Drug Delivery Systems Prepared by SCF Technology for Enhanced Bioavailability and Cellular Uptake of Model Antioxidant Compounds

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The cellular uptake of antioxidant compounds is one of the most important issues determining its effectiveness. In this work, the possibility of preparing innovative drug delivery systems that allow the improvement of bioavailability, cellular antioxidant activity and enhance cellular uptake of antioxidant compounds was investigated, using supercritical CO_2 technology. Quercetin and a natural tocopherol mixture were selected as model compounds for this study due to their potent antioxidant activity and relevant beneficial health effects. Carriers studied included hydroxypropyl-β-cyclodextrin and a biocompatible lipid, glyceryl monostearate frequently used as bioavailability enhancers of many hydrophobic oral drugs.

The particulate systems were prepared using PGSS® (Particles from Gas Saturated Solutions) technique. The supercritical process was applied using different composition ratios in order to determine the most promising formulations. Particles produced were analysed by Differential Scanning Calorimetry (DSC), Scanning Electronic Microscopy (SEM) and the drug loads were determined using HPLC UV/VIS with diode array. Moreover Caco-2 cells, used as model of intestinal barrier, were incubated for 1 or 3 hours in growth medium containing the antioxidant loaded particles. Cellular uptake of quercetin and loaded solid hybrid particles were indirectly evaluated through determination of intracellular reactive oxygen species (ROS) scavenging capacity (antioxidant activity). Methanolic extracts from Caco-2 monolayers were examined by HPLC analysis in order to determine cellular uptake of neat tocopherols and tocopherol-loaded hybrid particles. The bioavailability of quercetin and tocopherols (neat and when loaded in solid-hybrid particles) were evaluated using transepithelial permeability experiments.

Solid hybrid particles, in comparison with pure forms, allow the enhancement of intestinal transport to basolateral side of antioxidant compounds improving their bioavailability. Furthermore, the hybrid system Toc:HP-β-CD:GMS (1:5:10) reveals to be promising for intestinal cellular uptake of tocopherols.

Keywords: Antioxidant activity, Bioavailability, Cellular Uptake, Quercetin, Tocopherols, 2-(hydroxypropyl)-β- cyclodextrin, Glyceryl monostearate.

INTRODUCTION

The extent of the cellular uptake of antioxidant compounds is an important factor determining its effectiveness [1]. Several parameters as low aqueous solubility and the metabolism of antioxidant compounds have a major influence in the possibility of these compounds to be efficiently absorbed, enter the systemic circulation, cross cell membrane barriers and still hold the desired biological activity. The preparation of delivery systems has been highlighted as an efficient tool to overcome these difficulties providing new opportunities to low bioavailable active ingredients [2],[3],[4].

Tocopherol, also recognized as vitamin E, is an essential micronutrient for both human and animals. Recently, data available concerning to absorption of vitamin E have shown that no more than half of the doses ingested are absorbed [5]. Quercetin is a flavonol present in diverse vegetables and fruits with several recognized beneficial effects for human health with a low oral bioavailability [6].

In this work, the possibility of using supercritical fluid technology for the preparation of hybrid particulate drug delivery systems that allow an enhanced cellular uptake of antioxidant compounds was investigated. The bioactive compounds chosen as models were a natural mixture of tocopherols (Toc) and quercetin (QUER). Current methods of preparing these kind of particulate delivery systems often involve the use of multi-step processes, the need for organic solvents as well as severe operating conditions [7]. The possibility of using SCF technology for processing antioxidant compounds can bring some real advantages over conventional techniques since it avoids thermal degradation and decomposition due to low CO_2 critical temperature. In addition it prevents oxidation reactions due to the absence of light and oxygen during the process [8]

In this study, the carriers selected for solid hybrid particles formulation were mixtures of a hydrophilic cyclodextrin, ((2-hydroxypropyl)-β-cyclodextrin) and a biocompatible solid lipid: Glyceryl monostearate (with a hydrophilic-lipophilic balance (HLB) of 4)[9],[6],[10].

Hydrophilic cyclodextrins, as (2-hydroxypropyl)-β-cyclodextrin, can modify the rate of drug release and enhance drug transport across biological barriers; they are useful to form inclusion complexes with poorly water-soluble drugs, as quercetin, and consequently augment their dissolution characteristics and oral bioavailability [11],[12],[13].

Solid lipid formulations have been reported not only for the improvement of water dissolution, but also as efficient carriers to enhance the cellular uptake of some drugs [14]. Yuan *et al.* have recently pointed out their potential application for tumor cell targeting due to enhanced cellular uptake of paclitaxel loaded solid lipid nanoparticles [15]. Furthermore, as previously described [16] GMS (glyceryl monostearate) could attenuate the activity of the multiple resistant protein 2 (MRP2) and possibly other efflux transporters in Caco-2 cells correlated with quercetin efflux and respectively low bioavailability.

Herein, the possibility of producing hybrid ((2-hydroxypropyl)-β-cyclodextrin +GMS) delivery systems by PGSS® (Particles from Gas Saturated Solutions) was evaluated. The antioxidant, bioavailability and cellular uptake were studied using the Caco-2 cells as intestinal epithelial model. Caco-2 cell monolayers have been widely used as *in vitro* model to study intestinal absorption, for high throughput screening of drugs and excipients and cellular uptake. Several active transport systems located in the intestinal epithelium and enzymes achieve *in vitro* expression comparable to the native state. Furthermore, these cells spontaneously differentiate after reaching confluence in culture and exhibit apical and basolateral membrane demarcation by tight junctions and development of trans-membrane epithelial resistance [17]. Accordingly, in this study, Caco-2 cells were used to assess the

permeability and intracellular antioxidant activity of quercetin, and to evaluate the cellular uptake of tocopherols.

MATERIALS AND METHODS

1. *Materials*

Quercetin dihydrate 95+% (QUER), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) were purchased from Aldrich, Germany. Carbon dioxide (99,998 mol%, CAS [124-38-9]) was from Air Liquide. Lumulse® GMS (glyceryl monostearate) K was a gift from Lambent Technologies. Tocopherol (Toc) (a mix of naturally occurring d-alpha, d-beta, d-gamma and d-delta tocopherols) was purchase from Cognis, Germany. Methanol, HPLC grade was purchased from Lab-Scan, Poland; phosphoric acid *p.a* and Dimethyl sulfoxide (DMSO) were purchased from Panreac AC Quimica SAU, Spain. Acetonitrile HPLC grade was purchased from Sigma, Germany. 2',7'-dichlorofluorescin diacetate (DCFH-DA) and hydrogen peroxide (H_2O_2) purchase from Sigma-Aldrich (St Quentin Fallavier, France. Cells Antibiotic solution (10 g/L streptomycin and 10,000 U/ml penicillin) was purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640 medium, foetal bovine serum (FBS), L-glutamine 200 mM (100x) and trypsin–ethylenediamine tetraacetic acid solution were purchased to GIBCO (Invitrogen, Glasgow, UK).

2. *Preparation of the solid-hydrid particles*

Quercetin and Tocopherol solid hybrid particulate systems were produced using the PGSS® technique first described by Weidner *et al.* [18]. Briefly, it consisted in dissolving the carbon dioxide in the bulk of a mixture of substances at a certain pressure and temperature. This gas saturated solution is stirred and further expanded through a nozzle, into a collector were particles were recovered. For this study, carbon dioxide was fed by a liquid pump (Dosapro, Milton Roy MD140F3.2N3507, France) to a 500 cm^3 high-pressure stirred vessel (Parr instruments) containing the system (carriers+active principle) to be studied. The vessel was heated by a thin band heater (Watlow STB3J2J1), and after a determined stirring equilibrium contact time, the mixture was depressurized though a valve (Parker 4M4Z-B2LJ) and a 600μm nozzle (Spraying Systems Co.) to a 10 L collector. A schematic representation of the apparatus is represented in the Figure 1~~Figure 1~~~~Figure 1~~.

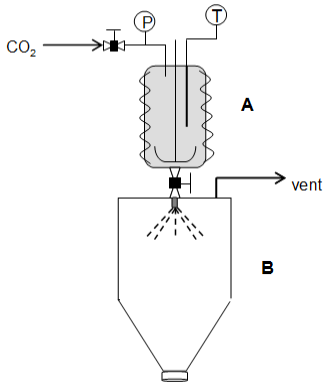

Figure 1 - Apparatus used in PGSS® experiments. A: High-pressure stirred vessel, B: collector with nozzle, TIC: temperature control.

3. Drug Load

Particles (1.3 mg) were dissolved in 3 mL DMSO (Dimethyl sulfoxide), sonicated for 5 min, and filtered with with 0.2 μm (Acrodisc® Syringe Filter, PALL). The quantification of QUER and Toc in the samples was determined by HPLC analysis at 35ºC using a LiChrospher C18 (5μm, 250mm×4mm i.d.) column from Merck with a guard column of the same type. The samples were injected using a 20 μL loop. A Surveyor equipment from Thermo Finnigan with a diode array detector (Thermo Finnigan - Surveyor, San Jose, CA, USA), and an electrochemical detector (Dionex, ED40) with a vitreous carbon electrode were used. The data acquisition systems were the Chromquest version 4.0 (Thermo Finnigan - Surveyor, San Jose, CA, USA) for the diode array detector and the software 4880 (Unicam 4880) for the electrochemical detection.

For QUER quantification the separation was carried out with a flow rate of 700 μL min^{-1} and the mobile phase consisted of a gradient mixture of eluent A (phosphoric acid, *p.a* 0.1%) and eluent B (acetonitrile, HPLC grade). The following gradient of eluents was used: 20 min with 80% eluent A and 20% eluent B; then 1 min with 42.4% eluent A and 57.6% eluent B; then 10 minutes with 80% eluent A and 20% eluent B.

For Toc quantification the separation was carried out with a flow rate of 1000 μL min^{-1} and the mobile phase consisted of a gradient mixture of eluent A (phosphoric acid, *p.a* 0.1%) and eluent B (acetonitrile, HPLC grade). The following gradient of eluents was used: 5 min with 50% eluent A and 50% eluent B; then 21 min with 2% eluent A and 98% eluent B; then 9 minutes with 50% eluent A and 50% eluent B.

4. Scanning electron microscopy (SEM)

Samples were coated with approximately 300 Å of gold in argon atmosphere, and particles morphology was observed by scanning electron microscopy (SEM) (Jeol, JSM-5310 model, Japan) at 20/25kV.

5. Differential Scanning Calorimetry (DSC)

The thermograms of the particulate delivery systems prepared by PGSS® were determined using a differential scanning calorimeter (Setaram, modelo DSC 131). Approximately 13mg of each sample was heated in aluminum pans from 25–300 ◦C and the scanning rate was conducted at 5ºC.min^{-1}under a N_2 gas stream.

6. Cell-based Assays

6.1. Cell Culture

Human colon carcinoma Caco-2 cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), and were routinely grown in RPMI 1640 supplemented with 10% of FBS and 2 mM of glutamine. A stock of the cell line was maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO_2 humidified atmosphere.

6.2. Cellular Uptake Experiments

6.2.1 Quercetin Intracellular Antioxidant Activity

To further understand cellular uptake of quercetin the intracellular antioxidant capacity of neat QUER and loaded solid hybrid particles were determined in Caco-2 cells monolayers. Caco-2 cells were seeded at a density of 2x10^4 cells/well, in 96 well plates and the medium was changed every 48 hours. The experiments were performed after reaching confluence ≈ 72 hours. Intracellular ROS scavenging capacity of QUER particles was evaluated following the formation of radical species after treatment with stress inductor, H_2O_2. The formation of intracellular ROS was monitored using the fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA), as described by Wang et al. [19]. Briefly, confluent Caco-2 cells were co-incubated during 1hour with 10 µM of neat quercetin or quercetin particles, and 25 µM of DCFH-DA. After that, medium was removed and the stress inductor (10 mM H_2O_2) was added to cells. The fluorescence (F) was measured at 0, 1 and 3 hours in a FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA) (λem= 538 nm, λex= 485 nm).The capacity of QUER and loaded solid hybrid particles in ROS inhibition was calculated as ((Ft,stress- F0)-(Ft-F0))/(Ft, stress-F0)×100 for the selected time points, were Ft, stress is the fluorescence at t hours for the control with stress inductor, Ft, is the fluorescence at t hours and F0 is the fluorescence at 0 hours. Results are expressed as % of ROS Inhibition.

6.2.2 Tocopherols Uptake

In order to evaluate cellular uptake of the Toc particle system, Caco-2 cells were seeded, at a density of 3x10^5 cells/well, in 6 well plate and grown in a humidified atmosphere 5% CO_2 at 37ºC and the medium was changed every 48 hours. The experiments were performed after 8 days after seeding. Medium was removed and the wells were rinsed with HBSS (pH=7.4; T= 37ºC). Neat Tocopherols mixture and the loaded hybrid particles (Toc:HP-β-CD:GMS

(1:5:10)) were added to the cells in HBSS and incubated at 37ºC; 5%CO_2 during 1 and 3h. After the incubation time, supernatant were removed and the cells were quickly rinsed twice with cold HBSS and afterwards 1mL of methanol was added to each well. Cells suspensions were removed and centrifuged at 10000g during 10min. The supernatant was collected and immediately frozen until tocopherol quantification. The quantification of Toc was performed by HPLC as described previously. The results are presented in terms of mass of tocopherols uptaked per mass of protein rinsed and presented as ppm of tocopherols per mg of protein (BSA).

6.3 Transepithelial Permeability Experiments

For all transport studies, Caco-2 cells were seeded in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4 mm pore size, Corning Costar Corp.) in 12-well plates at a density of 1.0×10^5 cells/well. The basolateral (serosal) and apical (mucosal) compartments contained 1.5 and 0.5 mL of culture medium, respectively (Figure 2~~Figure 2~~~~Figure 2~~).

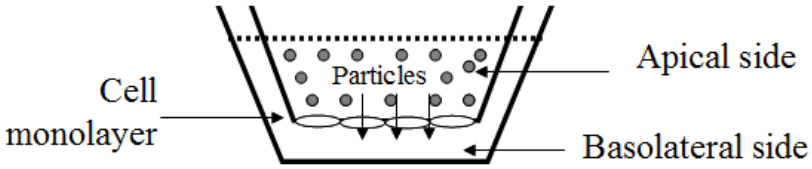

Figure 2 - Schematic representation of the Caco-2 cell monolayer seeded in Transwell® inserts.

Cells were allowed to grow and completely differentiate to confluent monolayers for 20-24 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2 mM of glutamine and 1% of streptomycin+penycilin (Gibco, Invitrogen)) three times a week for 24-28 days. Transepithelial electrical resistance (TEER) of cells grown in Transwell was measured using EVOM^{TM} EVOMX 85792.A0BG (World precision instruments, Florida, USA). Only monolayers with a TEER value higher than 500 Ωcm^2 were used for the permeability experiments.

Medium was removed and the inserts rinsed with Hank's buffered salt solution (HBSS) (pH=7.4; T= 37ºC). Neat Quercetin and tocopherols and quercetin and tocopherols loaded hybrid particles (QUER:HP-β-CD:GMS and Toc:HP-β-CD:GMS) were added in a solid form, to the apical side of ephitelial barrier containing HBSS. For all experiments, the final concentration in quercetin and tocopherols were 100 µM. Transepithelial transport was followed as a function of time. At 1, 2 and 3 h of incubation, samples were collected from the basolateral compartment and immediately frozen until analysis of quercetin and tocopherols by HPLC as described previously.

RESULTS

1. Drug Load

Operating conditions used in the preparation of quercetin particulate systems are summarized in Table 1. The p,T operating conditions were chosen accordingly to the lipid carrier S-L behavior in CO_2 [20]. The tocopherols particulate system prepared was a hybrid formulation (HP-β-CD:GMS) and the operating conditions and mass proportion used were based in the results achieved for quercetin systems.

Table 1 - Operating conditions used to prepare solid hybrid particles loaded with quercetin or tocopherols, HLB (hydrophilic–lipophilic balance) and drug load [(mg of drug /mg of particles) x 100].

Exp	*Systems Prepared*	*Mass proportion*	*Lipid HLB*	*Time (h)*	*T (ºC)*	*P (bar)*	*Drug Load (%)*
A	QUER:GMS	1:10	4	2	70	130	8.38
B	QUER:HP-β-CD	1:5	-				18.18
C	QUER:HP-β-CD:GMS	1: 2.5:10	4				0.85
D		1:5:10					4.98
E	Toc:HP-β-CD:GMS	1:5:10					8.80

From the results attained in quercetin binary systems it was possible to conclude that the drug had more affinity with HP-β-CD as entrapment efficiency was higher than for QUER:GMS system. Furthermore, for the hybrid formulations drug load significantly increases when the mass proportion of HP-β-CD increased suggesting that quercetin preferentially binds to it. This result indicates the existence of a possible interaction between the drug and the HP-β-CD where a possible inclusion complex was formed as previously reported [21]. In addition, in the DSC thermograms (data not shown) the absence of the quercetin and HP-β-CD peaks and the existence of one singular peak tend to confirm the complex formation.

As the main goal of the work presented was to prepare hybrid formulations for cellular uptake and bioavailability enhancement, the more propitious operating conditions attained in quercetin studies were chosen for the preparation of Toc particulate system. In this case, the drug load attained was higher than the expected. This result could be due to high Toc-GMS affinity and the mixture of carriers that still remains in the high-pressure vessel after depressurization is reacher in HP-β-CD.

2. *Scanning electron microscopy (SEM)*

The next figure**Erreur ! Source du renvoi introuvable.** shows the SEM micrographs of the prepared delivery systems.

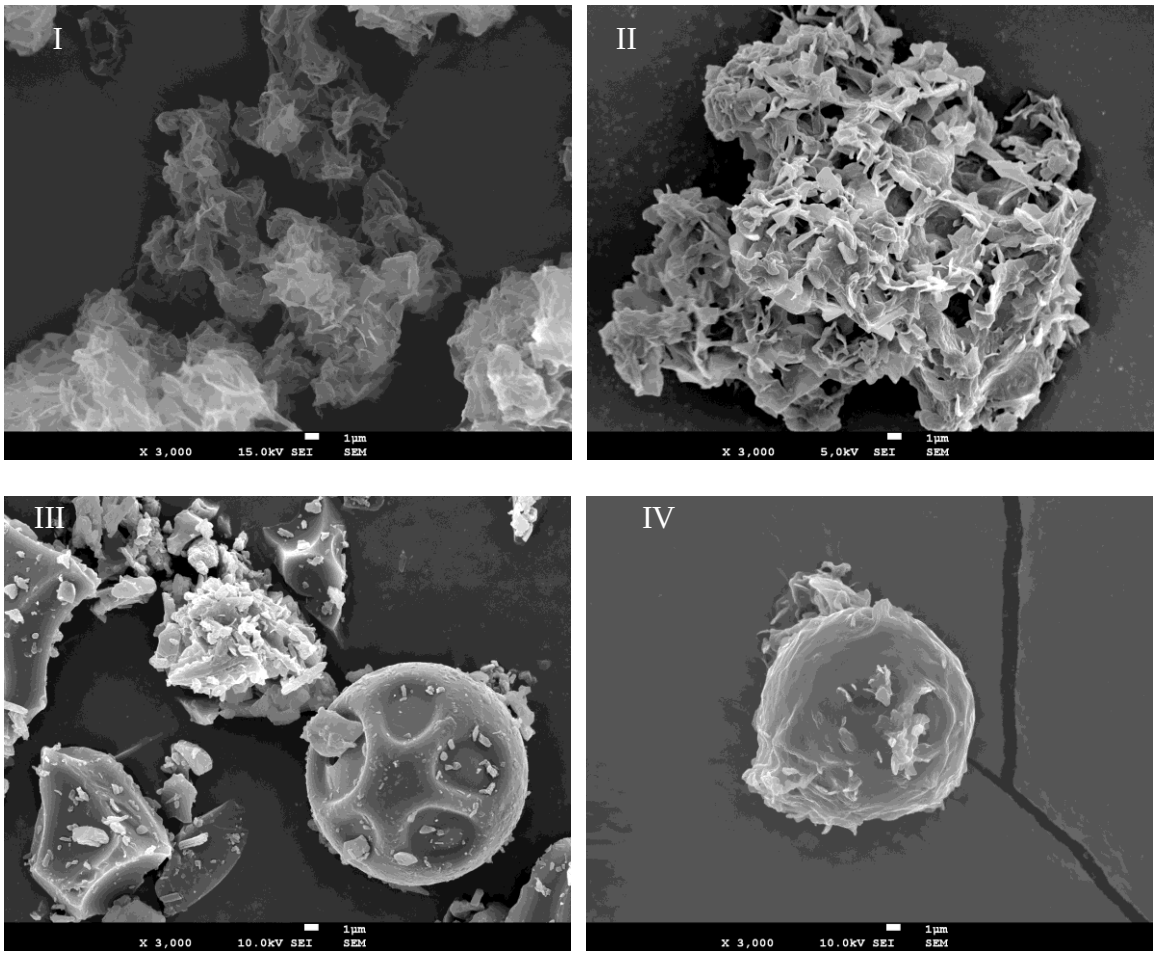

Figure 3 - SEM micrographs of the systems prepared: I) QUER:GMS (1:10)(A), II) QUER:HP-β-CD:GMS (1:5:10) (D), III) QUER:HP-β-CD (1:5) (B) and IV) Toc:HP-β-CD:GMS (1:5:10) (E).

The QUER:GMS (I) and QUER:HP-β-CD:GMS (II) particles have an extremely porous appearance with a surface consistency similar to flakes. On other hand, the QUER: HP-β-CD (III) mixture produced a particulate assorted where one can find a organized material with non porous and homogeneous surface with spherical morphology and another one, with a more porous appearance that can be considered a physical mixture of compounds.
In contrast, the loaded Tocopherols hybrid particles (IV) are lesser porous and present a smooth surface with spherical morphology.

3. Cellular Uptake

3.1 Quercetin Intracellular Antioxidant Activity

The formation of intracellular ROS after inducing oxidative stress with H_2O_2 was monitored using the fluorescent probe, DCFH-DA in the presence of neat QUER and QUER:HP-β-CD:GMS (1:5:10) The results obtained are presented in figure 4.

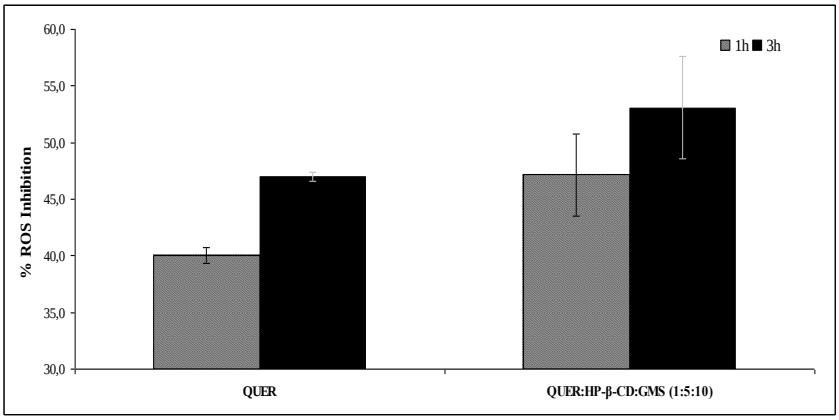

Figure 4 – Percentage of intracellular ROS inhibition when Caco-2 cells are incubated with the particulate system QUER:HP-β-CD:GMS (1:510) and neat quercetin (QUER) using a fixed concentration of 10μM of quercetin. ROS inhibition was calculated as ((Ft,stress- F0)-(Ft-F0))/(Ft, stress-F0)×100 for the selected time points, were Ft, stress is the fluorescence at t hours for the control with stress inductor, Ft, is the fluorescence at t hours and F0 is the fluorescence at 0 hours.

After 1 hour of oxidative stress induction both systems evaluated can inhibit the ROS formation. However for the solid-hybrid particulate systems studied the ROS inhibition was higher than for neat QUER. For the time-point of 3h, this effect continues but the ROS inhibition slightly increases. With this results is possible to conclude that QUER:HP-β-CD:GMS (1:5:10) can be uptake by enterocytes and suggests that a higher amount of quercetin was available. The result attained for neat QUER was expected since this compound was absorbed by passive diffusion. However, normally quercetin cannot reach systemic circulation due to efflux transporters.

3.2 Tocopherol Cellular Uptake Experiments

The tocopherols enterocytes uptake (neat and when loaded in solid-hybrid particles) was determined analyzing cellular methanolic extracts. The results obtained are presented in figure 5.

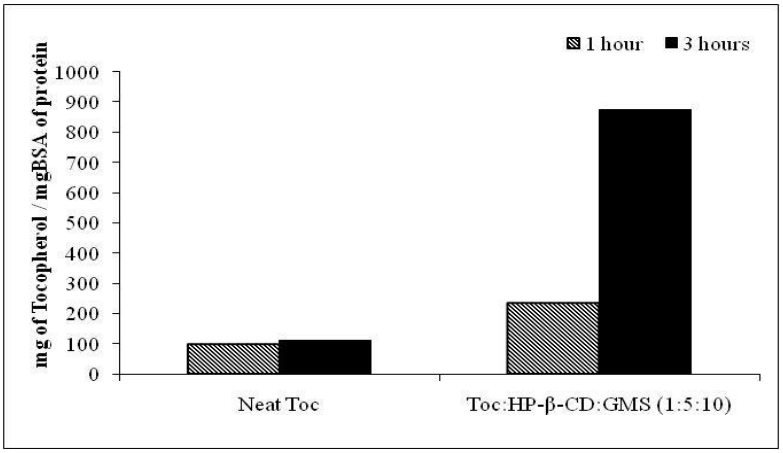

Figure 5 - Cellular uptake of tocopherol (mg) into Caco-2 cells when epithelial cells monolayer were incubated with neat Tocopherols mixture (Toc) and with Toc:HP-β-CD:GMS (1:5:10) solid-hybrid particulate system.

In previous studies the cellular uptake of tocopherol is enhanced by using emulsifiers [15]. These behavior is also observed for the solid-hybrid particulate system Toc:HP-β-CD:GMS (1:5:10). The hybrid system enhances the uptake of tocopherol in Caco-2 cell model presenting a 2 and 8 fold increase after 1 and 3 hours, respectively, compared with neat tocopherol.

4 Transepithelial Permeability Experiments

The permeability of quercetin and tocopherols through intestinal epithelial membrane were studied in order to determine their bioavailability. The results obtained in the transport experiments are presented in the next figure (Figure 6).

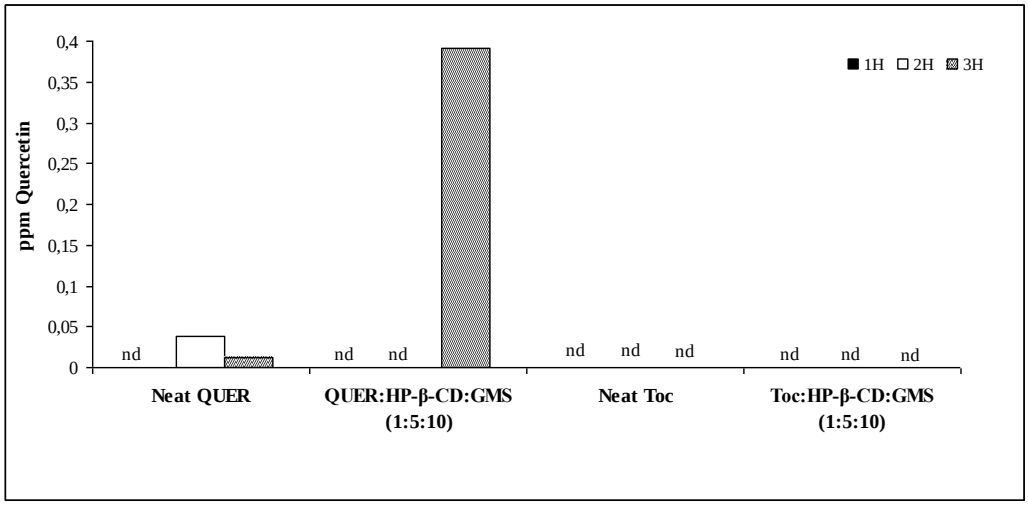

Figure 6 –Quercetin and tocopherols (represented in terms of ppm) present in monolayer basolateral side. (nd – non detected).

After 3 hours of exposure of epithelial intestinal barrier to the quercetin loaded solid-hybrid particles was possible to observe a significantly increase in quercetin present in basolateral side, in comparison with pure form. Furthermore, accordingly with previous work [4], was observed a time-prolonged effect during which the amount of quercetin in basolateral side (in case of neat quercetin tested) decreased. This fact was not observed for the loaded hybrid particles suggesting that the basolateral to apical efflux transport was avoided.On the contrary, for the tocopherols experiments (for the both systems) none transport across the intestinal epithelial barrier was observed at the indicated time-points. This result is not surprisingly since was previously described [22] that after overcome the enterocytes uptake, tocopherols where stored in the microsomal membranes, and release from these membranes are a complex mechanism dependent of intracellular apoB-containing lipoproteins or exogenous HDL that was not present in the serosal side of this experience. However, as the delivery of tocopherols to cells is the major problem for their secretion the increase of cellular uptake verified for the particulate system was a major achievement. Additionally, the incubation of Caco-2 cells with the systems studied did not affect TEER values, indicating that they did not compromise the barrier integrity.

CONCLUSION

This work demonstrated the viability of using SCF technology, in particular PGSS technique, to produce hybrid (HP-β-CD : GMS) delivery systems with promising application on bioavailability and celuular uptake enhancement of sensitive bioactive compounds, such as Quercetin and tocopherol.

The quantity of quercetin present in the several formulations depends on the quantity of HP-β-CD. These results combined with DSC thermograms reveal an existence of an interaction between the former active principle and the cyclodextrin.

A higher antioxidant intracellular response observed for system QUER:HP-β-CD:GMS (1:5:10), suggest an increase in the cellular uptake of quercetin, when compared to neat form. In addition the solid-hydrid system QUER:HP-β-CD:GMS(1:5:10) allows the enhancement of quercetin intestinal transport to basolateral side and the augment of QUER bioavailability, in comparison with neat form.

Additionally, the hybrid system Toc:HP-β-CD:GMS (1:5:10) reveals to be promising for intestinal cellular uptake of tocopherols. The results obtained for the cellular uptake and the transepithelial permeability experiments lead us to suggest that the tocopherols and loaded particles enter the cells where they remain stranded.

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