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RESEARCH PAPER

Influence of the Nanocomposite MgAl-HTlc on Gastric Absorption of Drugs: *In Vitro* and *Ex Vivo* Studies

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ABSTRACT

Purpose Furosemide (FURO) is a BCS class IV drug preferentially absorbed in the gastric environment. A previous study demonstrated that its intercalation into the lamellar inorganic matrix MgAl-HTlc, giving rise to MgAl-HTlc-FURO, improves its dissolution in acidic medium. As the gastric absorption of drugs can be hindered from the biological barriers mucus and gastric mucosa, the purpose of this work was to evaluate the effect of MgAl-HTlc on gastric pH, the possible modifications induced on mucus rheology and the influence on both artificial and biological membranes.

Methods Firstly the effect of growing MgAl-HTlc concentrations on gastric pH was evaluated. Both drug flux across the mucus layer and permeability across an artificial and biological membrane (gastric mucosa) have been studied as well.

Results The results highlighted that drug flux across gastric mucus is improved in presence of MgAl-HTlc-FURO and that MgAl-HTlc is able to modify mucus structure in a reversible manner. From permeability studies emerged that the use of a biological membrane is the most suitable for such studies and that MgAl-HTlc-FURO enhances FURO Papp.

Conclusions Data obtained suggest that MgAl-HTlc is a suitable material able to improve the biopharmaceutical properties of class IV BCS drugs.

KEY WORDS gastric mucosa · hydrotalcite · mucus · Papp · viscoelasticity

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INTRODUCTION

Oral delivery is the most convenient and widely accepted route of drug administration, especially for chronic therapies where repeated administrations are required. Oral formulations are largely used because of 1) patient compliance 2) low invasive character 3) easy administration and 4) low cost of manufacturing processes. However, numerous active pharmaceutical ingredients (APIs) are inadequate to be formulated as oral dosage forms because of low drug permeability across gastrointestinal (GI) membranes and/or poor solubility/dissolution in GI fluids (1–3). According to the Biopharmaceutics Classification System (BCS), orally administered drugs are labelled in four classes on the basis of their aqueous solubility/dissolution rate and permeability (4) and molecules belonging to class IV show problems of low and variable bioavailability due to both low solubility and low permeability.

It has been estimated that 10% of APIs in development and 40% of drugs already on the market belong to class IV of BCS (5) and, as the number of drugs displaying such problems is growing, many strategies have been proposed for drug absorption enhancement.

One of the most common approach is represented by the use of penetration enhancers (6), able to promote drug absorption by means of different mechanisms as modifications of: mucus rheological properties, membrane fluidity, prolonged residence time of drug in the absorption site and efflux pumps inhibitors (7).

In regards to mucus rheology (8), it is important to consider that mucus is an hydrogel, mainly constituted by mucin molecules secreted from goblet cells (large glycoproteins with negative charged net forming the mucus gel-like structure) and water. Mucus forms an adherent gel layer on the GI surface acting as lubricant and protective barrier against harmful agents, such as hydrogen ions and pepsin

(9,10). It has also been suggested that GI epithelial cells are protected from gastric acid attack by the hydrophobic lining of surface-active phospholipids present in the gastric mucus layer. A very important feature is the Unstirred Water Layer (UWL), placed near the absorbing cells in the GI lumen, showing a pH value in the range of 5.2–6.2, independent from the variable luminal pH. The mucus layer plays the main role in regulation of epithelial cell surface pH value. It has been proposed that its amphiphilic character is necessary for the maintenance of an acidic microclimate on mucosal surface (11). Mucus can reduce drug permeation avoiding drug diffusion to the underlying space until enterocyte surface (12) thus, when the mucus layer represents the rate limiting step for drug absorption, the use of systems able to modify its network structure is a possible and important approach in order to improve drug absorption (13).

In regards to membrane fluidity, this parameter can be increased by means of ingredients as fatty acids, able to interact with lipid components of enterocyte membrane and to induce the disruption of intercellular packing. In this way the molecules can cross the GI wall by using these new openings however, this mechanism can promote lipid extraction inducing an irreversible modification of the membrane (14).

Concerning the possibility to prolong drug residence time on its absorption site, recently excipients such as chitosan (15) and other mucoadhesive polymers (16) have been proposed as new approach to increase drug absorption, because of their ability to interact with mucus prolonging the contact time between drug and GI mucosa. These excipients show low toxicity and are able to promote drug flux across GI membrane avoiding epithelial layer damage (17,18). In the case of drugs which are substrate of efflux pumps (such as P-gp), displaying, for this reason a limited oral absorption, the realization of formulations containing molecules able to interact with the efflux pumps in place of the API, could be useful. In addition some ingredients (such as pluronic block copolymers, thiolated polymers), generally employed as excipients for oral dosage forms, can act as efflux pump substrates allowing a complete drug absorption (19–21).

In recent years, many kind of inorganic materials such as layered double hydroxides (HTlc) (22,23) have been successfully employed in drug delivery. The benefits in drug dissolution enhancement of APIs intercalated onto HTlcs galleries have been assessed by numerous studies (24–30). On the other hand, studies oriented to the investigation of HTlc effect on drug absorption in the GI tract have not been yet performed. In a previous study the model drug furosemide (FURO), labeled in BCS class IV, has been intercalated into MgAl-HTlc obtaining the nanocomposite MgAl-HTlc-FURO (in which the drug is homogeneously dispersed in molecular form between the HTlc interlamellar spaces) able to promote an improved drug dissolution in comparison to crystalline FURO (30).

Together to low solubility FURO shows low permeability too (31) and, because of its weak acidic nature, is preferentially absorbed in the stomach and upper intestine (32). For these reasons, FURO seemed to be a good model to study new strategies for gastric absorption enhancement of drugs. The objective of this research was to evaluate the effect of MgAl-HTlc on FURO permeability. As mucus layer covers gastric mucosa surface it can represent the first barrier for FURO absorption thus, *in vitro* studies have been performed in order to evaluate MgAl-HTlc effect on: i) drug flux across gastric mucus layer, ii) gastric pH, and iii) gastric mucus rheology.

Finally a new study has been proposed in order to measure FURO permeability across gastric mucosa.

MATERIALS AND METHODS

Materials

The intercalation product MgAl-HTlc-FURO, prepared following the procedure described in a previous work (30), had the exact formula $[Mg_{0.63}Al_{0.37}(OH)_2]$ $(FURO)_{0.27}$ $(OH)_{0.1}\cdot 0.56\ H_2O$ and a final drug loading of 55.60% (30). Gastric fluid with pepsin (European Pharmacopoeia, Ph. Eur. VII Ed.) was prepared by adding 8 mL of HCl (10 M) to a water solution of NaCl (2.00 g) and pepsine (3.20 g); the volume was adjusted to 1000 mL with deionized water (final pH=1.07). Krebs' ringer bicarbonate buffers at pH 3.00 and 7.40 (Sigma-Aldrich) had the following composition: D-glucose (1.80 g), KCl (0.34 g), NaCl (7.00 g), $NaH_2PO_4\cdot H_2O$ (0.21 g), $MgCl_2.6\ H_2O$ (0.11 g), Na_2HPO_4 (0.10 g) $NaHCO_3$ (1.26 g), Deionized water until 1000 mL. The pH values were adjusted using HCl (0.1 M) and NaOH (0.1 M). Cetrimide, n-propanol, D-glucose, pepsin, cholesterol were purchased from Sigma-Aldrich (Milan, Italy). Carbogen O_2/CO_2 (95%/5%) was supplied by Gas Tecnici (Foligno, Italy). Deionized water was obtained from reverse osmosis process with Milli Q System (Millipore, Roma, Italy). Other chemicals and solvents of reagent grade were used without further purification.

Methods

Mucus Samples Preparation

Porcine gastric mucus (PGM) was collected following the procedure described by Shaw *et al.* (8). Pig stomachs, obtained from Large White pigs weighing ~160–165 kg, were furnished by veterinary service of ASL N.1 Città di Castello (Umbria, Italy) and used within 12 h from pig sacrifice. Each stomach was opened along the greater curvature, inverted, any food content removed and finally

washed with NaCl 0.9% (wt./v) solution. The mucus was collected by gentle scraping using a smooth-faced spatula, ensuring no underlying mucosa was removed, from every region of the stomach, i.e. cardia, fundus, body and pylorus regions. The mucus was mechanically mixed with the spatula until it became visually homogenous and then stored overnight at 4°C before use (8).

In Vitro Flux Studies

The studies were conducted by using the flow through diffusion cell apparatus 4 (Farmacopea Ufficiale Italiana, F.U.XII Ed.) properly modified by introducing on the top of the diffusion chamber (standard cell for tablets) a cylindrical support (height 4 mm) provided by a steel net round base (diameter 12 mm) (G.P.A. Componenti Meccanici, Città di Castello, Italy) in which PGM was placed (33). Glass microfibre filters (GF/D 2.7 μm, Whatman GmbH, Dassel Germany) were used in order to have a stable mucus layer during the test and to prevent direct contact between the mucus and the filter chamber. PGM was placed on the support through a syringe to hold a 1 mm thick layer (surface 3.8 cm^2) and the sample (as simple powder) placed in the diffusion chamber. This experiment was performed on three different samples: crystalline FURO alone, physically mixed to MgAl-HTlc and MgAl-HTlc-FURO. Gastric fluid with pepsin (Ph. Eur. VII Ed.) thermostated at 37.0°C±0.5 was used as medium and pumped at 5 mL/min for 2 h. Samples were collected at predetermined time intervals for 2 h and filtered by using filter papers (0.45 μm, Whatman, England) by SWINNEX system (Millipore). FURO amount in each sample was determined by HPLC following the method reported in FURO monograph of the Ph. Eur. VII Ed. (see paragraph 2.7.), by using a drug calibration curve in gastric fluid with pepsin (r=0.9995). The experiments were carried out in triplicate and the error expressed as ± SD.

HTlc Effect on Gastric pH

A series of samples constituted by serial concentrations (growing) of MgAl-HTlc in gastric fluid with pepsin, in the concentration range 0.10 mg/mL–10 mg/mL (concentration increase 0.10 mg/mL), were prepared. Each sample was kept under magnetic stirring (600 rpm) for 4 h at room temperature then stored at 37.0°C±0.5 for 1 h before pH measurement (digital pHmeter HI 110, HANNA Instruments, USA). For each sample the experiment was performed in triplicate.

Rheological Studies

Rheological measurements were carried out by means of a rheometer Stresstech HR (Reologica Instruments, AB Milano, Italy) equipped with a cone-plate geometry (diameter of 40 mm and angle 1°). The samples for rheological measurements were prepared as follows. A fixed amount of PGM (2.00 g) was dispersed in 1 mL of gastric fluid with pepsin and incubated with three different MgAl-HTlc amounts (three samples) in order to obtain the following concentrations: 3.40 mg/mL; 4.20 mg/mL and 9.80 mg/mL. A MgAl-HTlc free sample was used as control. It was prepared by dispersing PGM (2.00 g) in gastric fluid with pepsin (1 mL). The obtained samples were characterized by viscometry (viscosity) and oscillation stress sweep measurements (viscoelasticity) at 37.0°C±0.5 in order to reproduce the physiological temperature conditions. The oscillation stress sweep measurements were carried out in two steps. At first the linear viscoelastic region has been measured working at the frequency of 1 Hz and varying the stress from 0.1 to 10 Pa. Then, the frequency sweep measurement was performed varying the frequency range, 1–10 Hz, and working at a fixed stress value, previously detected in the linear viscoelastic region.

Permeation Studies

Permeation studies were performed by using a Side-Bi Side cell (PermeGear, Inc. Bethlehem, PA.). A membrane (artificial or biological) was mounted between the two cells; the exposed surface area was 0.63 cm^2 and the reservoir volume 5 mL.

In the first experiment an artificial membrane was used, represented by nitro-cellulose 0.025 μm pore size, Millipore Italia S.p.A. (Milan, Italy) impregnated with a lipidic mixture consisting of Lipoid® E80 (1.70%) (Lipoid®, Ludwigshafen, Germany), n-octanol (96.2%) and cholesterol (2.10%) (Sigma-Aldrich, Milan, Italy) reaching a final weight increase of 95–105% (34).

In the second experiment, porcine gastric mucosa was used as biological membrane model. It was obtained from stomachs of Large White pigs weighing ~160–165 kg, furnished by veterinary service of ASL N.1 Città di Castello (Umbria, Italy) and used within 8 h from pig death. The serosa and outer muscle layer were stripped off with scalpel, then the mucosa was washed with NaCl 0.9% (wt./v) and mounted between the cells in order to orientate the apical side toward the donor chamber and the basolateral side toward the receptor chamber. The donor and receiver compartments were immediately filled with pre-warmed buffers circulated by a gas lift (95% O_2–5%CO_2) in order to guarantee tissue vitality. The flow rate of gas lift was adjusted to 10±2 mL/min^{-1} using a flow meter. The tissue was equilibrated for 10 min before adding the sample to the donor compartment.

Kreb's buffer pH 3.00 was used as donor medium in order to simulate the gastric environment and Kreb's buffer pH 7.40 was used as receptor medium the in order to simulate the plasmatic pH value. At predetermined

intervals, aliquots (200 μl) were removed from the receptor chamber and replaced by the same amount of fresh Kreb's buffer pH 7.40.

Both experiments (artificial and biological membrane) have been performed on two different drug samples: FURO alone and the composite MgAl-HTlc-FURO. In order to evaluate only the effect of MgAl-HTlc on FURO permeability, both samples were previously dissolved in the donor solution before starting the experiment. The amount of FURO dissolved in the donor compartment (5 mL) was 0.50 mg. In the case of MgAl-HTlc-FURO the dissolved amount was 0.90 mg (corresponding to 0.50 mg of FURO and 0.40 mg of MgAl-HTlc), calculated taking into account that the drug loading in the composite is 55.60%.

FURO amount was determined by HPLC following the method reported in Ph. Eur. VII Ed. FURO monograph (see paragraph 2.7.) by using a drug calibration curve in Kreb's buffer pH 7.40 (r=0.9992). The experiments were carried out in triplicate and the error expressed as SD.

Analytical Procedure

The amount of drug was determined by HPLC (Perkin-Elmer LC90J) following the method reported in FURO monograph of the Ph. Eur.(VII Ed.).

Stationary phase: RP8 column, Lichrospher® 100 (5 μm, Merck KGaA 64271 Darmstadt, Germany), mobile phase: potassium dihydrogen phosphate (0.20 g) and cetrimide (0.25 g) are dissolved in 70 mL of bidistilled water adjusting the pH to 7.00 with ammonia, at this solution n-propanol (30 mL) is added. Flow rate: 1 mL/min; Detection: UVspectrophotometer at 238 nm.

Data Analysis

The steady-state flux of the drug through PGM was determined from the following equation (35):

$$J = \frac{Q}{Axt} \tag{1}$$

where J represents the flux, Q is the drug concentration (μg) at the time t (min), and A represents the surface (cm^2).

Apparent permeability was calculated according to Eq. 2 (34):

$$Papp = \frac{dQ}{dt} \times \frac{1}{ACo} \tag{2}$$

where $Papp$ is the apparent permeability coefficient ($cm\ s^{-1}$), dQ/dt the amount of drug permeated per unit of time ($mg\ s^{-1}$), A the effective surface area of the artificial membrane (cm^2), and C_0 is the initial drug concentration in the donor compartment.

Statistical Analysis

The results are expressed as mean ± SD (n=3). Two-sample t test was used to compare the means of flux and permeability data at each time point and assess statistical significance. Results were considered statistically significant if $p<0.05$.

RESULTS AND DISCUSSION

HTlc Effect on Gastric Environment

The GI mucosal surface is covered by the mucus, a highly viscous and elastic gel which provides protection by pathogens, toxins, ultrafine particles, enzymatic injury and, in the case of the gastric mucosa, HCl of gastric fluid. However, various studies demonstrated that mucus can also represent a potential barrier to drug absorption (12,36,37).

Mucus gel constituted by water (~95%), salts, lipids such as fatty acids, phospholipids, cholesterol and proteins. In regards to the latter, the main component is represented by the glycoproteins mucins (38) which interact each other forming a final network responsible for mucus gel-like properties.

In Vitro Flux Studies

As FURO is preferentially absorbed in the stomach, it was interesting to evaluate if gastric mucus can represent an obstacle to drug diffusion from the gastric lumen to mucosal surface. With this aim, an *in vitro* method has been developed introducing a modification in the flow through diffusion cell apparatus 4 (F.U.XII Ed.). As shown in Fig. 1, a cylindrical support was mounted on the top of the dissolution chamber; it was provided by a steel net in which PGM (1 mm of thickness and surface 3.8 cm^2) was placed. Gastric fluid with pepsin (F.U. XII Ed.) was pumped at 5 mL/min in order to simulate GI fluid flow through the mucus layer covering the gastric mucosa. FURO as (i) crystalline powder alone, (ii) physically mixed to MgAl-HTlc and (iii) as composite MgAl-HTlc-FURO (in which FURO is intercalated between matrix lamellae) was placed as simple powder in the dissolution chamber. The gastric medium with pepsin was pumped for 2 h through the cell from the bottom to the top where the mucus was placed. Samples were collected at predetermined times and analyzed by HPLC (see Methods section) in order to measure FURO amount in each of them. Obtained data were elaborated in Eq. 1 in order to calculate the corresponding drug flux (J) values at each time. Analyzing the flux profile of each sample (Fig. 2) emerges that the values registered for the intercalation product MgAl-HTlc-FURO are statistically greater in comparison to the controls crystalline FURO alone and physically mixed to MgAl-HTlc in particular in the first 30 min (p=0.001).

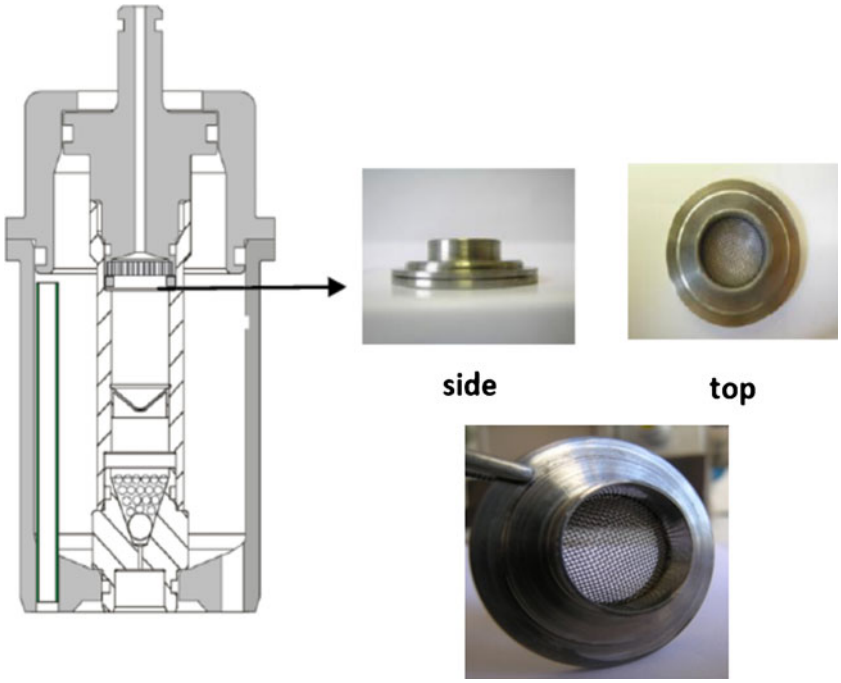

Fig. 1 Apparatus 4 for dissolution test (*left*) F.U. XII Ed. On the top of the dissolution chamber is introduced a steel cylinder (height 4 mm) with a steel net (*right*) where the PGM was placed.

The best performances observed for the intercalation product are mainly attributable to the enhanced drug dissolution. In fact, at a fixed time, the amount of drug released and dissolved from MgAl-HTlc-FURO is higher than those deriving from the drug in crystalline form both alone and physically mixed to MgAl-HTlc matrix. According to Fick's law, the increased amount of drug dissolved generates a concentrated solution at the mucus layer surface creating a driving force able to increase the amount of molecules that cross the mucus layer (high Q value in Eq. 1). However, a further difference can be recognized comparing the flux profiles deriving from crystalline FURO alone and physically mixed to MgAl-HTlc. In fact, highest values were obtained for the latter sample meaning that probably the HTlc inorganic matrix can exert a certain effect on FURO flux enhancement through PGM. On the basis of these results it was interesting to study the possible modifications that MgAl-HTlc can induce on PGM structure.

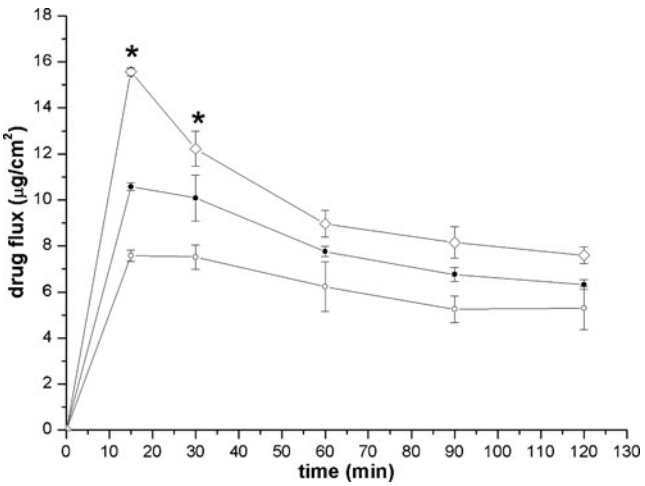

Fig. 2 Flux profiles through PGM in gastric fluid with pepsin at 37.0°C ± 0.5 ($n=3 \pm SD$). *Indicates that the means of drug flux data deriving from the MgAl-HTlc-FURO sample are significantly higher than that coming from FURO alone, $p=0.001$.

In these studies it was important to take into account that rheological properties of the mucus gel-like structure, due to mucin–mucin interactions, are dependent on pH changes. Studies focused on the evaluation of pH effect on mucus flow properties, in fact, report that its viscosity decreases as pH value increases (39). This is particularly important for PGM as in acidic conditions (stomach) it is present in gelled state displaying a protective action on gastric mucosa toward HCl.

HTlc Effect on Gastric pH

Together to flux studies, it was very interesting to investigate the influence of MgAl-HTlc on gastric medium pH. Previous studies (30,40) demonstrated that FURO intercalation into MgAl-HTlc promotes an increase of drug dissolution in acidic fluids. This aspect is important as FURO is preferentially absorbed in the stomach in which it displays the lowest solubility (31), because of its weak acidic nature. One of the main factors involved in drug dissolution improvement in acidic medium is represented by HTlc behaviour in these conditions. In fact, it is noteworthy that at low pH values (<4.00) HTlc undergoes to disruption (41,42) promoting the release of intercalated drug molecules. As consequence of MgAl-HTlc dissolution, magnesium and aluminum

hydroxides are converted in the corresponding chlorohydrates responsible for the a gradual increase of the microenvironmental pH. In order to investigate in a deep manner the relation between MgAl-HTlc concentration and the pH value changes, a new experiment was planned. Solutions containing growing MgAl-HTlc amounts have been prepared in gastric fluid with pepsin, thermostated at 37.0°C ±0.5 in order to obtain samples with different concentrations (see Methods section), then submitted to pH measurement. Each pH datum was correlated to the corresponding concentration value and reported in a graph (Fig. 3). The obtained profile shows that medium pH value increases proportionally to MgAl-HTlc concentration but under the concentration of 3.20 mg/mL acidic values are still maintained (1.70). Increasing MgAl-HTlc amount, obtaining concentration values between 3.20 mg/mL and 5.50 mg/mL, more interesting results can be recognized as the pH value increases from 1.70 to 3.91. For MgAl-HTlc concentrations higher than 5.50 mg/mL the pH reaches ~4.00. A further increase of MgAl-HTlc produces a plateaux in the range of 5.50 mg/mL-9.80 mg/mL. These aspects are very important as they prove MgAl-HTlc capability to maintain pH values near to 4.14 value also when used at high concentrations, making it a suitable antacid.

The antacid effect is generally due to partial neutralisation of gastric HCl and inhibition of the proteolytic enzyme, pepsin (43). However, numerous antiacids, actually available on the market, present the limit to provoke reflex acidity (at high concentrations) as consequence of the gastric pH increase, reaching values in the range of 4.00–5.00. In the case of MgAl-HTlc this phenomenon can be avoided as the reflex acidity could be obtained only using a large amount (>10 g) that exceeds the ordinary doses.

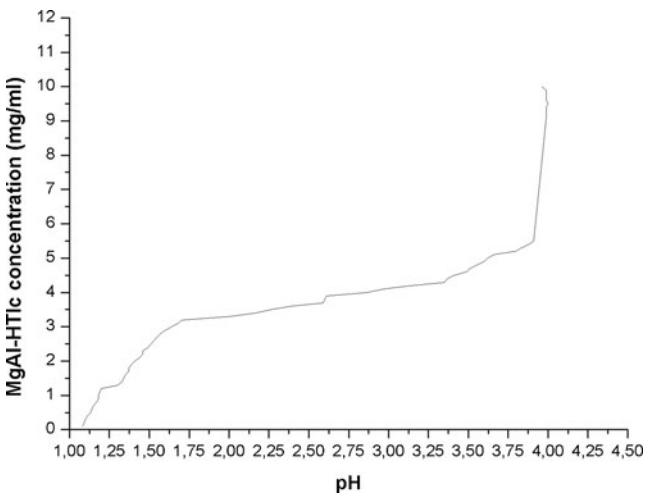

Fig. 3 Graphical representation of pH value modification *vs* MgAl-HTlc concentration in gastric fluid with pepsin (n=3).

Rheological Studies

After these studies and considerations, about MgAl-HTlc influence on gastric pH, the effect on gastric mucus rheological properties was investigated as well, taking into account that PGM is a non-Newtonian water insoluble gel showing a viscoelastic gel structure (44).

MgAl-HTlc effect on gastric fluid pH is an interesting starting point to investigate PGM rheological modifications induced by MgAl-HTlc. Taking into account that mucus viscosity decreases as pH increases (39), further studies have been performed in order to evaluate the effects of pH value changes induced from MgAl-HTlc on PGM flow properties (viscosity and viscoelastic properties). With this objective three sample have been prepared and submitted to viscometry and viscoelastic measurements.

The samples were represented from PGM treated with a MgAl-HTlc solution in gastric fluid with pepsin at three different concentrations in order to evaluate PGM viscosity and viscoelastic properties in relation to significant pH values: a) pH 2.70, deriving from MgAl-HTlc concentration 3.40 mg/mL; b) pH 3.90, deriving from MgAl-HTlc concentration 4.20 mg/mL and c) pH 4.14, deriving from MgAl-HTlc concentration 9.80 mg/mL.

Viscometry studies were carried out in order to investigate the effect of MgAl-HTlc presence on PGM flow properties (viscosity). The experiments were performed by means of a rheometer Stresstech HR equipped with a cone-plate geometry (Fig. 4) and working at 37.0°C±0.5 in order to simulate the temperature conditions of gastric lumen and using as control a sample constituted by a fixed amount of PGM alone in 1 mL of gastric fluid with pepsin. The rheogram

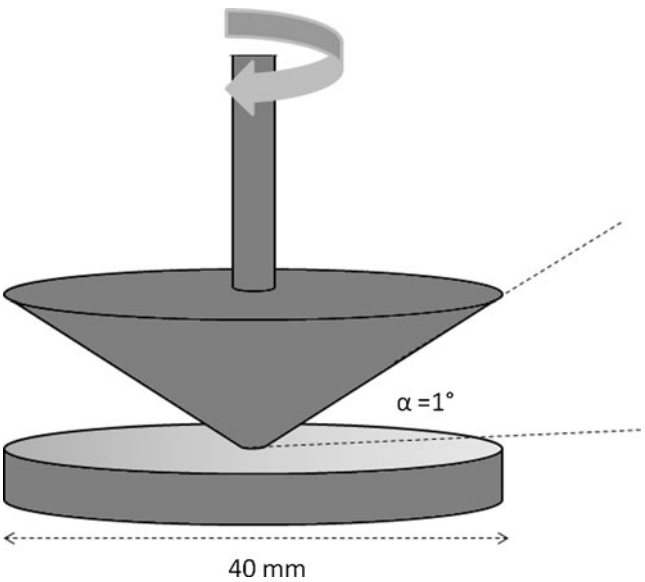

Fig. 4 Cone-plate geometry.

reported in Fig. 5 shows that the control has a low flow attitude due to the high viscosity whereas PGM samples, treated with MgAl-HTlc solutions, show better flow properties improving as MgAl-HTlc amount increases. From an accurate analysis of the obtained rheograms (Fig. 5), it is possible to recognize the minimum stress at which PGM starts to flow (yield value), for each sample, on the y axis. PGM treated with the lowest MgAl-HTlc amount shows a yield value similar to the control. In the case of other two samples, this value decreases as MgAl-HTlc amount increases obtaining $1.62 \cdot 10^{-1}$ Pa for the concentration of 4.20 mg/mL and $2.63 \cdot 10^{-2}$ Pa for the concentration of 9.80 mg/mL. These results suggest that MgAl-HTlc is able to modify PGM flow properties and that these modifications depends on MgAl-HTlc concentration. The modifications that MgAl-HTlc induces on PGM viscosity are explained from the well known gastric mucus susceptibility to pH value changes. As observed in the abovementioned studies, the increase of MgAl-HTlc concentration promotes the pH increase, pH 2.70 for 3.40 mg/mL; pH 3.90 for 4.20 mg/mL and pH 4.14 for 9.80 mg/mL, responsible for PGM viscosity decrease. These results are supported from literature data too (39,45) which demonstrate that the pH increase, associated to MgAl-HTlc dissolution and consequent magnesium and aluminum presence, could cause ionization of the carboxylic groups of mucin chains generating an enlargement of mucus meshes network and reducing its compactness (8,29).

As PGM displays a viscoelastic behaviour (44), the rheological characterization was extended to MgAl-HTlc effect on PGM viscoelastic properties. Also in this case PGM samples (2.00 g/mL) in MgAl-HTlc solutions in gastric fluid with pepsin (at the concentrations of: 3.40 mg/mL; 4.20 mg/mL and 9.80 mg/mL) have been submitted to viscoelastic measurements performed by using the above

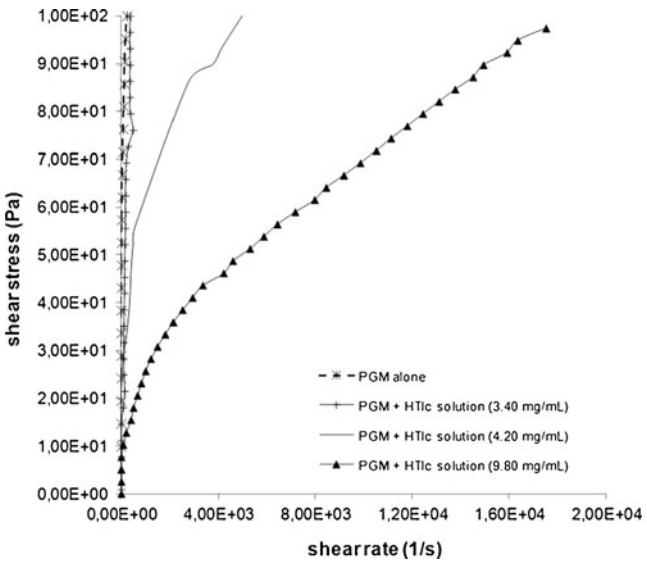

Fig. 5 Viscosity at 37.0°C ± 0.5 of PGM alone (control) and after treatment with three different MgAl-HTlc solutions (n=3).

mentioned rheometer. These studies are non-destructive oscillatory measurements and must be performed in two steps. At first the sample was strained by an increasing stress from 0.1 to 100 Pa, working at constant frequency of 1 Hz. The obtained profile allows to individuate the linear viscoelastic region in which it is possible to recognize the stress value useful to ensure an instantaneous recovery after the removing of the applied force. Then, oscillatory measurements were performed over a frequency range of 0.1–10 Pa and at constant stress, identified in the linear viscoelastic region (46,47).

Viscoelastic behaviour reflects the combined viscous and elastic response of semisolid materials under mechanical stress. A viscoelastic material is characterized from: i) elastic (storage) modulus (G′) that represents the elasticity of material, namely the material ability to store energy, ii) viscous (loss) modulus (G″) which expresses the material ability to dissipate energy (47,48), iii) the loss tangent (tan δ) representing the G″/G′ ratio which offers information about the structure of the network system. As the loss tangent approaches zero, the elastic structure of the system predominates, whereas, if the loss tangent exceeds the unity, the system is considered to be viscous.

Fig. 6 shows the rheograms relative to G′ and G″ moduli of all the samples. Data coming from the viscoelastic measurements performed on the control, PGM alone (graph not reported), showed that the elastic modulus (G′) prevails (tan $\delta < 1$) on G″ obtaining high values for both moduli (average $G' = 3.25 \cdot 10^{7}$ Pa; average $G'' = 5.00 \cdot 10^{5}$ Pa). The prevalence of G′ modulus on G″ is maintained in PGM samples treated with MgAl-HTlc (Fig. 6). In comparison to the control, lower values G′ and G″ have been registered for all the samples (G′ value average $< 3.25 \cdot 10^{1}$ Pa; G″ value average $< 1.28 \cdot 10^{1}$ Pa) meaning that MgAl-HTlc is able to modify mucus viscoelastic properties (Table I). It must be underlined that G′ and G″ moduli decrease is strictly related to MgAl-HTlc concentration in fact, the highest G′ and G″ moduli were observed for PGM sample treated with a MgAl-HTlc concentration of 9.80 mg/mL. In regards to the remaining samples (MgAl-HTlc concentrations of 3.40 mg/mL and 4.20 mg/mL) the respective G′ and G″ profiles were similar. The differences observed in these studies can be ascribed, also in this case, to the different pH values of MgAl-HTlc solutions. The highest concentration 9.80 mg/mL has a final pH of pH 4.14 meaning that, in comparison to the lower pHs of the remaining two samples, in these conditions the mucin chains carboxylic groups undergo to ionization, phenomenon that provokes the enlargement of mucus meshes network reducing its compactness.

As mentioned before, the G′ prevalence on G″ modulus is maintained also after treatment with MgAl-HTlc. This is an important point that must be highlighted as it

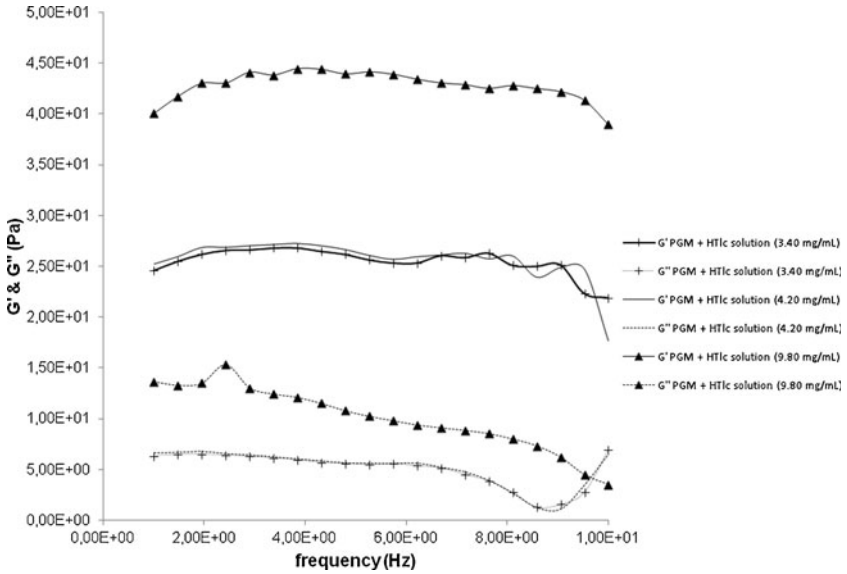

Fig. 6 G′ and G″ profiles registered at 37.0°C ± 0.5 for PGM treated with three different MgAl-HTlc solutions (n=3).

demonstrate that HTlc does not induce an irreversible modification on mucus structure so that its protective action toward mucosal surface is not impaired.

Taking into account data coming from literature (49) and the results deriving from rheological studies and MgAl-HTlc effect on gastric pH, some considerations must be done: i) HTlc dissolution in acidic environment produces a local increase of pH which can contribute to reduce mucus viscosity obtaining mucin chains relaxation; ii) mucin viscosity decreases in presence of specific ions as aluminium and magnesium (coming from HTlc dissolution); iii) the mucus elasticity also increases with relation to greater ion valency: high concentrations of multivalent cations, such as magnesium, can collapse the mucus gel entirely and facilitate reversible cross-links between mucin monomers.

Permeation Studies

FURO is a weak acid and for this reason it is preferentially absorbed in the stomach (32); a previous work demonstrated that FURO intercalation into MgAl-HTlc is able to improve drug dissolution in acidic conditions increasing the amount of molecules available to be absorbed (30). As FURO is classified as poor permeable drug (class IV BCS) it was interesting to build and to perform *in vitro* assays able to predict FURO apparent permeability (Papp) across gastric mucosa; useful to evaluate if this property can be modified in presence of the products coming from MgAl-HTlc dissolution in acidic medium (Mg and Zn chloroidrates). It must be considered that numerous *in vitro* models are available for intestinal absorption assessment as employment of cell lines (e.g. Caco-2 cells (50), everted gut sac, isolated and perfused intestinal segments, isolated biological membranes (51), but the spectrum of available models to investigate gastric absorption of drugs is restricted.

At first an the vitro permeation method, proposed by Corti *et al.* (34), based on an appropriate artificial membrane, has been performed. In order to simulate the composition of biological barriers a nitrocellulose membrane (pore size of 0.025 μm) was impregnated by a lipidic mixture, n-octanol and cholesterol (see Methods section) and mounted between the two chambers of a side-Bi-side diffusion cell. The experiment was performed introducing the Kreb's buffer pH 3.00, in which the drug (crystalline FURO alone or intercalated in MgAl-HTlc) was previously dissolved, in the donor chamber. Contemporaneously the Kreb's buffer pH 7.40 was placed in the receptor chamber. At predetermined intervals, aliquots of 200 μl were removed from the receptor chamber and analyzed by HPLC. Drug concentration was measured and each value was inserted in Eq. 2 in order to calculate the corresponding Papp. The experimental values of drug Papp, in presence or not of MgAl-HTlc, have been plotted *vs* time (Fig. 7).

Table I Viscoelasticity Values Obtained from the PGM Alone (Control) and Treated with Three Different MgAl-HTlc Concentrations

Sample	G′ (Pa) ± SD	G″ (Pa) ± SD	tan δ ± SD
Control	$3.25 \cdot 10^{7} \pm 1.27$	$5.00 \cdot 10^{5} \pm 1.08$	$1.54\ 10^{-2} \pm 0.06$
PGM + MgAl-HTlc 3.40 mg/mL	$2.55 \cdot 10^{1} \pm 1.33$	$5.03 \cdot 10^{0} \pm 1.68$	$1.97 \cdot 10^{-1} \pm 0.06$
PGM + MgAl-HTlc 4.20 mg/mL	$2.57 \cdot 10^{1} \pm 2.08$	$5.10 \cdot 10^{0} \pm 1.75$	$2.00 \cdot 10^{-1} \pm 0.07$
PGM + MgAl-HTlc 9.80 mg/mL	$4.28 \cdot 10^{1} \pm 1.42$	$1.01 \cdot 10^{1} \pm 3.16$	$2.34 \cdot 10^{-1} \pm 0.07$

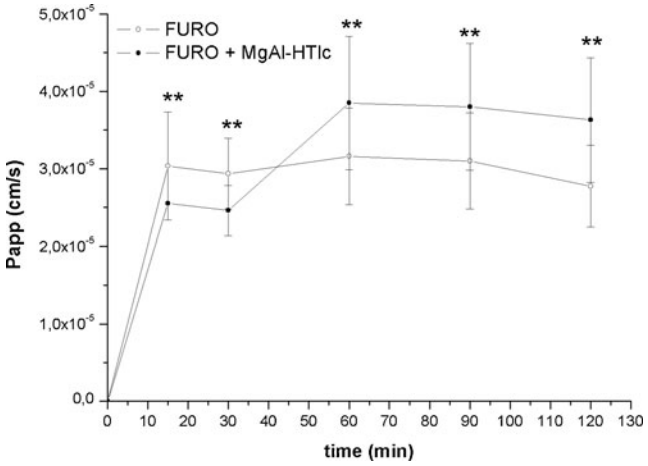

Fig. 7 Papp profiles *vs* time of FURO alone and in presence of MgAl-HTlc obtained using an artificial membrane ($n=3 \pm SD$). **Indicates that the means of FURO Papp data in presence or not of MgAl-HTlc are not significantly different, $p=0.11$.

From a first analysis of the graph results that after the first 60 min Papp of FURO alone prevails then, after this period, an increase of Papp values is observed for FURO in presence of MgAl-HTlc. In fact, in the case of FURO alone after 20 min the Papp becomes constant ($3.00 \cdot 10^{-5}$ cm/s) and this value is maintained for all the experiment. In the case of FURO in presence of MgAl-HTlc Papp increases gradually (after 30 min $2.55 \cdot 10^{-5}$ cm/s) reaching $3.85 \cdot 10^{-5}$ cm/s after 60 min, value kept until the end of the experiment.

By comparing the data of both samples at each time point resulted that Papp values of FURO alone do not show a statistical difference compared to the results obtained in presence of MgAl-HTlc ($p=0.11$). Thus, two different conclusions can be drawn: or the artificial membrane is not able to discriminate the different Papp of FURO alone and in presence of MgAl-HTlc, or MgAl-HTlc does not affect FURO Papp. This study was not satisfactory as it was necessary to find a most efficient *in vitro* method able to simulate the gastric barrier.

Therefore, in order to reproduce the physiological conditions it was necessary to execute the same assay by using a biological membrane (gastric mucosa). instead of an artificial membrane. In the choice of the most appropriate source of this material it was important to consider that pig tissues are most similar to human tissues in comparison to other animal model used such as the rat (52). Thus, a new experiment was planned and performed by using fresh porcine gastric mucosa deriving from the stomachs of Large White pigs, intended for the food chain. The gastric mucosa was accurately separated from the underlying layer and then mounted between the two chambers of the side-Bi-side diffusion cell rapidly filled with the donor and acceptor medium. In this assay the survival of biological membrane was guaranteed by a O_2/CO_2 (95%/5%) gas mixture circulated in the medium solutions. The new experiment was carried out in the same conditions of the previous study and the Papp values, deriving from the samples analyses, FURO alone and in presence of MgAl-HTlc, have been plotted *vs* time and the resulting profiles reported in Fig. 8. The comparison of Papp values of both sample at each time point revealed that the profiles obtained from FURO in presence of MgAl-HTlc are statistically greater compared to that coming from FURO alone ($p=0.01$). The Papp of FURO alone increases gradually reaching a value of $8.80 \cdot 10^{-7}$ cm/s after 90 min then decreases to $5.19 \cdot 10^{-7}$ cm/s at 120 min. In regards to FURO in presence of MgAl-HTlc the graph shows a gradual increase of Papp reaching the value of $1.32 \cdot 10^{-6}$ cm/s after 120 min.

These satisfactory results can be mainly ascribed to the effect that MgAl-HTlc dissolution exerts on gastric pH. Considering that the experiment has not been performed in sink conditions, it can be hypothesized that FURO concentration in the donor compartment increases until the drug solubility values is reached. FURO solubility in acidic environments is low because of its weak acidic nature thus, in these conditions drug precipitation can occur and this could explain the decrease of Papp at 120 min. In regards to FURO in presence of MgAl-HTlc the precipitation process is avoided as its gradual dissolution in acidic medium generates an increase of pH value, able to maintain FURO in solution.

Then Papp data registered in this second assay (Fig. 8) have been compared to the data coming from the experiment performed through the artificial membrane (Fig. 7) and some differences can be highlighted.

1) Papp values of both samples (FURO alone and in presence of MgAl-HTlc) are lower using the porcine mucosa than artificial membrane. These results can be explained considering that the biological membrane

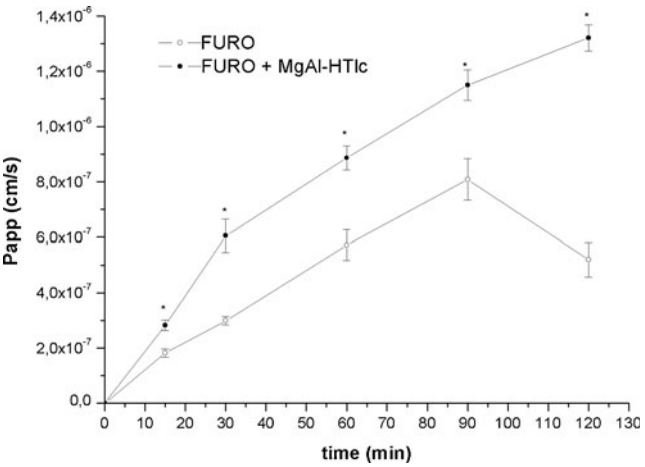

Fig. 8 Papp profiles *vs* time of FURO alone and in presence of MgAl-HTlc obtained using porcine gastric mucosa ($n=3 \pm SD$). *Indicates that the means of FURO Papp data in presence of MgAl-HTlc are significantly higher than that coming from FURO alone, $p=0.01$.

shows a complex structure and a complex chemical composition representing a tangible barrier to drug diffusion in comparison to artificial membrane.

2) The Papp profile coming from FURO alone is different from that deriving from FURO in presence of MgAl-HTlc. The low p values obtained indicate the good reproducibility of the proposed method and that the biological membrane is more discriminating than the artificial membrane.

The results coming from the performed permeation assays suggest that there is not a good correlation between the two methods. This can be explained considering that the artificial membrane (*in vitro* model), despite the treatment with the lipid mixture, is not able to reproduce the complex structure of the GI barrier. On the other hand, the use of a biological membrane (*ex vivo* model) such as the porcine gastric mucosa, shows a high suitability for studying the permeation properties of a poor absorbable drug as FURO. Taking into account these considerations it is possible to state that data coming from *in vitro* and *ex vivo* studies are not well correlable, thus, in the case of FURO, the use of an artificial membrane is not suitable to predict its absorption in gastric environment.

CONCLUSIONS

BCS class IV drugs suffer from both low solubility and low permeability responsible for their low and variable bioavailability. In recent years numerous technological approaches have been proposed with the aim to solve these problems and an interesting approach is represented by the use of inorganic matrices, in particular the lamellar anionic clay hydrotalcite (HTlc). Numerous scientific studies demonstrated its effectiveness and usefulness on drug dissolution enhancement. The present research had the aim to evaluate the possible effect of the hydrotalcite (MgAl-HTlc) on drug gastric absorption using as model the diuretic furosemide (FURO), a BCS class IV drug preferentially absorbed in the stomach. The performed studies had the objective to evaluate if the co-administration of MgAl-HTlc with FURO can enhance its gastric absorption. This aspect has been evaluated considering the two possible barriers for gastric drug absorption: mucus and mucosa.

1) Before to reach the gastric mucosa surface the drug must cross the mucus layer. Thus, from *in vitro* flux studies through pig gastric mucus, it was observed that FURO flux is enhanced, in presence of MgAl-HTlc, in comparison to crystalline drug alone. The reasons of these results can be resumed in two important points: i) in acidic conditions MgAl-HTlc undergoes to disruption/dissolution provoking an increase of the microenvironmental pH. It was established a relationship between MgAl-HTlc concentration and pH value increase. ii) the modification of gastric fluid pH induces the ionization of mucin chains carboxylic groups inducing an enlargement of the network.

This last aspect must be correlated to mucus rheological properties. In fact, growing MgAl-HTlc concentrations produce an increase of pH provoking a proportional decrease of mucus viscosity and, at the same time, an increase of its elastic properties.

2) FURO permeability has been evaluated both through artificial and biological (pig gastric mucosa) membrane which resulted the most appropriate model for such studies. The results obtained revealed that also in this case MgAl-HTlc presence induces an increase of FURO Papp.

This is an interesting finding in the development of simple and efficient delivery systems as the intercalation into MgAl-HTlc of a drug with poor biopharmaceutical properties can solve problems as poor solubility and poor permeability. The use of MgAl-HTlc is advantageous as the intercalation procedure does not modify drug chemical structure and, after administration, the mechanisms involved in drug liberation and absorption do not compromise the equilibrium of physiological mechanisms.

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