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Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 572–580
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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The inclusion complexes of hesperetin and its 7-rhamnoglucoside with (2-hydroxypropyl)-β-cyclodextrin

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Received 26 November 2004; received in revised form 12 May 2005; accepted 12 May 2005
Available online 27 June 2005

Abstract

The effect of (2-hydroxypropyl)-β-cyclodextrin (HP-β-CyD) on the solubility properties and spectroscopic features of hesperetin and its 7-rhamnoglucoside, hesperidin, was qualitatively and quantitatively investigated in water, by means of UV–vis absorption and fluorescence spectroscopy. The stoichiometric ratios and stability constants describing the extent of formation of the complexes have been determined by phase-solubility measurements; in both cases type-A_L diagrams have been obtained (soluble 1:1 complexes). The higher degree of interaction showed by hesperetin may be attributed to the higher hydrophobicity and smaller size of the aglycone molecule, which therefore exhibits a greater affinity for the CyD and fits better into the cavity. The effect of molecular encapsulation on the two flavanones antioxidant activity was afterwards evaluated by means of different biological assays, concerned to the different mechanisms of in vivo action. The protection efficacy was in all cases higher for the complexed drugs, with respect to the free ones; these results are of great interest for their potential usefulness in pharmaceutics.

Keywords: (2-Hydroxypropyl)-β-cyclodextrin; Hesperetin; Hesperidin; UV–vis spectroscopy; Fluorescence spectroscopy; Binding constant; Solubility measurements; Biological assays

1. Introduction

Cyclodextrins (CyDs) have widely proved their usefulness as tools to generate aqueous drug solutions without the use of organic co-solvents, surfactants, or lipids, as formulation adjuncts, which increase dissolution rates and oral bioavailability of solid drug complexes [1,2].

Because of the solubility limits and the safety concerns with native cyclodextrins, numerous chemical modifications of the cyclodextrins have been made and newer derivatives are constantly being developed. Since each CyD hydroxyl group differs in its chemical reactivity, the randomness of position and type of substitution causes the resultant CyD to be amorphous, which contributes to its greatly enhanced aqueous solubility compared to the crystalline parent. This property of amorphousness of the chemically modified CyDs (CMCyDs) may also be helpful to overcome problems of toxicity and realize efficient drug delivery systems [3,4].

The hydrophobic cavity interior and limitation of molecular mobility inside the CyDs cavity can perturb certain photophysical properties of included guest molecules [5–7]. Several works have been focused on the study of photophysical processes of organic molecules inserted in CyDs cavity, such as room-temperature phosphorescence [8,9], excited-state proton transfer [10], femtochemistry [11] and molecule sensing [12]. Among the different analytical methods, which have been applied for the analysis and characterization of

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doi:10.1016/j.jpba.2005.05.009

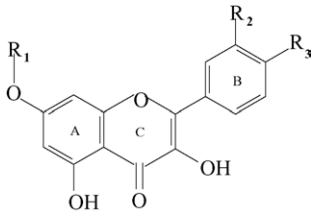

Fig. 1. The chemical structures of the flavanones: hesperetin (R_1 = H, R_2 = OH, R_3 = OCH_3) and hesperidin (R_1 = rhamnosylglucoside, R_2 = OH, R_3 = OCH_3).

CyDs inclusion complexes, UV–vis and fluorescence spectroscopy are the most widely used, because of the significant analyte signal increment that is frequently induced [13,14]. Fluorescence enhancements have been reported for a large number of organic molecules, either in the presence of native or modified β-CyD [15,16].

Among naturally occurring *Citrus* flavonoids, hesperidin and hesperetin (Fig. 1) have been reported to exert a wide range of pharmacological effects.

Hesperidin possesses antioxidant [17], blood lipid lowering [18] and anti-carcinogenic activities [19]. In addition, they have been found to improve venous tone, enhance microcirculation, assist healing of venous ulcers and are used for the treatment of chronic venous insufficiency [20], hemorrhoids [21] and the prevention of post-operative thromboembolism [22]. Epidemiological studies have shown an inverse correlation between the intake of dietary flavonoids and death from coronary disease [23,24]. Dietary hesperidin is deglycosylated into hesperetin by intestinal bacteria prior to absorption [25]. The very poor solubility of these flavanones in water yet imposes considerable limitations to pharmaceutical use. The solubility of hesperidin in water has been estimated to be 20 ppm or less [26,27] and is also indicated by the use of this solvent to remove impurities from precipitated hesperidin [28].

Formulations that improve the solubility of these drugs may, therefore, provide improved therapeutic options for patients.

Our interest was here devoted to improve the physicochemical properties of hesperetin and its glycoside hesperidin, by interaction with HP-β-CyD. The efficacy of protection of these flavanones against oxidative damage was demonstrated by means of different in vitro tests (bathophenanthroline test; comet assay; lipid peroxidation) and was further improved after complexation with (2-hydroxypropyl)-β-cyclodextrin.

Different analytical techniques, such as UV–vis absorption and fluorescence spectroscopy, were employed for a throughout investigation of the structural characteristics of such supramolecular aggregates, which exhibited distinct spectroscopic features and properties from both “guest” and “host” molecules.

The stoichiometry and equilibrium constants for complexes formation, which permitted a quantitative description of the solubility behaviour, were determined by phase-solubility studies.

2. Experimental

2.1. Chemicals

The following reagents and solvents were used: hesperetin (3′, 5, 7-trihydroxy-4-methoxyflavanone, $C_{16}H_{14}O_6$, FW 302.3); hesperidin (hesperetin-7-rhamnoglucoside, $C_{28}H_{34}O_{15}$, FW 610.6) and all chemicals for the biological activity tests were supplied by Sigma–Aldrich Chemie Gmbh (Germany); (HP-β-CyD, FW ≈1170.0, mp ≈278 °C decreasing, degree of substitution ≈0.6), from Fluka Chemie (Switzerland). They were employed without any further purification. Water used throughout the study was double-distilled and deionised, then filtered trough 0.22 μm Millipore filters (USA). All solvents employed in the spectrophotometric analyses were of spectroscopic reagent grade, from Merck (Germany). All other materials were HPLC grade.

2.2. Apparatus and procedure

The UV–vis absorption spectra were obtained with a Perkin-Elmer UV–vis double beam spectrophotometer mod. Lambda 45, equipped with a PC for data processing (software: UV-Win Lab, from Perkin-Elmer, Norwalk, USA). Ten-millimeter pathlength rectangular quartz cells (Hellma) were employed in the 200–500 nm spectral range (scanning speed 60 nm/min; slit = 2). Concentrations of the samples to be measured were adjusted so that the extinction values did not exceed $E = 1.0$ at a given wavelength. Baseline was established for each measurement placing in the reference compartment an aqueous solution of HP-β-CyD at the same concentration of the sample. All the data shown represent the average of, at least, three determinations. UV–vis measurements were performed to obtain a calibration curve of both flavanones, i.e. hesperetin ($0.0–3.0 \times 10^{-5}$ M) and hesperidin ($0.0–3.0 \times 10^{-4}$ M). Also, the samples obtained from solubility measurements were analysed, to assess the effect of HP-β-CyD ($0.0–9.0 \times 10^{-3}$ M) on the UV absorption spectra of the two drugs.

The fluorescence spectra were recorded using a Perkin-Elmer LS-45 luminescence spectrometer (UK) with 3.0 nm band-width for excitation and emission; the instrument was interfaced with a PC computer for signals reading. The optical density of the samples at the excitation wavelengths 360 and 370 nm (hesperetin and hesperidin, respectively) did not exceed 0.15. The samples were placed in rectangular quartz cuvettes of 10.00 mm pathlength (Perkin-Elmer), all measurements were carried out at room temperature (25 ± 0.1 °C). Spectra were recorded in triplicate and data averaged.

Fluorescence emission intensities of the samples obtained from solubility measurements were measured ($0.0–9.0 \times 10^{-3}$ M HP-β-CyD added to aqueous excess of both flavanones).

2.3. Solubility measurements

Phase-solubility studies were performed with a Haake C25 thermostated bath, equipped with a Haake F6 controller which allowed an accuracy of ±0.01 °C. Excess of flavanones were added to unbuffered aqueous solutions of HP-β-CyD ($0.0–9.0 \times 10^{-3}$ M) in 10 ml capped tubes, then sonicated in a water bath (Bandelin RK 514, Berlin, Germany) for 15 min. Tubes were sealed to avoid changes due to evaporation and magnetically stirred for 4 days in a thermostated bath at 25 ± 0.01 °C, shielded from light to prevent any degradation of the molecules. After the equilibrium was reached, suspensions were filtered through Sartorius Minisart®-SRP 15 PTFE 0.45 μm filters (Germany). An aliquot from each vial was withdrawn by 1 ml glass syringe (Poulten & Graf GmbH, Germany) and assayed spectrophotometrically to evaluate the amount of flavonoid dissolved. Experiments were carried out in triplicate; solubility data were averaged and used to calculate the binding constants for hesperetin and hesperidin/HP-β-CyD complexes formation, by UV–vis as well as by fluorescence spectroscopy.

2.4. Preparation of the solid complexes

Inclusion complexes of the two flavanones were obtained by the co-evaporation method. Weighed hesperetin (30 mg) and hesperidin (91.7 mg) were added to 30 ml solutions of HP-β-CyD (6 and 9 mM, respectively) in purified water. In particular, even though a 1:1 stoichiometry was obtained for both complexes, an excess of cyclodextrin was employed to increase the yield of complexation, as widely reported in literature.

Stirring was carried out for 2 days, under controlled temperature (25 ± 0.01 °C). After that time, solutions were filtered through Whatman® PTFE 0.22 μm filters and water was evaporated under vacuum (30 °C), obtaining the solid complexes (hesperetin/HP-β-CyD, yield 62%; hesperidin/HP-β-CyD, yield 54%) which were washed with a little water, dried up to constant weight and kept into desiccator. Uncomplexed drug had been filtered prior to solution evaporation, thus obtaining the solid complexes, still containing an excess of free HP-β-CyD. The amount of drug in the complexes was determined by UV measurement, from the estimated molar extinction coefficient of the complexes.

2.5. Biological activity tests

2.5.1. Production and detection of hydroxyl radical

The oxidation extent of deoxyribose is related with $OH^{\bullet}$ production. In this test we have quantified the deoxyribose degradation product, measuring the production of malondialdehyde (MDA), by its condensation with thiobarbituric acid [29]. The assays were carried out incubating 0.1 mM EDTA, 0.1 mM ascorbic acid, 20 mM $FeCl_3$, 10 mM KH_2PO_4 (pH 7.4), 2.8 mM deoxyribose and 2.8 mM H_2O_2 in the presence of the tested compounds for 60 min at 37 °C. In order to evaluate the role of Fe^{2+} chelation in the protective effect exerted by HP-β-CyD, EDTA has been excluded from assay mixture in a set of samples treated with HP-β-CyD. One percent thiobarbituric acid and 2.8% trichloroacetic acid (0.5 ml each) were added to 0.5 ml of the reaction medium and heated to 100 °C for 15 min. MDA levels were measured spectrophotometrically at 532 nm.

2.5.2. Auto-oxidation of Fe^{2+}

Ferrous ion is spontaneously oxidized to ferric ion, and this auto-oxidation has been evaluated by measuring the Fe^{2+}-bathophenanthroline disulphonate complex in presence of HP-β-CyD.

The test has been developed on the basis of Yoshino et al. [30] with slight modifications. Nine hundred and eighty microliters of 10 mM Tris–HCl (pH 7.1) were added with 10 μl of HP-β-CyD (0.3 or 1.2 μM final concentration); the reaction was started by addition of 10 μl of 0.05 mM $FeSO_4$; the mixtures were incubated at 37 °C. Five hundred microliters of 1 mM bathophenanthroline disulphonate were added at appropriate intervals and the absorbance has been measured at 540 nm.

2.5.3. Comet assay

The single cell gel electrophoresis was performed as described by Singh et al. [31] on human leucocytes from peripheral venous blood. Freshly collected whole blood was incubated for 1 h at 37 °C with 10 μM benzo[a]pyrene (BaP) to induce DNA damage. Hesperidin and hesperetin (free and complexed, 15 and 60 μM final concentrations) were pipetted immediately before BaP. Frosted microscope slides (prepared in triplicate per sample per experiment) were covered with 140 μl of 0.75% regular melting agarose; a second 110 μl layer of 0.5% low melting point agarose mixed with leucocytes incubation mixture was pipetted out. After solidification, a third layer of 110 μl low melting point agarose was pipetted out on the slides and allowed to gel at 4 °C. Slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl pH 10, 1% Sarkosyl with 1% (v/v) Triton X-100 and 10% (v/v) DMSO) and kept at 4 °C overnight. Slides were then placed in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min to allow DNA unwinding; electrophoresis was conducted in the same solution for 25 min at 0.6 V/cm. Finally, after washing (3× 5 min in 0.4 M Tris–HCl buffer, pH 7.5), slides were stained with 50 μl propidium iodide (2.5 mg ml^{-1}). Comets were viewed under a fluorescence Nikon E800 microscope with a 200× objective. Hundred cells were randomly selected in each slide and scored from 0 to 4 on the basis of comet tail length; undamaged cells (score 0) looked like an intact nucleus without tail, while

damaged cells appeared as comets. Final score was calculated by the formula: score = (n cells scored 1) + ($2 \times n$ cells scored 2) + ($3 \times n$ cells scored 3) + ($4 \times n$ cells scored 4).

2.6. Statistical analysis

Statistical analysis was carried out to study the significance of the results of biological activity tests comparing free and complexed hesperidin and hesperetin with the controls. The Dunn test, a non-parametric multiple comparison test based on Kruskal–Wallis rank sums, was performed to analyse Comet scores.

3. Results and discussion

3.1. UV–vis spectroscopy studies

A calibration curve was obtained for hesperetin and hesperidin, by dissolving increasing amounts of the drugs, exactly weighed, in the desired volume of purified water. A plot of the measured UV absorbances at the appropriate wavelength against the molarity of the analytes gave straight lines in the concentration range from 0.0 up to 3.3×10^{-6} M, for hesperetin ($R^2 = 0.998$) and from 0.0 up to 3.6×10^{-5} M, for hesperidin ($R^2 = 0.995$). At higher concentrations a plateau was observed, meaning that the solubilities of the two flavanones were exceeded. Hesperetin and hesperidin maximum water solubilities (S_0) at $25 \pm 0.1\,^{\circ}$C were, therefore, found to be 3.3×10^{-6} M (0.001 mg ml^{-1}) and 3.6×10^{-5} M (0.022 mg ml^{-1}), respectively. The last finding is in very good agreement with what reported by the Merck Index [27]. Experiments were carried out in triplicate, each data point represents the mean ±0.00057 S.D. for hesperetin (calculated R.S.D. = 2.13%) and ±0.0007 S.D. for hesperidin (calculated R.S.D. = 0.56%).

The effect of HP-β-CyD on the UV absorption spectra of the flavanones was quantitatively investigated by holding the concentration of the guest constant $2 \times S_0$ (i.e., 7.2×10^{-5} M hesperidin and 6.6×10^{-6} M hesperetin) and varying the host concentration between 0.0 and 9.0×10^{-3} M (solubility measurements). The absorption maxima were progressively shifted to longer wavelengths in the presence of the HP-β-CyD (from 285 to 289 nm for hesperetin; from 283 to 286 nm for hesperidin; Fig. 2), while the absorption intensities increased as a function of the HP-β-CyD concentration. In the case of hesperetin, however, a well-defined plateau was observed at 5.0×10^{-3} M HP-β-CyD; at this point, the molecule must be fully complexed.

The plots of solubilised drugs against the cyclodextrin concentration showed good linearity, as described in Section 3.3, suggesting the formation of 1:1 inclusion complexes, for both flavanones.

3.2. Fluorescence studies

The same drug solutions obtained by solubility measurements were excited at 370 nm (hesperetin/HP-β-CyD) and 360 nm (hesperidin/HP-β-CyD), where the optical density did not exceed 0.15. The fluorescence emission was measured, as a function of the cyclodextrin concentration. Again, the observed fluorescence intensities linearly increased with the increasing of the HP-β-CyD concentration, supporting the formation of 1:1 inclusion complexes in both cases (see Fig. 3).

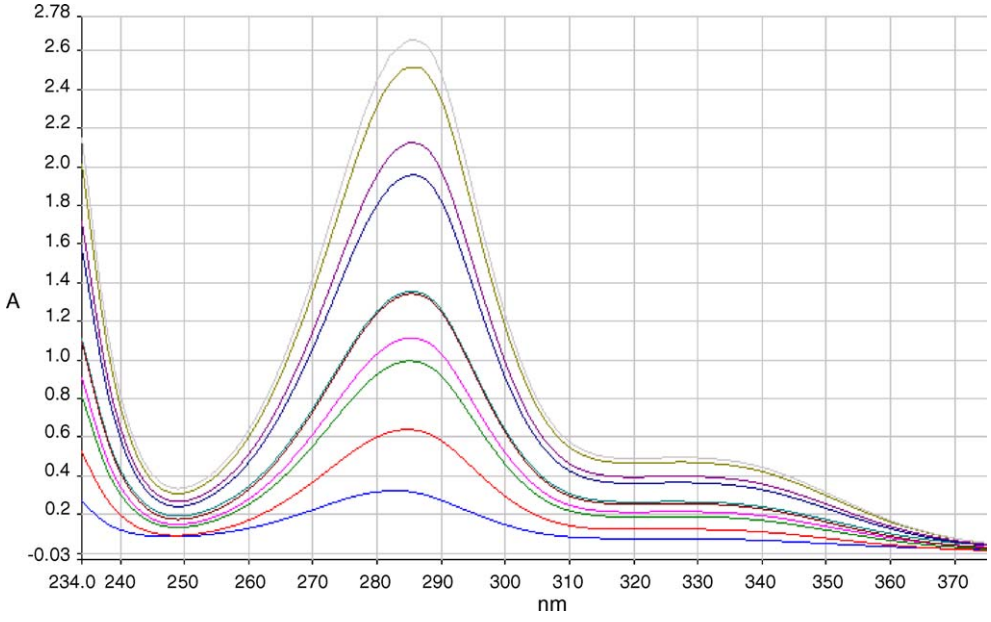

Fig. 2. UV absorption spectra of hesperidin (7.2×10^{-5} M) in the presence of increasing concentrations of HP-β-CyD (0.0–9.0×10^{-3} M).

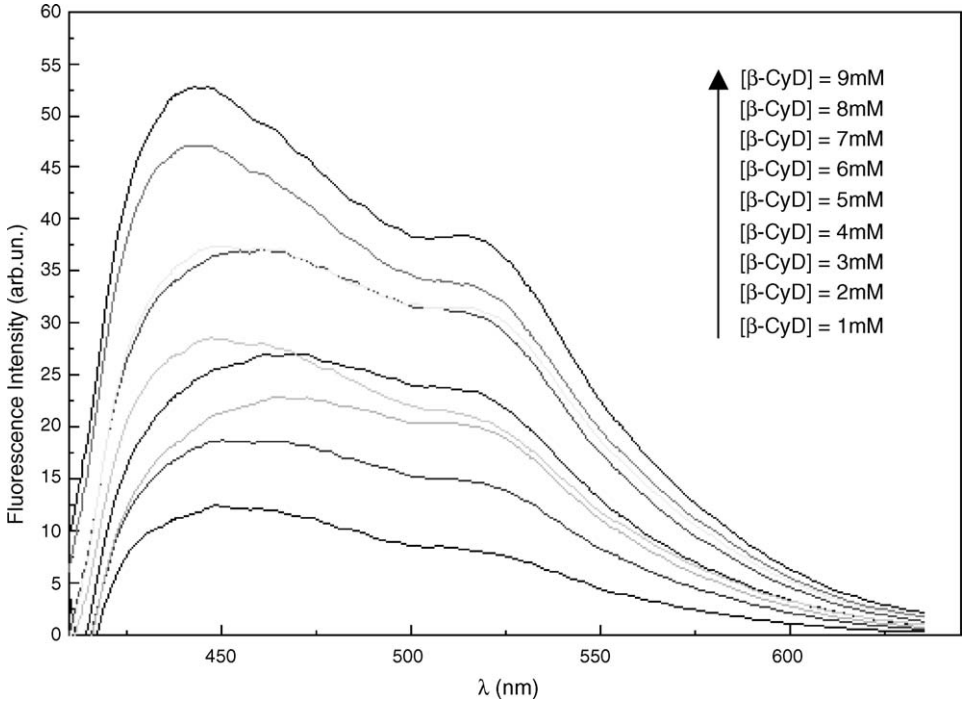

Fig. 3. Emission spectra of hesperidin (1.63×10^{-5} M, excited at 360 nm) in presence of increasing concentrations of HP-β-CyD.

3.3. Binding constants

The binding constant for a 1:1 complex is:

$$K = \frac{[S \cdot CyD]}{[CyD][S]} \quad (1)$$

in which [S·CyD], [CyD] and [S] represent the equilibrium concentrations of the complexed substrate, free cyclodextrin and free substrate, respectively.

Eq. (1) requires careful consideration when, as in the present case, the substrate is not fully soluble and amounts

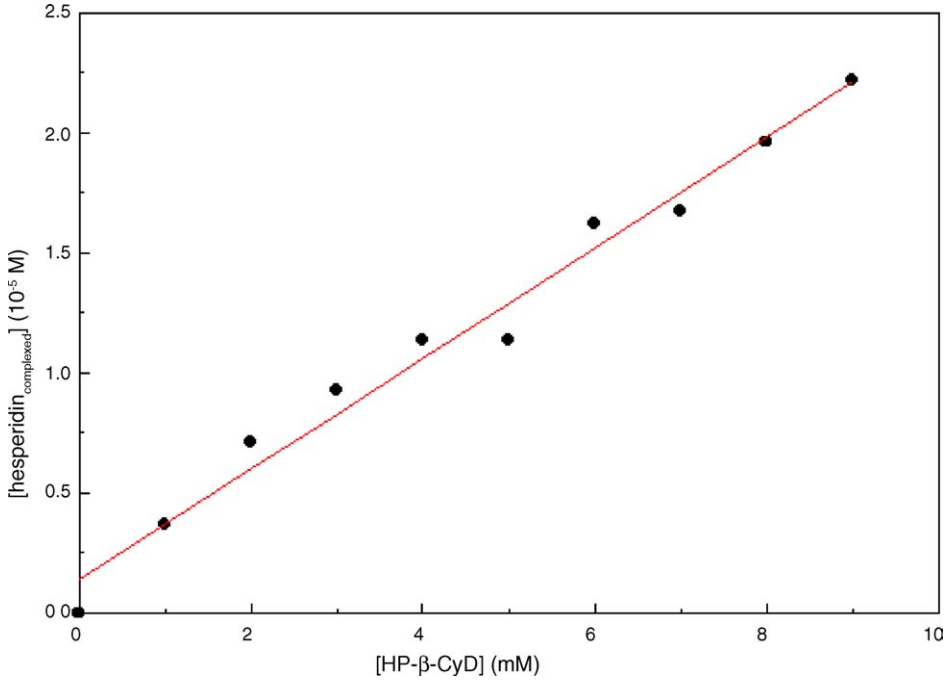

Fig. 4. Fluorescence determination of the amount of complexed flavanones as a function of HP-β-CyD concentration at 25 ± 0.1 °C. The differences in the fluorescence emission at 440 nm (for hesperidin, excited at 360 nm, shown in figure) and 480 nm (for hesperetin, excited at 370 nm) were normalized for the measured extinction molar coefficient of the complexes.

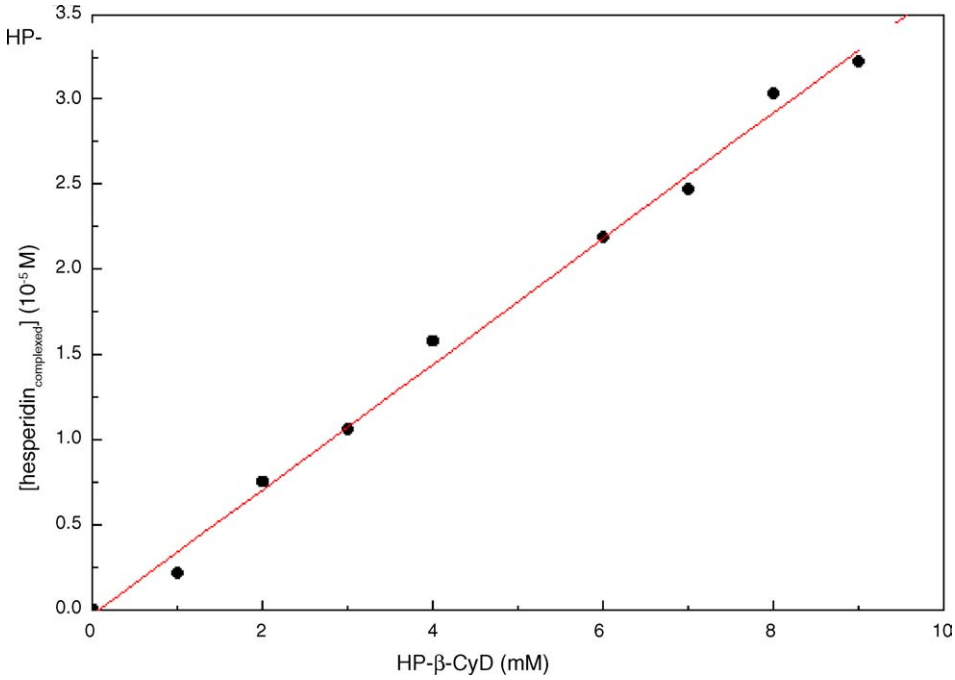

Fig. 5. UV–vis determination of the amount of complexed flavanones as a function of HP-β-CyD concentration at 25 ± 0.1 °C. The differences in the UV–vis absorbance intensities (at 360 and 370 nm for hesperidin and hesperetin, respectively) were normalized for the measured extinction molar coefficient of the complexes.

that exceed its solubility are used. In this case the binding constant can be calculated from the phase solubility technique, which allows for the evaluation of the affinity between cyclodextrin and substrate, according to the method reported by Higuchi and Connors:

$$K = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \quad (2)$$

where S_0 is the solubility of the substrate and the Slope parameter is obtained from the straight-line portion of the plot of complexed substrate concentration, [S·CyD], against cyclodextrin concentration. In order to obtain [S·CyD] from the experimental quantities, it has to be considered that the substrate concentration, C_S–S_0 (C_S being the analytical substrate concentration), which is not soluble in the aqueous phase, acts as a reservoir for the formation of the complex when the cyclodextrin is added. The following calculation is performed for the fluorescence measurements, but it is reproducible for the UV–vis measurements simply changing fluorescence intensity with absorbance.

Let us consider that I_F is the integrated area of the fluorescence spectrum for which all the substrate concentration, C_S–S_0, is in the complexed form, and I_0 the integrated area of fluorescence spectrum of the substrate when the analytical cyclodextrin concentration is $C_{CyD} = 0$ (the use of the area instead of the maximum minimizes errors due

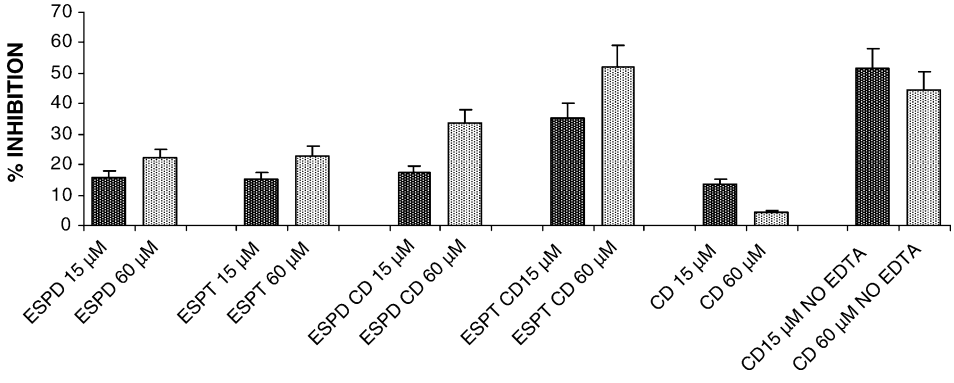

Fig. 6. Effect of 15 and 60 μM free and complexed hesperidin (ESPD), hesperetin (ESPT) and HP-β-CD as hydroxyl radical ($OH^{\bullet}$) scavengers on deoxyribose oxidation. Each bar represents the mean ± S.D. of MDA production in three experiments, each in triplicate. Results are reported as % I of $OH^{\bullet}$ production in respect to untreated samples (0%).

to peak shifts or shape changes). In the presence of a given amount of HP-β-CyD, at the analytical concentration of C^i_{CyD}, the fluorescence intensity of the solution increases because of the increased amount of complexed substrate. It will be $I_i = I_0 + f_C[S{\cdot}CyD]_i$, with $f_C = (I_F - I_0)/(C_S - S_0)$. Therefore, the concentration of the complexed substrate can be obtained from the fluorescence intensity values:

$$[S \cdot CyD]_i = \frac{(I_i - I_0)(C_S - S_0)}{(I_F - I_0)}.$$

In the case of both hesperidin ($S_0 = 3.6 \times 10^{-5}$ M) and hesperetin ($S_0 = 3.3 \times 10^{-6}$ M), plotting this quantity as a function of the total concentration of HP-β-CyD, a linear behaviour is obtained, indicating a 1:1 stoichiometry of the complexes. From the slope of the linear fit the estimated binding constants, using Eq. (2), are $K = 60 \pm 20\ M^{-1}$ ($\log K = 1.81$, $R^2 = 0.98$, slope = 0.0023), for the hesperidin/HP-β-CyD inclusion complex (see Fig. 4), and $K = 19000 \pm 4000\ M^{-1}$ ($\log K = 4.28$, $R^2 = 0.93$, slope = 0.059), for the hesperetin/HP-β-CyD inclusion complex.

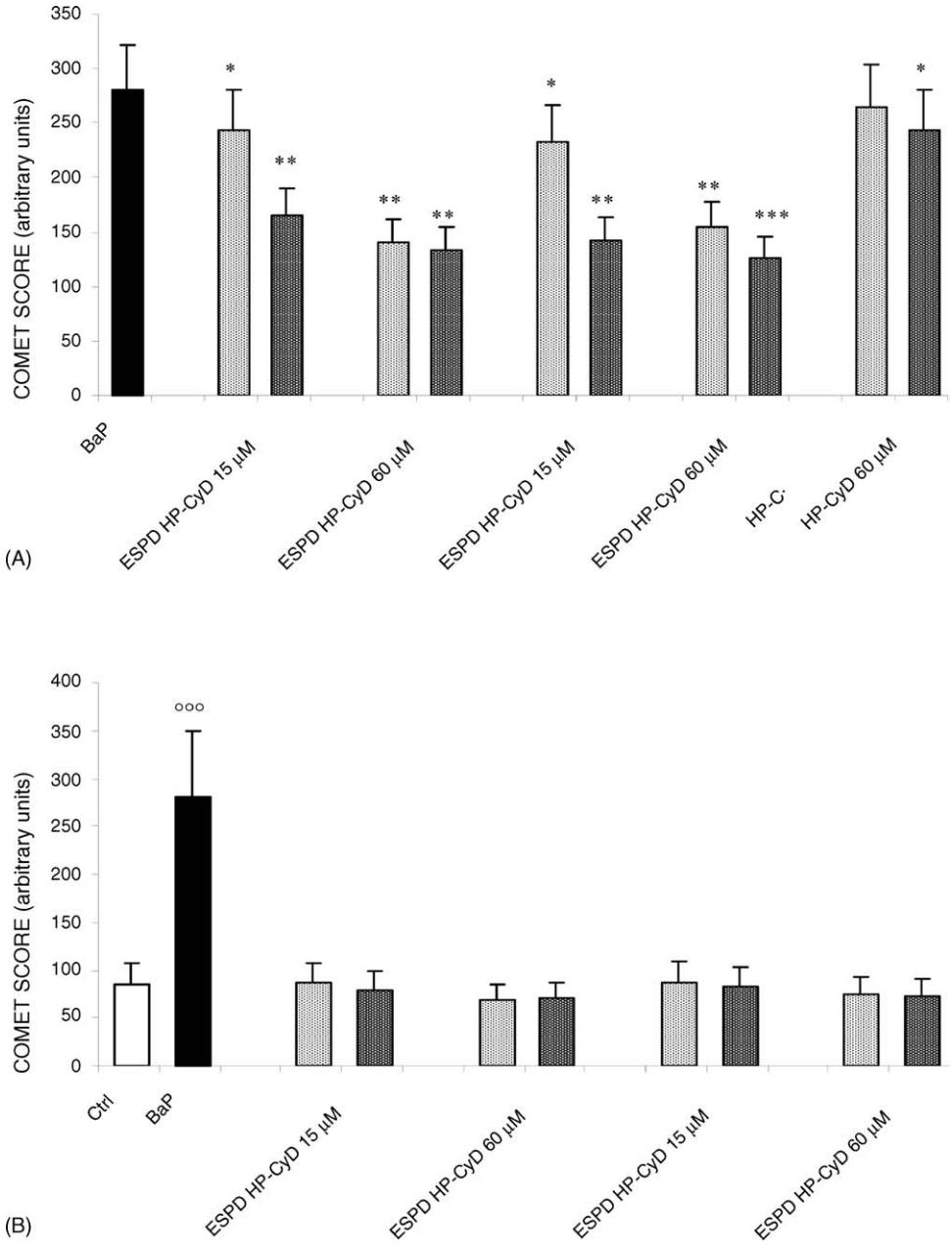

Fig. 7. Effect of 15 and 60 μM free and complexed hesperidin (ESPD), hesperetin (ESPT) and HP-β-CyD on BaP-mediated DNA damage (A) and on undamaged human leucocytes (B) by comet assay. Each bar represents the mean of three experiments, each in triplicate, with 1 S.D. error bars shown. By means of Dunn's test, the comet score of the treated cells was compared to that of untreated but damaged cells ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$); BaP damage and the effect of the free and complexed flavanones were compared to undamaged control cells ($^{\circ\circ\circ}P < 0.001$).

An analogous procedure using absorbance values gives $K = 90 \pm 30\ M^{-1}$ ($\log K = 1.95$, $R^2 = 0.99$, slope = 0.0037), for the hesperidin/HP-β-CyD inclusion complex (see Fig. 5), and $K = 21000 \pm 4000\ M^{-1}$ ($\log K = 4.32$, $R^2 = 0.98$, slope = 0.067), for the hesperetin/HP-β-CyD inclusion complex. Experiments were carried out in triplicate, each data point represents the mean ±0.0032 S.D. for hesperetin (calculated R.S.D. = 3.59%) and ±0.00127 S.D. for hesperidin (calculated R.S.D. = 1.54%).

A higher complexation efficacy was obtained for hesperetin, as clearly revealed by the higher numeric value of the binding constant. This can be easily explained in terms of a much smaller molecule, considerably less polar than the glycoside (hesperidin), hence exhibiting greater affinity towards the hydrophobic cavity of the cyclodextrin.

3.4. Hydroxyl radical production and Fe^{2+} auto-oxidation

The percentage of inhibition (% *I*) of $OH^{\bullet}$ production by the test compounds was calculated from Abs_{532} values according to the formula:

$$\%\, I = \left[\frac{(A_0 - A_S)}{A_0}\right] \times 100$$

where A_0 is the absorbance of the damaged control and A_S corresponds to samples treated with antioxidant compounds.

As described in Fig. 6, the antioxidant activity exerted by free flavanones is enhanced by complexing them with HP-β-CyD. This effect is dose-dependent and the 60 μM concentration demonstrates almost 50% more of activity than 15 μM one. Free HP-β-CyD does not express more inhibition of hydroxyl radical production than free and complexed flavanones. The elimination of EDTA from the assay mixture with free HP-β-CyD, aiming to assess its role in this test, determines a percentage of inhibition higher than that of the HP-β-CyD/EDTA system.

3.5. Comet assay

The results obtained from the single-cell gel electrophoresis of human leucocytes are shown in Fig. 7. Decreased score corresponds to decreased damage and indicates a protective effect of antioxidant compounds. Concentrations were normalized for free flavanone molarity (15 and 60 μM free hesperidin and hesperetin) in the BaP/cells reaction mixture. Reduced DNA damage in stressed cells was observed with all antioxidant compounds tested and it was more pronounced with complexed flavanones compared to free compounds; this difference was more evident at 15 μM concentration, while 60 μM provided effective protection even with free flavanones (Fig. 7A). Treatment with both concentrations of HP-β-CyD did not provide significant protection against DNA oxidative stress. Both free and complexed flavanones did not induce damage per se (Fig. 7B).

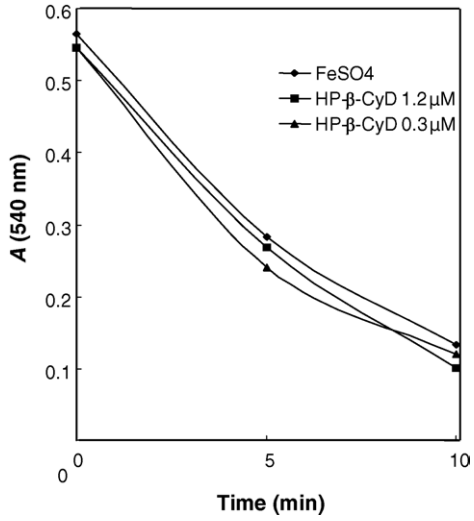

Fig. 8. Effect of HP-β-CyD on Fe^{2+} auto-oxidation. The absorbance measures Fe(II)-bathophenanthroline disulphonate complex without HP-β-CyD (♦) and with HP-β-CyD (■) 1.2 and (▲) 0.3 μM.

4. Conclusions

The inclusion complexes of hesperetin and hesperidin with (2-hydroxypropyl)-β-cyclodextrin were obtained by co-evaporation from aqueous suspensions of the host and guest molecules. The effect of cyclodextrin complexation on the UV–vis absorption and fluorescence properties of the two flavanones was, therefore, studied in aqueous solution, and an increased apparent solubility was in both cases observed in the medium. Particularly, the dependence of both the absorption and emission intensities on HP-β-CyD concentration suggested that 1:1 soluble complexes were responsible for the spectral changes observed, since type-A_L diagrams were obtained for both flavanones. The concentration of the complexed substrates was obtained from fluorescence, as well as from UV–vis intensity values. Thus, plotting these quantities as a function of the total CyD concentration, the associated binding constants were estimated from the slope of the linear fit. The two techniques employed showed quite a good agreement; linearity and reproducibility were satisfactory. Both the free molecules and the complexes were afterwards tested in different biological assays, concerned to the different mechanisms of in vivo action. The hydroxyl radical is one of the most aggressive oxidants. This species can be efficiently generated via the Fenton reaction from Fe(II)-EDTA in the presence of hydrogen peroxide and a reducing agent as ascorbic acid. The $OH^{\bullet}$ can diffuse and abstract hydrogen atoms from deoxyribose substrate inducing its oxidation. The presence of free and complexed flavanones at both the tested concentrations (60 and 15 μM), prevents deoxyribose oxidation acting as radical scavengers, as suggested by the reduced percentage of inhibition.

In particular, the complexed flavanones showed at both the concentrations a higher % *I*, more pronounced with HP-β-CyD-hesperetin; this result can be well explained by a

significantly better solubility in the aqueous medium of the complexes in respect of free flavanones.

In absence of EDTA, the radicalic reaction is partially prevented. Fe^{2+} is partially chelated by the HP-β-CyD, as suggested by the bathophenanthroline test (Fig. 8) and the reduced deoxyribose oxidation.

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