AAPS PharmSciTech, Vol. 14, No. 2, June 2013 (© 2013)
DOI: 10.1208/s12249-013-9926-4

Research Article

Improving Oral Bioavailability and Pharmacokinetics of Liposomal Metformin by Glycerolphosphate–Chitosan Microcomplexation

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Received 12 September 2012; accepted 12 January 2013; published online 8 March 2013

Abstract. The purpose of this study was to develop a new delivery system capable of improving bioavailability and controlling release of hydrophilic drugs. Metformin-loaded liposomes were prepared and to improve their stability surface was coated with chitosan cross-linked with the biocompatible β-glycerolphosphate. X-ray diffraction, differential scanning calorimetry, as well as rheological analysis were performed to investigate interactions between chitosan and β-glycerolphosphate molecules. The entrapment of liposomes into the chitosan-β-glycerolphosphate network was assessed by scanning electron microscopy and transmission electron microscopy. Swelling and mucoadhesive properties as well as drug release were evaluated *in vitro* while the drug oral bioavailability was evaluated *in vivo* on Wistar rats. Results clearly showed that, compared to control, the proposed microcomplexes led to a 2.5-fold increase of metformin T_{max} with a 40% augmentation of the AUC/*D* value.

KEY WORDS: chitosan; controlled release; *in vivo* bioavailability; liposomes; oral delivery.

INTRODUCTION

Oral administration of drugs is central in the development of pharmaceutical research due to its extensive applications to most patients. With the aim to improve the oral bioavailability, several strategies have been proposed to reduce dosing frequency and/or gastrointestinal side-effects of many drugs. A possible approach to achieve sufficient uptake and transport of orally administered drugs consists in the use of particulate carriers made from mucoadhesive polymers (1–3). Mucoadhesive nano- and micro-particulate dosage forms can increase drug bioavailability thanks to their ability to interact with the mucus layer that covers the surface of epithelial cells in the gastrointestinal tract, thus, increasing the residence time of the drug at the absorption site and enhancing its absorption and bioavailability (3–6). Moreover, after oral administration, nano- and micro-sized particles, thanks to their reduced size, can spread out throughout the length of the small intestine where they allow the drug to achieve a controlled and reproducible release with improved absorption and reduction of potential side effects. In biomedical field, these particles are promising systems for drug delivery provided they are nontoxic and biocompatible. Indeed, polymer encapsulation strategy offers a high stability of systems in contact with biological fluids, protects the drug from adverse external conditions and controls its release in the upper intestine (3,6–8).

Mucoadhesive polymers have also been used to protect and stabilize liposomes by coating (8–11). Among the various employed polymers, chitosan (CH) has been shown to possess good mucoadhesive properties in the hydrated form. Moreover, it has already successfully used to coat liposome formulations (10–14). Surface modification of liposomes with CH has been shown to prolong their residence time in the gastrointestinal tract of rats in comparison with uncoated ones, thanks to ionic interactions between positive amino groups of the polymer and the negative mucus gel layer (11,15,16).

In the present study, mucoadhesive CH-β-glycerolphosphate microcomplexes (GP/CH microcomplexes) containing metformin-loaded liposomes were prepared and characterized, aiming to control oral metformin (Met) release and improve its bioavailability and intestinal absorption. Met, an anti-hyperglycaemic agent orally used in the treatment of non-insulin-dependent diabetes mellitus, was chosen as a hydrophilic model drug characterized by low oral absorption and bioavailability. Liposomes were made of phosphatidylcholine, which would facilitate the passage of Met through biological membranes while CH was used to protect the phospholipid vesicles and retain the microcomplexes on the intestine membrane (12,17,18). Liposomes need to be protected from the harsh environment in the stomach because they are destabilized by

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low pH, lipases and bile salts. In fact, phosphatidylcholine liposomes could withstand at low pH values but they are destabilized in these conditions and drug release increases as the pH decreases (19). Whereas, the exposure to bile salts or pancreatic lipase induces a disruption of the liposomes and a consequent significant leakage of entrapped drug. Bile salts intercalate into the bilayer structure causing a destabilization of vesicles which results in the formation of new lipidic structures involving bile salts. Moreover, various lipases are able to degrade the lipids allowing a consequent disintegration of the softer bilayers (20–22). In particular, Whitmore and Wheeler reported a complete loss of entrapped marker when the liposomes were incubated with 1% bile acids solution (23), while Chiang and Weiner observed a total loss of entrapped carboxyfluorescein after 1 h in the presence of a mixture of bile salts and lipase at pH 2 (24).

Effective oral drug delivery with stable liposomes can be achieved using surface modified vesicles. However, CH alone is unable to protect drug-loaded liposomes in the gastric environment because of its high solubility at acid pH. Crosslinkage is a common method used to increase biomaterial stability in the gastrointestinal tract (25,26). Therefore, in the present study, β-glycerolphosphate (GP), a non-toxic and biocompatible molecule, was used to crosslink the CH by ionotropic gelation. The crosslinkage of CH-coated liposomes leads to formation of GP/CH microcomplexes: single liposomes or vesicle clusters are enveloped by the GP/CH coating (27–29). Noteworthy, the experimental setting did not require the use of organic solvents, thus, reducing the risk of toxicity due to residual solvents in the final formulation. Non-crosslinked CH-coated liposomes were used as control.

The first objective of the present work was to prepare and characterize GP/CH microcomplexes and, secondly to evaluate the effect of microcomplexation on the *in vitro* and *in vivo* Met-controlled release. More specifically, the mmicroscopic interactions of the complex components were evaluated by X-ray diffractography (XRD) and differential scanning calorimetry (DSC). Complex morphology and structure was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The macroscopic properties of the resulting microcomplexes were evaluated: size distribution, zeta potential, encapsulation efficiency and rheological behaviour. Moreover, swelling and mucoadhesive properties as well as the drug release were studied *in vitro*, while the oral bioavailability and intestinal absorption of Met were evaluated *in vivo*.

MATERIALS AND METHODS

Materials

Metformin hydrochloride was purchased from Galeno (Potenza, Italy). A commercial mixture of phospholipids (Phospholipon® 50, P50, with 45% phosphatidylcholine and 10–18% phosphatidylethanolamine, maximum 3% triglycerides, maximum 0.25% D,L-a-tocopherol and the remaining part (37%) lipids: fatty acids, glycolipids, phosphatidylinositol etc) and hydrogenated soy phosphatidylcholine (Phospholipon® P90H) were kindly supplied by AVG (Milan, Italy) and Lipoid GmbH (Ludwigshafen, Germany). CH (CH) with an average molecular weight of 7.5×10^5 g/mol (75–85% deacetylated), β-glycerophosphate, dextrose, cholesterol and all other products were of analytical grade and were purchased from Sigma Aldrich (Milan, Italy). Phosphate buffer solution (PBS) pH 7 was obtained from Carlo Erba Reagents (Milan, Italy).

Preparation of Microcomplexes

Liposomes were prepared by mixing 2 g of P90H, 0.4 g of P50 and 0.1 g of cholesterol. The phospholipid mixture was swollen with 10 mL of metformin (Met, 100 mg/mL) and dextrose (50 mg/mL) PBS solution, at 51°C for 12 h (30,31). Dispersion was sonicated in a Soniprep 150 apparatus (MSE Crowley, London, United Kingdom) at 51°C for 2 min. CH (10 mg/mL) was dissolved in aqueous acetic acid (5 mg/mL) solution. In order to prepare CH-liposome microcomplexes, Met-loaded liposomes (25 mL) were added dropwise to CH (75 mL) solution under continuous stirring and then, a GP aqueous solution was added to CH-liposome dispersion (GP/CH 8/1 W/W). Samples were dispersed using an Ultra-Turrax mixer, and then sonicated at 51°C for 2 min (32). CH-coated liposomes in the form of dried powder were obtained by spray-drying, using a Mini Spray Dryer Büchi 190 (Büchi, Flawil, Switzerland) with a standard 0.7 mm nozzle. Inlet temperature, spray flow and compressed spray air flow (represented as the volume of the air input) were set at 140°C, 6 and 10 mg/mL, respectively.

Characterization of Microcomplexes

Morphology of dried GP/CH-liposome microcomplexes was evaluated using a Hitachi S-4800 (Monocomp, Madrid, Spain) scanning electron microscope (SEM) at 2 kV. Liposome and microcomplex dispersions were observed by a Jem1010 (Jeol Europe, Paris, France) transmission electron microscope at 80 kV, as previously reported (33).

The average size and polydispersity index (PI) of liposomes were measured using a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, United Kingdom). Sample size and PI were detected by means of dynamic light backscattering using a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25°C. Zeta potential was calculated using M3PALS, a second generation PALS (Phase Analysis Light Scattering) system, which measures the particle electrophoretic mobility in a thermostated cell. The microcomplex size was measured using a Malvern Mastersizer 2000 version 5.1 (Malvern Instrument, Worcestershire, United Kingdom). Dried samples were dispersed in an appropriate amount of Millipore water and measurements were carried out immediately and 24 h after rehydration. Size and size distribution were constant during this time. The size distribution of each sample was measured at least three times. Average particle size was expressed as volume mean diameter ($d_{4,3}$) in micrometers. The span was also calculated. It is defined as $(d_{(v,90)}-d_{(v,10)})/d_{(v,50)}$, where $d_{(v,90)}$, $d_{(v,10)}$ and $d_{(v,50)}$ are the diameters at 90%, 10% and 50% cumulative volume, respectively. Thus, the span gives a measure of the range of the volume distribution relative to the median diameter.

The drug encapsulation efficiency (E%) of the formulations after purification was expressed as the percentage of the

ratio of actual and theoretical amounts of Met loaded in the carriers. Each dispersion (1 ml) was purified from non-encapsulated drug by dialysis against dextrose (10 mg/ml) in PBS solution (1 L) at 5°C for 2 h using Spectra-Por® membranes (12–14,000 MW cut-off, 3 nm pore size, Spectrum Laboratories, Inc., Rancho Dominguez, CA, United States), which were appropriate to allow dissolution and consequent removal of the non-entrapped, Met (pH 7, solubility 0.9 mg/ml) (34). The total amount of encapsulated Met was determined by HPLC after disruption of vesicles with Triton X-100. Drug content was analyzed at 235 nm using a liquid chromatograph Alliance 2690 (Waters). The column was a C18 (60 Å, 4 μm, Waters) and the mobile phase was PBS at pH 6, delivered at a flow rate of 0.8 mg/mL.

Evaluation of Drug-Microcomplex Interactions

X-ray diffractograms were recorded with Bragg–Brentano geometry on a Bruker AXS D5005 (Bruker AXS GmbH, Karlsruhe, Germany) in the 2θ range from 5° to 80°, in steps of 0.02° at 6 s per step.

Differential scanning calorimetry studies were performed using a DSC model 821e (Mettler Toledo International Inc., Barcelona, Spain). The samples (2–5 mg) were scanned in sealed aluminium pans under nitrogen atmosphere. DSC thermograms were scanned in the first heating run at a constant rate of 10°C/min and a temperature range of 0–500°C. DSC thermograms of liposomes, polymers and complexes were recorded.

Rheological Analysis

A Thermo Haake Rheostress 1 rheometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) with data acquisition software (RheoWin 2.94) and a circulator for sample temperature control was used for rheological analysis. Dried powders were dispersed in water (60 mg/ml) and loaded on the rheometer plate. Samples were allowed to rest for at least 300 s prior the analysis. The exposed edges of the sample were covered with silicone oil to prevent evaporation of water during measurement. All measurements were performed in triplicate at 37°C. In all cases, the oscillatory test was carried out using a cone-plate geometry (2°, 35 mm diameter). In order to determine the linear viscoelastic range, stress sweeps at a frequency of 1 Hz were performed for all the studied systems. Frequency sweep tests were carried out from 0.01 to 10 Hz, at 1 Pa, for both systems. The oscillatory parameters used to compare the viscoelastic properties of all the systems were the storage modulus (G'), the loss modulus (G'') and loss tangent ($tan\ \delta = G''/G'$).

Swelling Studies

In order to assess the swelling degree of CH-coated liposomes and GP/CH microcomplexes, dried powders were placed in a tube with 10 mL of buffered solution, first at pH 1.2 (45 min) and then at pH 7.4 (up to 24 h). During the experiments, samples were magnetically stirred and thermostated at 37.0±0.5°C. At specific time intervals, samples were centrifuged at 1,000 rpm for 5 min. The swelling medium was withdrawn, and the tubes were weighed. The swelling ratio (S_w) of the test samples was calculated according to the following equation:

$$\left[S_w = \frac{W_t - W_{t0}}{W_{t0}} \times 100\right] \quad (1)$$

where W_t is the weight of the swollen test sample and W_{t0} is the weight of the dried test sample.

In Vitro Release Study

The *in vitro* release profile of Met from dried GP/CH microcomplexes was assessed in buffered solution at pH 1.2 and in buffered solution at pH 7.4 using a dissolution rotating basket apparatus (US Pharmacopeia), which consists of a cylindrical basket held by a motor shaft. The basket holds the sample and rotates in a round flask containing the dissolution medium (35). Samples (150 mg) were dispersed in 2.5 mL of distilled water and placed in a dialysis tube. The tube was transferred in the basket and immersed in the dissolution medium, thermostated at 37.0±0.5°C. Drug release was assessed for 24 h (45 min at pH 1.2 at 200 rpm, and afterwards at pH 7.4 at 100 rpm). At scheduled time intervals, 1 mL of the medium was withdrawn and replaced with fresh medium to ensure sink conditions. The drug content in the samples was determined by HPLC (see "Characterization of Microcomplexes". All experiments were performed in triplicate

Preparation of Gastrointestinal Tissues and Mucoadhesive Tests

Wistar rats (13 weeks old) were fasted for 24 h in order to minimize the content of their gastrointestinal tract, and thus facilitate the washing step. Rats were sacrificed by intraperitoneal administration of 60 mg/kg of Dolethal® and their gastrointestinal tissues (*i.e.* stomach, duodenum, jejunum, ileum and colon) were excised according to the Principles of Laboratory Animal Care approved by the Ethics Committee of the University of Valencia. Each tissue was slowly washed with a large amount of 0.9% NaCl saline solution and then immediately used. The mucoadhesion study was carried out using a universal tensile tester LR 50K (Lloyd Instruments, Bognor Regis, United Kingdom). The stainless steel plate (L-shape) was inserted into the upper and lower jaws of the instrument, so that surface plates were facing each other. Rat gastrointestinal tissue was stuck to the upper surface of the plate with glue, while swelled sample was placed to the lower plate with buffer solution and thermostated at 37°C. Twenty microliters of PBS (pH 7.4) were spread on the contact surface between sample and tissue. The upper jaw with tissue stuck on the plate was then slowly lowered up to touch the sample surface. No external force was applied. The sample was kept in contact with the tissue for 10 min, after which the upper jaw was slowly raised at a rate of 10 mm/min. Mucoadhesion was assessed using different swelled samples (as a function of time) on specific parts of the gastrointestinal tract: after 45 min on the stomach, after 60 min on the duodenum, after 90 min on the jejunum, after 120 min on the ileum and after 150 min on the colon. All experiments were performed in triplicate. The total amount of force involved in the withdrawal of

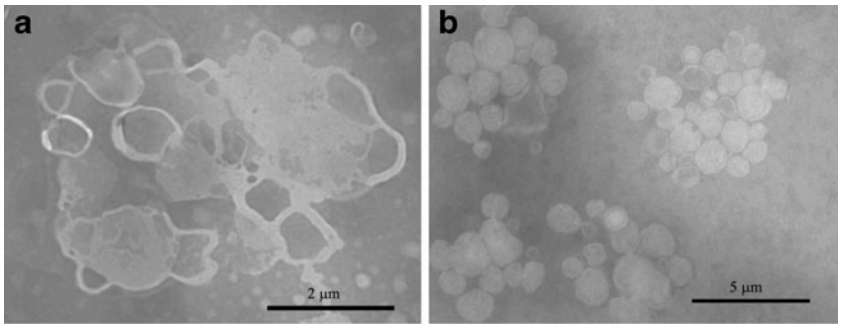

Fig. 1. TEM images of **a** CH-coated liposomes and **b** GP/CH microcomplexes

the tissue from the sample (work of adhesion, W_{ad}) was calculated from the area under the force *versus* distance curve.

Oral Bioavailability Study

All the pharmacokinetic studies reported here adhered to the Principles of Laboratory Animal Care and were approved by the Ethics Committee of the University of Valencia (Spain) according to RD 1201/2005. Male Wistar rats weighing 250–300 g were used in all the experiments. Twenty-four hours before drug administration, animals were subjected to jugular vein cannulation with medical-grade silicon tubing. Prior to surgical intervention, a dose of 30 mg/kg of Dolethal® (sodium pentobarbital) was administered by intraperitoneal injection. Following surgery, animals were fasted overnight with water freely available, until drug administration the following day. Rats (n=20) were randomly allocated into four groups: two groups were used as a reference, they received a Met solution (200 mg/kg) administered either intravenously (iv) or orally, while the other two groups received an oral dose of the prepared formulations. The tested formulations were administered in a range of dosage between 200 and 300 mg/kg. After administration, blood samples (0.2 mL) from the jugular vein cannula were withdrawn into heparinized syringes at scheduled time points. In all cases, the total blood extracted did not exceed 1 mL/day. The volume of removed blood was replaced with the same volume of saline solution. After collection, each blood sample was centrifuged at 3,000 rpm for 5 min, and the plasma transferred to new polypropylene tubes and stored at −20°C until assay for drug content. The method proposed by Porta *et al.* (36) was used to determine concentration of Met in plasma samples.

The area under the curve (AUC) was calculated by the trapezoidal rule extrapolation method; the AUC for the data points portion was calculated by adding up the trapezoids, the area from the last datum point to time infinity was estimated by dividing the last measured plasma concentration by the terminal phase rate constant.

The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were read directly from the experimental data. AUC and C_{max} were divided by the dose administered to normalize data and compare among the different formulations. Finally, absolute bioavailability, F, was calculated as the ratio of AUC/D of the tested formulation and AUC/D of the iv administration

Statistical Analysis of Data

Results are expressed as the mean ± standard deviation. Analysis of variance and Bartlett's test for homogeneity of variance were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, United States). Post hoc testing (P<0.05) of multiple comparisons was performed using the Scheffe test. Differences were considered significant at the 0.05 level of probability (p).

Pharmacokinetic parameters were compared by means of the non-parametrical test Kruskal–Wallis (p<0.05 was considered statistically different).

RESULTS AND DISCUSSION

Preparation and Characterization

In the present work, liposomes were prepared by using a combination of two different phospholipid commercial mixtures: hydrogenated phosphatidylcholine (P90H) and soy phosphatidylcholine (P50). The use of hydrogenated phospholipids

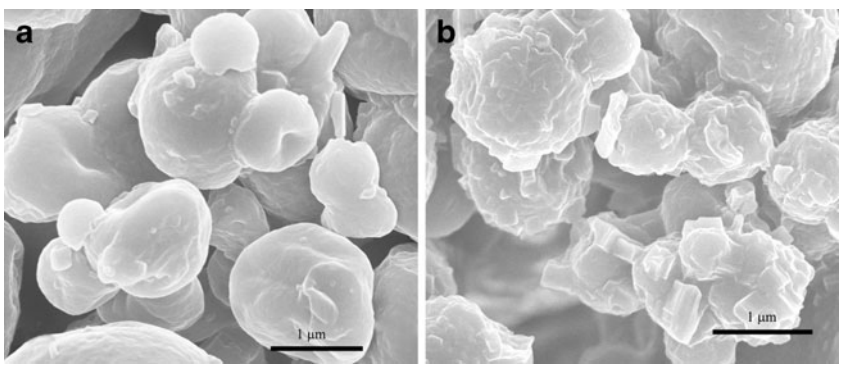

Fig. 2. SEM images of **a** CH-coated liposomes and **b** GP/CH microcomplexes

increases bilayer transition temperature, thus, allowing vesicles to overcome stability problems (37,38). The P90H/P50/cholesterol ratios were optimized to obtain small liposome size and high vesicle stability. Liposomes were prepared by swelling the lipid mixture in PBS for 12 h and then sonicating the dispersion (39). To further improve stability as well as to achieve a mucoadhesive formulation, the surface of P90H/P50 liposomes was coated with CH. Successively, the polymer was crosslinked with GP to obtain the GP/CH microcomplexes, by the ionic interactions between positively charged amino groups of chitosan and negatively charged GP molecules (29,40). The crosslinkage treatment of chitosan coating may improve carrier stability and ability to control drug delivery. Moreover, one of the main advantages of this crosslinkage procedure is that it is performed without using any organic solvent or toxic reagent. Vesicle dispersions were spray-dried to obtain microparticles.

Chitosan-coated liposomes (so-called chitosomes) were previously prepared by several authors and their effective formation was confirmed using several techniques such as SEM and TEM (11,14,41–43).

During this study, formation of vesicles was evaluated by TEM analysis. Photomicrographs of liposomes showed the presence of oligolamellar vesicles with spherical shape and homogeneous size distribution (data not shown). TEM images of rehydrated CH-coated liposomes and GP/CH microcomplexes confirmed the effective presence of bilayered vesicles in the samples although the bilayer structure was surrounded by the polymer chains. The presence of polymer layers surrounding the liposomes allows the formation of less homogeneous systems probably containing either single vesicles or vesicle clusters (Fig. 1a, b).

SEM images support TEM results: CH-coated liposome samples seem to be formed by single or associated coated vesicles. The shape is almost spherical with smooth surface. On the contrary, GP/CH microcomplexes showed a more irregular shape and with a rough surface (Fig. 2a, b).

The influence of the polymer on the physico-chemical properties of liposomes is reported in Table I. Liposomes (166±7 nm) were small in size and homogeneously dispersed. Mean size increased greatly when liposome surface was coated with CH (4,061 nm) or GP/CH (4,730 nm) and the coating process led to less homogeneous systems (span>1). These results are probably the consequence of an aggregation process that involved vesicles or vesicle clusters only partially coated by the polymers (14,44,45). As shown in Table I and Fig. 2, the poorest homogeneous system was the one prepared with GP/CH that, owing its less amount of the amine groups, led to particles with the lowest positive zeta potential value (+11 mV) and, therefore, to an aggregation process during the spray drying procedure. Indeed, as written above, TEM and SEM pictures support this suggestion since particles are irregularly shaped with clear evident aggregation phenomena.

Zeta potential of liposomes was negative (−22 mV) due to their composition. In fact, the primary components of P50 and P90H are phosphatidylcholine and hydrogenated phosphatidylcholine, respectively. These compounds are zwitterionic molecules that contain phosphate and choline functional groups (isoeletric point is ~pH 4) (46 and related references). When they aggregate to form bilayered vesicles, electric charge distribution at the membrane interface is a function of the organization of negative and positive residues of the phospholipids and the consequent binding and orientation of counterions. Depending on pH, protons or hydroxide ions neutralize phosphate or choline groups changing the vesicle surface charge. At pH near neutrality (~7) the zeta potential of phosphatidylcholine vesicles is clearly negative (~ −22) (41,47). Moreover, P50 a commercial mixture also containing fatty acids, could contribute to the negative value of zeta potential (48).

Negative charges of liposomes allowed electrostatic interactions to occur with the positively charged polymeric CH molecules. The interaction between liposome surface and CH led to a change of the zeta potential toward positive values, which further confirmed the presence of the hydrophilic polymer on the liposome surface. CH was highly protonated and CH-coated liposomes showed a strong positive charge (+56 mV). On the other hand, as written above, the use of GP/CH led to a zeta potential value of only +11 mV, as a consequence of the crosslinking process that had reduced the free amino groups capable of contributing to the surface charge of these microcomplexes.

As expected, liposomal Met *E*% values were low (10± 3%, Table I) due to the high hydrophilicity of the drug. However, the polymer layer on the surface of liposomes was associated with an increased Met *E*%, which reached 25% in the case of GP/CH microcomplexes, while it was only 15% for CH-coated liposomes (Table I).

Differential scanning calorimetry and X-ray diffraction analyses were carried out to assess the potential interactions among drug, phosphatidylcholine, CH and GP.

Thermal curves of each component and the prepared formulations are reported in Fig. 3. Met thermogram showed a sharp endothermic peak at the temperature corresponding to its melting point (T=220°C) and a degradation peak at 330°C. GP and Met-loaded liposomes showed a dehydration peak around 100°C due to water loss. In the case of CH, an endothermic peak around 100°C, corresponding to water evaporation, and an exothermic peak at 315°C, referring to polymer degradation, were observed (49).

In thermograms of CH-coated liposomes and GP/CH microcomplexes, the exothermic peak corresponding to polymer degradation was shifted and much smaller than that of the pure chitosan. However, thermograms of CH-coated liposomes showed new peaks at around 200–270°C probably due to the electrostatic interactions between the CH and the phosphatidylcholine moieties. In GP/CH microcomplex thermogram, an abrupt change in the thermal behaviour of the CH was evident in the range 340–490°C, which may indicate the interaction between CH and GP molecules.

Table I. Uncoated Liposomes, CH-Coated Liposomes and GP/CH Microcomplexes: Mean Diameter, Polydispersity Index (PI) or Span, Zeta Potential (ZP) and Encapsulation Efficiency (*E*%) Values (n=6)

	Size (nm)	PI	Span	ZP (mV)	*E* (%)
Liposomes	166±7	0.12		−22±1	10±3
CH-coated liposomes	4,061±1,500		2.31	+56±1	15±5
GP/CH microcomplexes	4,730±1,600		1.77	+11±1	25±4

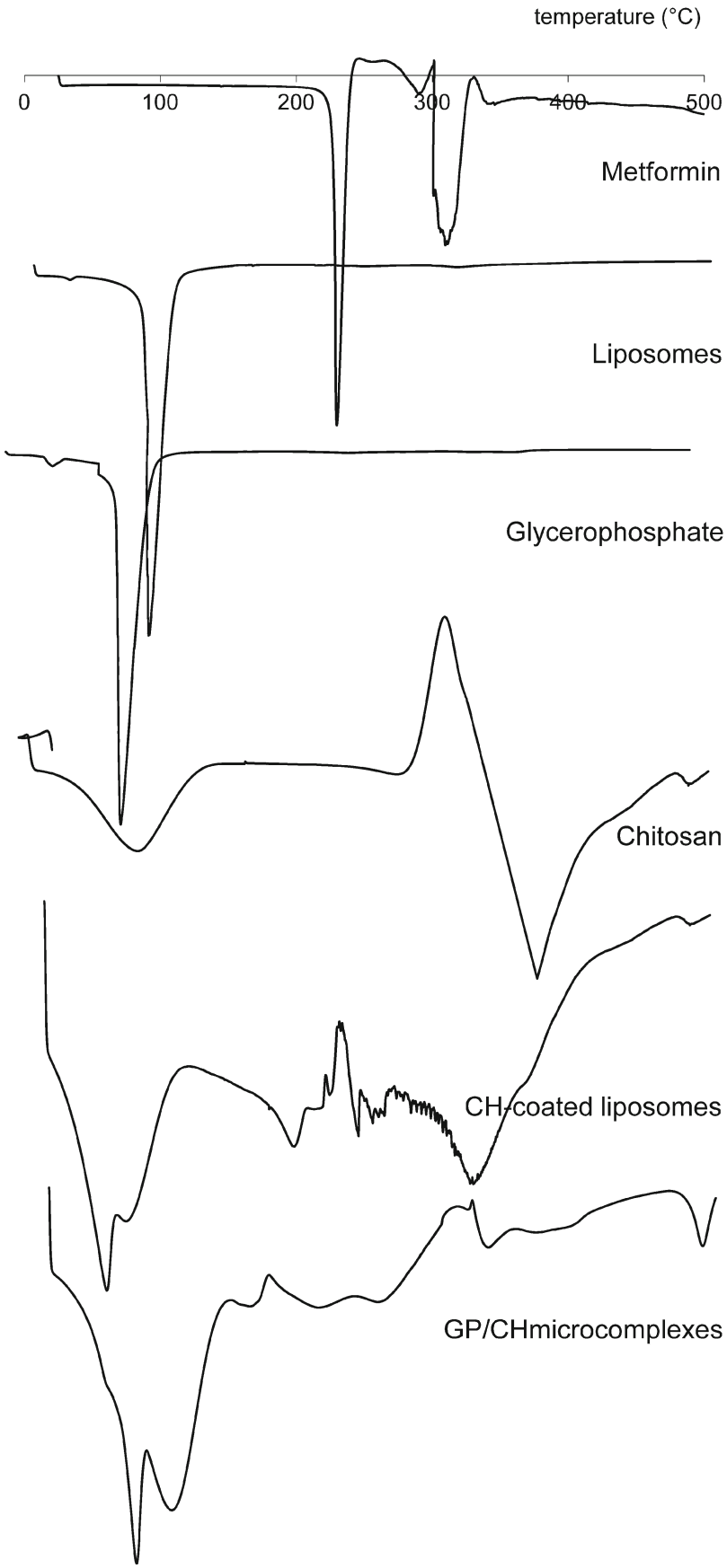

Fig. 3. Differential scanning calorimetry of Met, Met-loaded liposomes, GP, CH, CH-coated liposomes and GP/CH microcomplexes

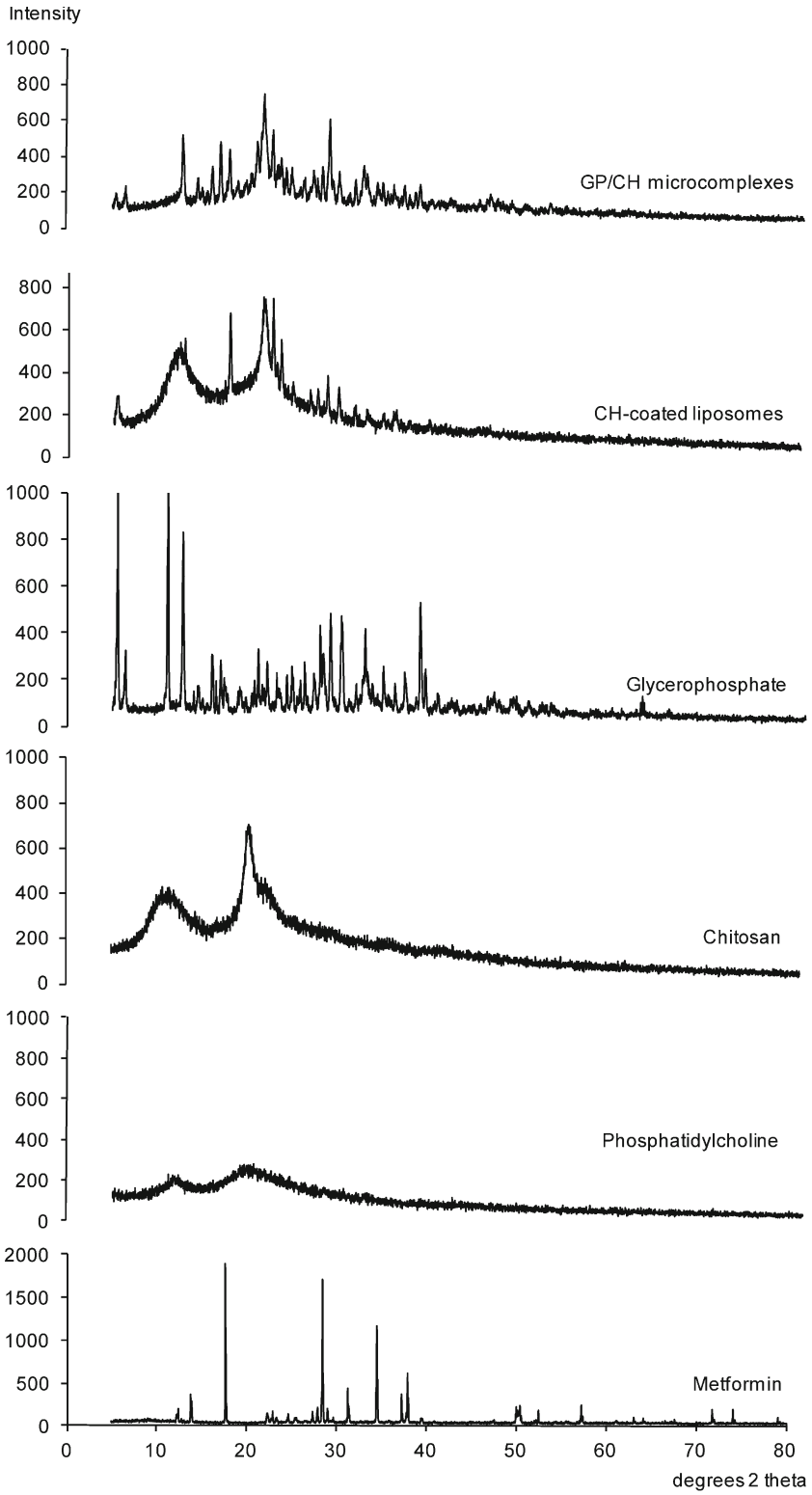

Fig. 4. X-ray powder diffraction patterns of Met, CH, phosphatidylcholine, CH-coated liposomes and GP/CH microcomplexes

The X-ray powder diffraction patterns of the single components and microcomplexes are reported in Fig. 4. Met revealed three distinct important peaks at 18, 28 and 34° 2θ; characteristic of its crystallinity. CH and phosphatidylcholine showed two large diffraction peaks in the range 8°–25° 2θ. When Met was encapsulated in the CH-coated liposomes, the diffraction patterns were similar to those of the drug and CH alone, confirming their unmodified presence in the microcomplex. Detection of any change in CH or phosphatidylcholine spectra was not possible due to overlapping of their peaks.

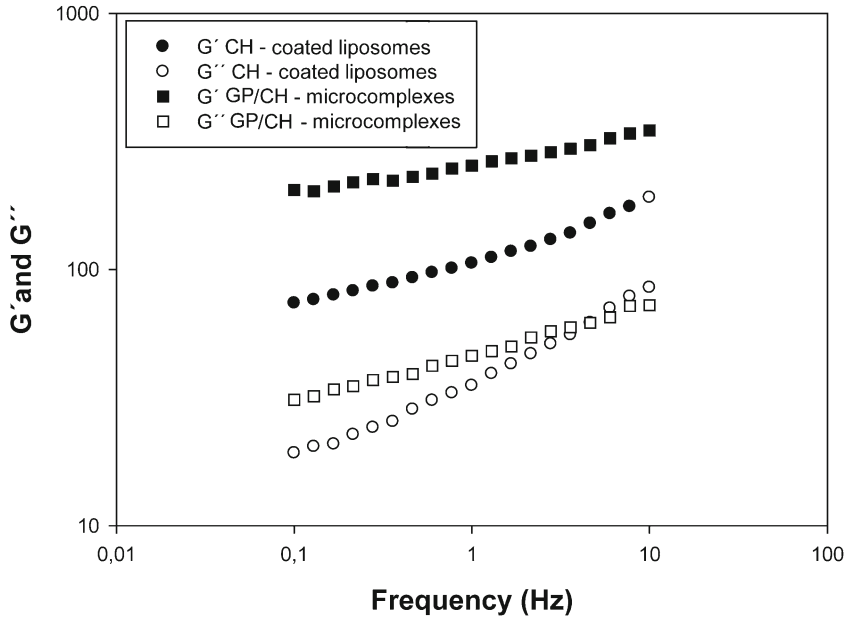

Fig. 5. Rheological analysis. Magnitude of the storage modulus (G') and loss modulus (G'') of CH-coated liposomes and GP/CH microcomplexes as a function of the frequency (Hz). G' (*filled symbols*) and G'' (*open symbols*)

When the drug was encapsulated in the GP/CH microcomplexes, the CH peak at 12° 2θ disappeared while the one at 21.5° 2θ was less intense whereas the Met peaks were visible. This behaviour suggests the presence of chemical interactions between CH and GP, thus, confirming polymer crosslinkage.

Rheological Analysis

In the attempt to evaluate the complexation of CH in the formulations, their rheological properties were investigated. The experimental values of mechanical spectra for CH-coated liposomes and GP/CH microcomplexes, obtained in the region of linear behaviour, are shown in Fig. 5. Both formulations showed a predominance of elastic over viscous behaviour (G' was greater than G'') and the loss tangent value was always lower than 1. This behaviour is typical of three-dimensional networks and indicates the formation of a new microstructured organization like a true gel. This was especially evident in GP/CH microcomplexes that showed loss and, especially storage modulus higher than those of CH-coated liposomes, due to the crosslinkage of CH amino groups with the phosphate groups of GP. In the CH-coated liposomes there was a predominance of elastic behaviour due to CH/phosphatidylcholine electrostatic interactions forming a microstructured system. On the contrary, a plain CH gel (without Met liposomes) showed a typical behaviour of non-structured system, in which the absence of complexation caused the predominance of viscous over elastic properties (data not shown).

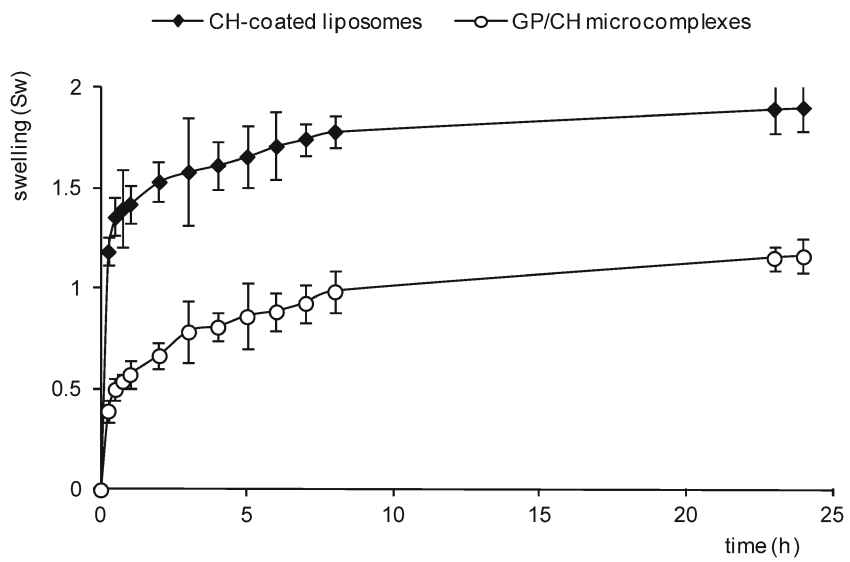

Fig. 6. Swelling ratio (S_w) of CH-coated liposomes and GP/CH microcomplexes in buffer solution at pH 1.2 for 45 min and then at pH 7.4 up to 24 h

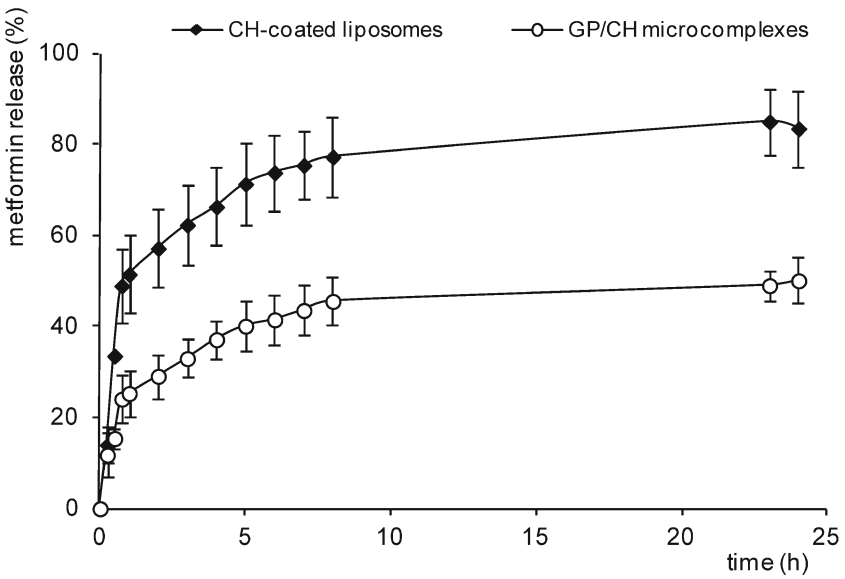

Fig. 7. *In vitro* Met release from of CH- and GP/CH microcomplexes at pH 1.2 for 45 min and then pH 7.4 up to 24 h (n=3)

Swelling Studies

As expected, bearing in mind the chemical properties of CH, CH-coated liposomes showed a considerable water uptake and relate swelling ratio. When immersed in a buffer solution at pH 1.2, in 15 min samples were able to uptake an amount of water corresponding 4-fold their original weight while at pH 7.4 the swelling ratio and rate were lower: in 1 h, the water uptake was approximately 35% of their original weight (Fig. 6).

At pH 1.2, the swelling ratio of GP/CH microcomplexes was 50% lower than that of CH-coated liposomes and, after 15 min, they absorbed an amount of water equivalent to their original weight as a consequence of the reduced ability of the crosslinked CH to interact with the aqueous medium in acidic environment, thus, reducing water penetration rate into the system in comparison with CH-coated liposomes. At pH 7.4, the swelling ratio of GP/CH microcomplexes increased slightly with a swelling rate such as that of CH-coated liposomes. However, it did not allow a high water uptake capable of generating over-hydrated structures that could cause an immediate drug release and loss of the polymer mucoadhesive properties. Indeed, at the end of the experiments GP/CH microcomplexes' S_w was almost a half than that of CH-coated liposomes.

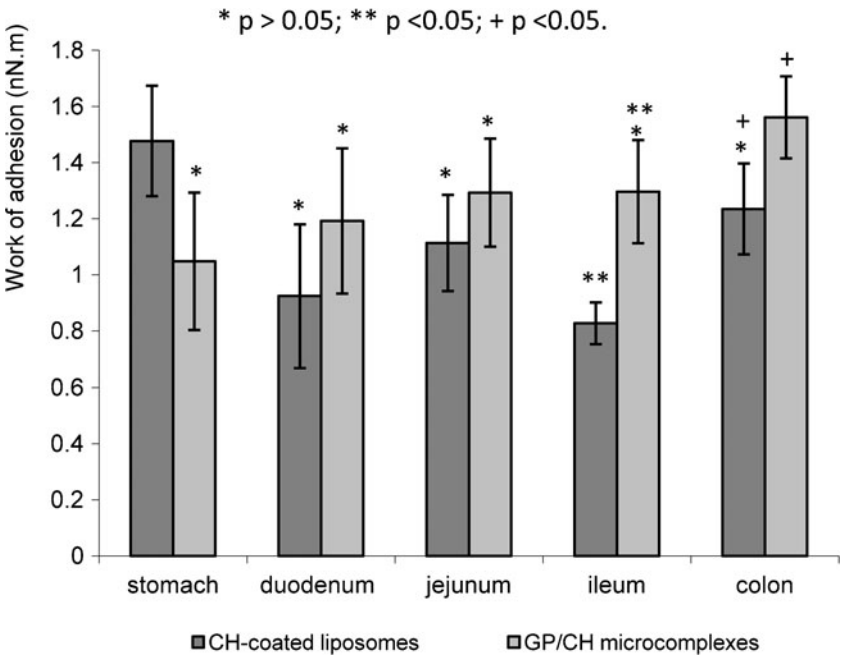

Fig. 8. *Ex vivo* mucoadhesive performance of samples: work of adhesion (W_{ad}) is the total amount of force involved in the tissue (stomach, duodenum, jejunum, ileum and colon) withdrawal from the CH-coated liposomes or GP/CH microcomplexes dispersion (n=3)

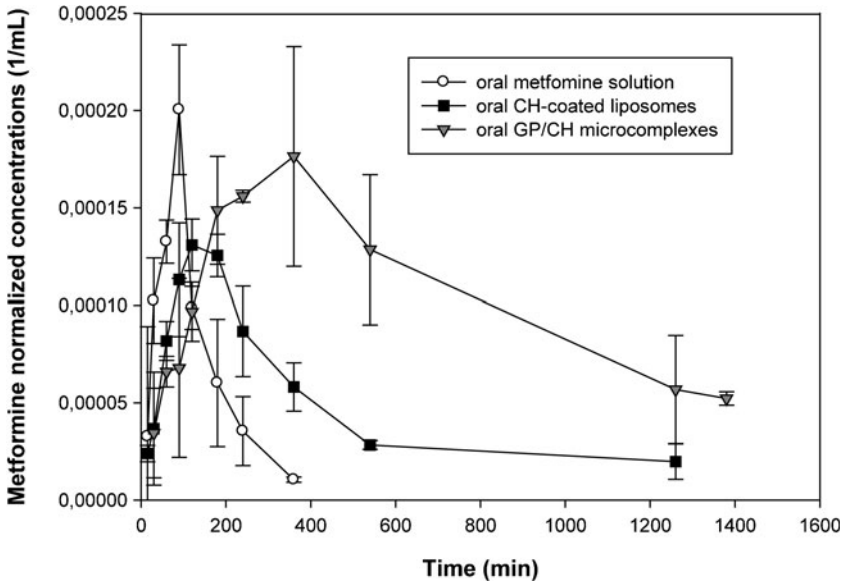

Fig. 9. Mean plasma concentration–time profiles of Met after oral administration (n=5)

In Vitro Release Study

Drug release from hydrophilic matrices is thought to be affected by swelling behaviour of samples. As shown in Fig. 7, Met release from both formulations was faster at pH 1.2 (up to 45 min), than at pH 7.4. The amount of drug released from CH-coated liposomes in 45 min in acidic medium reached 49% of the used dose. At pH 7.4 the Met release was strongly reduced, and it became almost constant during time. Using GP/CH microcomplexes, the drug release at pH 1.2 reached only 24% of the loaded dose, and further decreased at pH 7.4. According to the swelling behaviour of the samples, Met release from swollen CH-coated liposomes was always higher than that from GP/CH microcomplexes. At the end of the experiments, the former released 85% of the administered dose, while the latter only 50%. It is well known that drug release from hydrophilic matrices is affected by the property of the used polymer such as water affinity and consequent swelling properties. The GP crosslinkage reduces the polymer water affinity as a consequence of both the reduced number of free amino groups capable of interacting with water and the crosslinkages that avoid polymer dissolution. Therefore, swelling is reduced and the polymer cannot dissolve in the aqueous medium. As a consequence, the drug diffusion from the GP/CH microcomplexes decreased in comparison with CH-coated liposomes.

Mucoadhesive Properties

The mucoadhesive properties of CH- and GP/CH microcomplexes were evaluated using stomach and intestine tissues of rats. Figure 8 shows the influence of the contact time between coated liposomes and gastrointestinal mucosa on the work of adhesion (W_{ad}). Mucoadhesion process is a consequence of interactions between the mucus layer and mucoadhesive polymers and it is greatly dependent on polymer structure and charge density. Positively charged polymers, such as chitosan, can form polyelectrolyte complexes with negatively charged mucins and exhibit strong mucoadhesion depending on polymer charge. Polymer structure is also important to modulate the mucoadhesion interactions: the crosslinking of water-soluble polymers could restrict the system over hydration and polymer dissolution, improving mucoadhesion.

CH-coated liposomes have a higher mucoadhesion in the stomach than in the duodenum, jejum and ileum. This behaviour could be due to the combination of its high positive zeta potential (+56 mV) and its uncrosslinked structure that allows a strong mucoadhesion at short time (stomach) but also a faster hydration of its chains that will form weak gels that readily dissolve.

GP/CH microcomplexes exhibited a good mucoadhesion in ileum and colon higher than that of CH-coated liposomes probably because at short time (stomach) its low zeta potential

Table II. Pharmacokinetic Parameters of Met After Single Intravenous (Aqueous Solution) and Oral Administration (Aqueous Solution, CH-Coated Liposomes and GP/CH Microcomplexes): Total Area Under the Plasma Concentration–Time Curve (AUC), Peak Plasma Concentration (C_{max}), Time to Reach C_{max} (T_{max}) and Extent of Absolute Oral Bioavailability (F)

Route of administration	Formulation	T_{max} (min)	C_{max}/D (L^{-1})	AUC/D (minL^{-1})	F (%)
IV	Aqueous solution			261±36	
Oral	Aqueous solution	70±30	0.43±0.07	60±14	20±8
	CH-coated liposomes	*140±30	0.13±0.01	63±5	23±4
	GP/CH microcomplexes	*180±50	0.20±0.05	99±2	*38±5

Parameters have been normalized by the dose
*p<0.05 (statistical differences from oral solution)

(+11 mV) led a weak mucoadhesion. However, at longer times, the slower hydration of the crosslinked polymer favoured the interpenetration of its chains with the mucus in the later part of the gastrointestinal tract, *i.e.* ileum and colon. Moreover, they showed mucoadhesion to colon mucosa stronger than to stomach and small intestinal mucosa, probably due to the lack of villi in the colon, which is however, rich of goblet cells that produce mucine thus facilitating mucoadhesion (11).

Hence, crosslinked GP/CH microcomplexes may be capable of increasing drug residence time in the distal intestine and the amount of drug absorbed in this tract.

Oral Bioavailability Study

The *in vivo* plasma concentration *vs* time profiles after oral administration of aqueous solutions of Met or prepared formulations, are shown in Fig. 9. The Met dosage range was 200–300 mg/kg. The total area under the plasma concentration *vs* time curve from time zero to time infinity (AUC), peak plasma concentration (C_{max}) normalized for the dose (D), time to reach C_{max} (T_{max}) and extent of absolute oral bioavailability (F%) of the drug are summarized in Table II.

After oral administration of the Met solution, its intestinal absorption was rapid (T_{max}=70 min) and no lag time was detected, although a high variability was observed (Fig. 6 and Table II). The drug pharmacokinetic parameter (T_{max}) obtained from the CH-coated liposomes and GP/CH microcomplexes revealed that Met absorption was delayed (T_{max} from 70 to 140 and 180 min, respectively, $p<0.05$), drug AUC/D increased (from 60 to 99 minL^{-1}, $p<0.05$) only using crosslinked formulation whereas C_{max}/D was lower (1/3 and ½, respectively) than that obtained after solution administration.

Moreover, the normalized AUC of Met-loaded GP/CH microcomplexes showed a statistically significant increase ($p<0.05$) in oral bioavailability of the drug (F% from 20 to 38). Therefore, results showed that only GP/CH microcomplexes were actually able to improve the Met absorption and bioavailability. Taking into account that Met absorption is partly mediated by a saturable carrier (50,51), the improvement in absorption when the drug was encapsulated in GP/CH microcomplexes could have been due to the mucoadhesive properties of this complex, which may have facilitated a prolonged residence of Met in the preferential absorption site, as well as to a slow release, which would have helped to avoid saturation of the transporter. This phenomenon may guarantee a sustained hypoglycaemic effect of the drug and, in addition a reduction of side effects (abdominal discomfort, nausea and diarrhoea) which occur at high drug concentrations in the gastrointestinal tract (51).

Although the experimental conditions of this work have not been applied to other drugs yet, it is important to highlight that the behaviour observed here with metformin could be extrapolated to other drugs with similar physico-chemical properties (*i.e.*, molecular weight, charge, solubility, compatibility with phospholipids). One important aspect is the interaction between the drug and the excipient used in the formulation, which would determine encapsulation efficiency, stability and drug release, as can be observed in this work. On the other hand, once the drug is encapsulated, surface properties that determine parameters such as mucoadhesion, are mainly dependent on the polymer used as coating and not on the drug itself.

CONCLUSION

A new liposome/crosslinked chitosan–glycerolphosphate microcomplex has been designed for oral administration of hydrophilic drugs. This microcomplex based on Met loading vesicles was coated with GP/CH to protect the vesicles and control drug release. Indeed, results have shown an increased stability of the formulation in the intestinal environment, and a prolonged drug release. Characterization of the rheological and physico-chemical properties of GP/CH microcomplex confirmed an adequate complexation of CH with GP that enveloped the vesicles in a gel network and positively affected microcomplex physico-chemical properties and drug release. The *in vivo* oral bioavailability of Met suggests that GP/CH microcomplexes are effective carriers of the highly water-soluble antihyperglycaemic drug, thus, allowing its controlled delivery and improved oral availability.

ACKNOWLEDEGMENTS

This work was partially supported by MIUR grants (PRIN 2008) and “Azioni Integrate Italia-Spagna” 2009. Sardegna Ricerche Scientific Park (Pula, CA, Italy) is acknowledged for free access to facilities of the Nanobiotechnology Laboratory. The authors gratefully thank the Electronic Microscopy section of SCSIE (Servei Central de Suport a la Investigació Experimental, Universidad de Valencia, Spain) for the analysis performed.

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