resulting in an increase in the levels of direct bilirubin (Rasmussen et al., 1976; Robertson and Brodersen, 1991; Martin et al., 1993). Propylparaben, a paraben preservative, is able to bind to albumin and decreases the levels of direct bilirubin. However its mechanism of action is unclear (Rasmussen et al., 1976). Using fluorescence spectroscopy, Ahmad et al. (2004) showed at pH 7 an increase in the binding of bilirubin to HSA up to 5.0 M urea.

In plasma, direct bilirubin is mainly the conjugated form, whereas in bilirubin–HSA solution, it is the unconjugated bilirubin. Cucurbitacins E and D decreased unbound bilirubin concentration in bilirubin–HSA solution and direct bilirubin concentration in plasma, suggesting that they can increase the binding of both forms of bilirubin.

The comparison of ratios UBC_{CucE}/UBC_0 and DBC_{CucE}/DBC_0 determined respectively in bilirubin–HSA solution and plasma of healthy subjects showed that cucurbitacin decreased bilirubin levels in plasma better than in bilirubin–HSA solutions. The difference between plasma and bilirubin–HSA solution was not related to difference in HSA concentration which is equal in plasma samples to 39±0.2 g/L determined by the bromocresol green method (albumin-Kit). The presence of endogenous compounds in plasma, especially those that can affect the binding of bilirubin, could explain, at least in part, the difference between the results obtained in bilirubin–HSA solution and those found in plasma.

Besides, results obtained in plasma from subject presenting a hyperbilirubinemia (unconjugated hyperbilirubinemia) showed that Cuc E (100 μM) decreased direct bilirubin concentration



Cuc	C_1,C_2 bond	R
D	C_1— C_2	H
E	C_1 = C_2	Acetyl
I	C_1 = C_2	H

Scheme 1. Structures of cucurbitacins D, E and I.

(mainly conjugated form of bilirubin) from 40 (control) to 19 μM (plasma incubated with Cuc E) and decreased the concentration of unconjugated bilirubin (obtained by difference: total bilirubin concentration-direct bilirubin concentration) from 160 (control) to 83 μM (plasma incubated with Cuc E). Thus, Cuc E modifies more the level of unconjugated bilirubin than that of the conjugated form.

The effects of Cuc are related to their structures represented in Scheme 1. On the one hand, Cuc E has similar structure to Cuc I, except its acetyl group on C26. On the other hand, Cuc D and Cuc I differ only in structure regarding the bond between C1 and C2, which is single in Cuc D and double in Cuc I. Consequently, the presence of a double bond between C1 and C2 and the absence of an acetyl group at C26 are essential to prevent Cuc from modifying the binding of bilirubin to albumin.

The hydrophobicity of cucurbitacin is not probably the factor that can control the decrease of bilirubin levels. In fact, Bartalis and Halaweish (2005) showed that Cuc E is more hydrophobic than Cuc I which is more hydrophobic than Cuc D. Similar conclusions were also obtained in our laboratory. The retention times obtained by HPLC using C18 Column (10 μm, 250 × 10 mm) and mobile phase consisted of acetonitrile/methanol/H_2O (30, 40, 30) were respectively 21, 23 and 55 min for cucurbitacins D, I and E (results not shown).

Conclusion

Our study has demonstrated that cucurbitacins E and D interact with albumin leading to increase in the binding of bilirubin to albumin regardless to whether the bilirubin is conjugated or unconjugated. Based on fluorescence spectral results it can be concluded that the cucurbitacin produced rearrangement in domain II leading to an increase in the hydrophobic microenvironment of tryptophan and to the binding of bilirubin to protein.

The binding of other endogenous and exogenous compounds to albumin could be also influenced by the presence of these cucurbitacins. Cuc I was the only cucurbitacin that did not modify the binding of bilirubin. It could be considered less capable to affect the interaction of small molecules with albumin than cucurbitacins E and D.

Acknowledgment

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