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# *In vitro* and *ex vivo* studies on Diltiazem hydrochloride-loaded microsponges in rectal gels for chronic anal fissures treatment

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**Abstract**

Diltiazem hydrochloride, topically applied at 2% concentration, is considered effective for the treatment of chronic anal fissures, althought it involves several side effects among which anal pruritus and postural hypotension. To test the hypothesis that a sustained delivery system of diltiazem hydrochloride may be helpful for the treatment of chronic anal fissures, in the present study we evaluated the potential of gels containing diltiazem hydrochloride entrapped in microsponges. Such microsponges were based on Eudragit RS 100 and the effect of some formulation variables was assessed by a $2^3$ full factorial screening design. An optimized formulation of diltiazem hydrochloride microsponges was dispersed in Methylcellulose 2% or Poloxamer 407 20% and the resulting gels (*micro-l*-diltiazem hydrochloride 2%) were subjected to *in vitro* drug release, *ex vivo* permeability and drug deposition after application on porcine rectal mucosa. The results showed a prolonged release up to 24 h from *micro-l*-diltiazem hydrochloride at 2% in the gels. The permeation tests revealed up to 18% higher drug retention on the mucosal tissue after 24 h by the *micro-l*-diltiazem hydrochloride 2% gels compared to conventional diltiazem hydrochloride gels at 2%. These results suggest that diltiazem hydrochloride-loaded microsponges dispersed in rectal gels may be useful to overcome some limitations of conventional local chronic anal fissure therapy.

*Keywords*: Diltiazem hydrochloride; Chronic anal fissures; Microsponges; Factorial screening design; Rectal gels; Permeation.

Diltiazem hydrochloride (PubChemCID: 62920);

Dichloromethane (PubMedCID: 6344);

Polyvinyl alcohol (PubChem CID: 11199);

Methylcellulose (PubChem CID: 6850753);

Poloxamer 407 (PubMedCID: 24751);

Disodium hydrogen phosphate (PubChem CID: 24203);

Potassium dihydrogen phosphate (PubChem CID: 516951);

Sodium hydrogen carbonate (PubChem CID: 516892).

*Abbreviations*

| | |
|---|---|
| *c*-diltiazem hydrochloride gel | Diltiazem hydrochloride-loaded conventional gel; |
| Mc-based *c*-diltiazem hydrochloride | Diltiazem hydrochloride-loaded conventional gel prepared with Methylcellulose (Mc); |

| | |
|---|---|
| Px-based *c*-diltiazem hydrochloride | Diltiazem hydrochloride-loaded conventional gel prepared with Poloxamer 407 (Px); |
| *Micro-l*-diltiazem hydrochloride gel | Diltiazem hydrochloride-entrapped in microsponge formulation (F 9)-loaded gel; |
| F9 loaded Mc-based *micro-l*-diltiazem hydrochloride | Diltiazem hydrochloride-entrapped in microsponge formulation (F 9)-loaded gel prepared with Methylcellulose (Mc); |
| F9 loaded Px-based *micro-l*-diltiazem hydrochloride | Diltiazem hydrochloride-entrapped in microsponge formulation (F 9)-loaded gel prepared with Poloxamer 407 (Px). |

## 1. Introduction

Chronic anal fissure is recognized as a common and painful proctologic disease which can bring about even serious impairment of quality of life (Altomare et al., 2011). This pathological condition is very probably related to hypertonia at the internal anal sphincter (IAS) and, therefore, the objective of chronic anal fissures clinical treatment is focused to reduce such IAS pressure (Altomare et al., 2011; Canelles et al., 2015). Although lateral internal sphincterotomy still remains the gold standard of treatment for the disease, it is associated to potential complications including fecal incontinence (Motie and Hashemi, 2016; Vaithianathan and Panneerselvam, 2015). Among the various medical therapeutic alternative approaches, the use of calcium channel blockers, as diltiazem hydrochloride (Figure 1S) or nifedipine topically applied, is considered effective enough even though the patients often show a high risk for recurrence after the end of therapy (Altomare et al., 2011; Canelles et al., 2015). However, a comparative study of medical *versus* surgical approaches clearly showed that, despite good response to medical treatment, surgical procedure is more effective (Motie and Hashemi, 2016). It follows that the medical approach, particularly that with diltiazem hydrochloride could be of choice in patients who are not willing to undergo surgery. On the other hand, according to the Guidelines from the American Society of Colon and Rectal Surgeons, medical approaches of anal fissures are recommended as first line therapy, specifically with pharmacological agents as calcium channel blockers topically formulated (Sahebally et al., 2017; Brady et al., 2017). Moreover, there are studies suggesting that the use of diltiazem hydrochloride at the concentration of 2% for treatment of chronic anal fissures involves headache, anal pruritus and postural hypotension as frequent side effects (Canelles et al., 2015; Hashmi and Siddiqui, 2009). These adverse effects lead to patient non-compliance and it is further heightened from the needing of frequent administrations (2-3 times per day) of the calcium channel blocker drug, likewise due to diltiazem hydrochloride short half-life (3.2±1.3 h) and its high water solubility (Stepanovs et al., 2016). In an attempt to increase patient compliance and improve the results of diltiazem hydrochloride based medical approach, we hypothesized that a modified delivery system able to release the calcium channel blocker in a sustained manner may be helpful to reduce both side effects and administration frequency. We reasoned, indeed, that a drug sustained delivery system may be useful both to avoid the achievements of drug levels corresponding to adverse effects and to reduce the administration number.

To accomplish a sustained delivery system of diltiazem hydrochloride, there are several options including the prodrug or conjugate approach as well as the use of micro- or nano-particulate systems. Among all, microencapsulation is recognized as a valuable strategy because several advantages can be attained including biocompatibility, stability, better compliance, and sustained release pattern that allows to reduce the toxicity and dosage frequency (Bale et al., 2016). Several types of microparticulate delivery systems have been to date developed such as microspheres, microcapsules and microbeads and their feasibility for diltiazem hydrochloride delivery has also been well documented in the literature (Al-Zoubi et al., 2016; Cetin et al., 2012). In this context, a notable step forward was reached in 1987 by Won who introduced the so-called microsponge delivery systems (MDS) to overcome some disadvantages exhibited by classic microspheres (Won, 1987). In particular, unlike classic microspheres, MDS are constituted by spherical cross linked spongy particles which possess a porous surface and have been used mostly for topical applications and even for oral administration (Srivastava and Pathak, 2012). Several preparative methods of microsponges

have been reported in literature among which the liquid-liquid suspension polymerization and the quasi emulsion solvent diffusion method are the most employed.

The aim of the present study was to evaluate the potential of gels containing diltiazem hydrochloride entrapped in microsponges intended for the treatment of chronic anal fissures. To the best of our knowledge, this approach based on the non-dihydropyridine calcium channel blocker diltiazem hydrochloride and on microsponge technology is unprecedented in the literature. For this purpose, we designed microporous diltiazem hydrochloride-loaded microsponges based on Eudragit RS 100 prepared by the quasi emulsion solvent diffusion method (Srivastava and Pathak, 2012; Kumari et al., 2016). Since this method requires poor water solubility of the drug to be loaded in particles (Srivastava and Pathak, 2012; Kumari et al., 2016), the following considerations were taken into account: diltiazem hydrochloride is commercially available as hydrochloride salt and belongs to Class I of Biopharmaceutical Classification System (BCS), possessing high solubility and high permeability (Stepanovs et al., 2016; Trapani et al., 2004). Instead, diltiazem free base belongs to Class II of BCS with limited water solubility and high permeability (Stepanovs et al., 2016; Trapani et al., 2004) and, therefore, a temporary conversion of the hydrochloride salt to free base may be used as an approach to incorporate this calcium channel blocker into modified-release microsponges *via* the aforementioned technique in a satisfactory way.

The quasi emulsion solvent diffusion method is technologically simple (Figure 1S), but the choice of formulation variables is crucial for the characteristics of the resulting particles. Among the different Eudragit polymers, Eudragit RS 100 was selected since it is well known that this copolymer leads to microsponges of smaller size and satisfactory release rate control (Devrim and Canefe, 2006). According to manufacturer information, Eudragit® RS 100 is a copolymer of ethyl acrylate, methyl methacrylate and a low content of methacrylic acid ester with quaternary ammonium groups (trimethylammonioethyl methacrylate chloride). To evaluate the effect of the drug:polymer weight ratio, the volume of the organic solvent [dichloromethane (DCM)] employed and the surfactant Poly(vinyl alcohol) (PVA) used on the production yield, drug entrapment efficiency and particle size, a $2^3$ full factorial screening design was carried out taking into account the relevance of such statistical approach in the development of microsponges as drug carriers (Crcarevska et al., 2015). The compatibility of diltiazem with formulation components was established by Fourier transform infra-red (FT-IR) spectroscopy and differential scanning calorimetry (DSC). Shape and surface morphology of the microsponges were examined using scanning electron microscopy (SEM). The *in vitro* drug release kinetics from selected microsponges were studied in phosphate buffer at pH 5.5 and pH 7.4. Finally, an optimized formulation was used to obtain diltiazem hydrochloride-loaded microsponges dispersed in two types of hydrogel bases containing Methylcellulose 2% or Poloxamer 407 20% as gelling agents. These gels were compared to conventional diltiazem hydrochloride 2% gel (*c*-diltiazem hydrochloride 2%) for *in vitro* drug release, *ex vivo* permeability and drug deposition after application on porcine rectal mucosa.

## 2. Materials and methods

### *2.1. Materials*

Diltiazem hydrochloride 99.9% was purchased from Puho Pharmaceuticals Co. Limited, China, Ammonio methacrylate copolymer (type B) (Eudragit RS 100) was a kind gift from Evonik Industries AG (Germany). Poloxamer 407 (Lutrol F-127) was a gift from BASF (Germany). Other reagents and materials were purchased as follows: Poly(vinyl alcohol, Mw 49.000 Da) – (Sigma Aldrich, USA); Methylcellulose high viscosity (Metolose 90SH – 30000) – Synthapharm Ges, F. - Germany; Dichloromethane (DCM, HPLC grade > 97.8%) and anhydrous Ethanol 99.5% were purchased from (Fisher Chemical, Germany); Disodium hydrogen phosphate dehydrate, Sodium chloride, Potassium dihydrogen phosphate and Sodium hydrogen carbonate were purchased from Thermo Fisher Scientific (USA); Sulfuric acid 96% was provided by Panreac, Spain; Dialysis membranes were provided from a local hospital (10 kDa molecular weight cutoff); Polyethersulfone (PES) 0.22 μm pore size syringe filter were provided by Filters Fioroni (Spain).

## *2.2. Methods*

### *2.2.1. Quantitative determination of diltiazem*

Calibration curves for the quantitative determination of diltiazem hydrochloride and diltiazem base were obtained in distilled water, in two buffer media (pH values equal to 5.5 and 7.4) as well as in anhydrous ethanol, by spectrophotometric analysis on UV/Vis spectrophotometer Rayleigh UV-9200 (China). Absorption maxima were found for all above-mentioned media in the wavelength range of 238 nm-244 nm (ethanol: 244 nm, distilled water: 240 nm, buffer pH 5.5: 240 nm, buffer pH 7.4: 238 nm). The curves were linear in the concentration range of 1.5 – 45.0 μg/mL ($R^2 > 0.999$).

### *2.2.2. Diltiazem hydrochloride conversion from hydrochloride salt to base*

Diltiazem hydrochloride was converted to the corresponding free base by treatment with $NaHCO_3$ as previously described (Ivanova et al., 2017). Briefly, 10 g of diltiazem hydrochloride were dissolved in distilled water (100 mL) under magnetic stirring. To this solution, $NaHCO_3$ was slowly added in excess to the stoichiometrically calculated amount (1.85 g) and precipitation of the water insoluble diltiazem base occurred. The resulting slurry was stirred for further 15 min and then diltiazem base was isolated through filtration and washed with distilled water.

The structural assignment of diltiazem base was confirmed by: *i)* Gas Chromatography-Mass spectroscopy (GC-MS, Hewlett-Packard 5995c GC-MS low-resolution spectrometer); *ii)* comparison of the melting point with the one from an authentic sample (106.6 °C), being the melting point of diltiazem hydrochloride of 213.1 °C (https://www.drugbank.ca); *iii)* comparison of FT-IR functional groups with the ones from an authentic sample. Diltiazem base was kept in desiccator for at least 24 h until further use.

### *2.2.3. Solubility studies*

For diltiazem hydrochloride and free base, water solubility tests were carried out at 37 °C. Test compound was added to 10 mL distilled water or PBS pH7.4 under continuous stirring. The mixture was kept under stirring for 24 h. Undissolved fractions were detected at the end of the test for both diltiazem forms. Samples from the supernatant were withdrawn, filtrated and analyzed spectrophotometrically for diltiazem content as reported in *Section 2.2.1.*

### 2.3. Preparation of diltiazem-loaded microparticles with microsponge-like morphology

The microsponges containing diltiazem were prepared by the quasi emulsion solvent diffusion method (Ivanova et al., 2017). Formulations were prepared with variable drug:polymer ratios, organic phase volume (dichloromethane, DCM) and surfactant concentrations (PVA). Briefly, PVA was dissolved in 100 mL distilled water at 80 $^{o}$C to stabilize the external aqueous phase. Diltiazem base and polymer Eudragit RS100 were dissolved in DCM and added dropwise to the cooled external phase while kept under continuous stirring at 650 rpm. The mixture was stirred for 3 h in a laboratory hood and then attached to a vacuum system for another hour to eliminate residues of DCM. Microsponges were isolated by centrifugation (5 min at 4000 rpm), following filtration through stainless steel wire cloth, 400 mesh, and multiple washing with distilled water. Drug unloaded microsponges were also prepared and taken as control. Each formulation was repeated-three times and all samples were dried in desiccator at room temperature for 24 h. Characterization and analysis of particles was done at this stage. In the case of microsponges from the optimized formulation F 9, the entrapped diltiazem base was converted back to the corresponding hydrochloride salt using the HCl generating chamber (Figure 2S) according to the procedure reported in Supplementary Material. Once this conversion was made, these diltiazem hydrochloride loaded microsponges were dispersed in Methylcellulose 2% or Poloxamer 407 20% hydrogel bases for the studies on porcine rectal mucosa (see below).

#### 2.3.1. Production yield

After 24 h stay in desiccator, each sample of microsponges was accurately weighted. Production yield (P.Y.) was presented as percent, using Equation 1:

$$\text{P.Y.\%} = \frac{\text{Actual weight of microsponges}}{\text{Theoretical total weight of microsponges (drug + polymer)}}.100 \qquad \text{Eq. 1}$$

#### 2.3.2. Drug entrapment efficiency

To determine diltiazem entrapment efficiency in microsponges, test solutions were prepared according to the following procedure. An appropriate amount of microsponges, theoretically containing 10 mg of polymer Eudragit RS 100, was accurately weighted, transferred to a 100 mL volumetric flask and dissolved in 5 mL of DCM. Further dilution with anhydrous ethanol up to the mark was performed and, then, 1 mL of stock solution was transferred to 10 mL volumetric flask and again diluted to up the mark with anhydrous ethanol to obtain a final concentration of diltiazem between 3-30 mg/L.

Reference solution was prepared as follows: 10 mg of Eudragit RS 100 were accurately weighed, transferred to a 100 mL volumetric flask, dissolved in 5 mL of DCM and further diluted up to the mark with anhydrous ethanol. One mL of stock solution was transferred to 10 mL volumetric flask and diluted up to the mark with anhydrous ethanol.

Diltiazem concentration was measured by UV spectrophotometry at $\lambda$ = 244 nm of test solution against reference solution, using calibration curve of diltiazem in anhydrous ethanol.

Entrapment efficiency (E.E.) was presented as percent, using Equation 2:

$$\text{E.E.\%} = \frac{\text{Actual drug content in particles}}{\text{Theoritical drug content in particles}}.100 \qquad \text{Eq. 2}$$

Each measurement was carried out in triplicate.

*2.3.3. Experimental design*

Three formulation factors including drug:polymer w:w ratio ($X_1$), DCM volume ($X_2$) and PVA concentration ($X_3$) were evaluated for their effects on Production Yield (P.Y.%, $Y_1$), Drug Entrapment Efficiency (E.E.%, $Y_2$), and particle size (P.S., $Y_3$) of the prepared microparticles by a $2^3$ full factorial screening experimental design using Statgraphics Centurion XVI.II software (StatPoint Technologies, Inc., Warrenton, VA, USA). Table 1 shows the independent variables, their levels and studied responses. The dependent variables (*i.e.,* P.Y.% ($Y_1$), E.E.% ($Y_2$), and P.S. ($Y_3$)) were evaluated and polynomial equations were generated by applying one-way analysis of variance (ANOVA) ($p < 0.05$). To demonstrate graphically, the influence of each factor on responses, three-dimensional response surface plots were generated. Multiple regression analysis was used to assess the responses employing the following Equation 3:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \qquad \text{Eq. 3}$$

Eq. 3 shows the quantitative effects of the independent variables ($X_1$, $X_2$ and $X_3$) and their interaction ($X_1X_2$, $X_1X_3$ and $X_2X_3$) on the selected dependent variable (Y); $b_0$ is the intercept and $b_1$, $b_2$ and $b_3$ represent the regression coefficients for the polynomial equation. Significance was set at $p < 0.05$ in the model for the effects of all factors. A factor is considered to significantly influence a selected response if the $p$ value differs from 0 and is < 0.05. To graphically show the independent variables which significantly affect a dependent variable, standardized Pareto charts were used. In this charts a vertical reference line at $p$ value of 0.05 is showed for which an effect exceeding this line is statistically significant. Moreover, to graphically show the effect of changing two independent variables on the selected variable Y when the third one is kept at an intermediate value, 3D response surface plots were built.

[Insert Table 1]

*2.4.Characterization of microparticles*

*2.4.1. Thermal analysis by Differential Scanning Calorimetry (DSC)*

DSC studies were carried out with a Mettler Toledo DSC 822e STARe 202 System equipped with a DSC Mettler STARe Software. About 5 mg of solid sample were placed in an aluminium pan and hermetically sealed. The scanning rate was of 5 °C/min under a nitrogen flow of 20 mL/min, and the temperature range was from 25 to 250 °C. The calorimetric system was calibrated by using indium (purity 99.9%) and following the procedure of the Mettler STARe Software. Each experiment was carried out in triplicate.

*2.4.2. FT-IR spectroscopy*

FT-IR spectroscopic analyses were performed using a Perkin-Elmer 1600 FT-IR spectrometer (Perkin-Elmer, Milan, Italy) on samples dispersed in KBr and compressed in pellets by hydraulic press. The range examined was 4000–400 $cm^{-1}$ with a resolution of 1 $cm^{-1}$.

*2.4.3. Scanning electron microscopy (SEM)*

The size, morphology and surface characteristics of the microparticles were evaluated by SEM analysis. All the samples were stuck on double-face carbon adhesive stubs and coated with a 10 nm palladium. The morphological reproducibility was assessed by means of replicate analyses for each sample. The electron microscope images were taken by a Sigma Zeiss FE-SEM at 5-10 kV, 30 μm aperture. The statistical analysis of the microsponges was made by ImageJ Software.

*2.5. In vitro drug release study from diltiazem containing microsponges*

Diltiazem containing microsponges were subjected to *in vitro* drug release test as described in European Pharmacopoeia Dissolution Apparatus 2 (Paddle apparatus), using 7-station Compact Dissolution Tester PT-DT 70 (Pharma test, Germany). An accurately weighed amount of microparticles equivalent to 5 mg of diltiazem was placed in a dialysis bag and dispersed in 1 mL of dissolution medium constituted by PBS pH 7.4 at 37 $^{o}$C and rotation speed of 30 rpm. The dialysis bag was then attached to the paddle and immersed into 400-mL dissolution medium. *In vitro* drug release behavior was also investigated in phosphate buffer at pH 5.5, temperature of 37 $^{o}$C and rotation speed of 30 rpm. Samples from dissolution medium were withdrawn at scheduled times and diltiazem released was spectrophotometrically determined. Each experiment was carried out in triplicate.

*2.6. Preparation of microsponge-loaded and conventional diltiazem hydrochloride 2% gels*

For these studies microsponges from the optimized formulation F 9, after the treatment with the HCl generating chamber (Supplementary Material) were used.

Two common gel bases were used – Methylcellulose high viscosity 2% (Mc) and Poloxamer 407 20% (Px). Methylcellulose was added to hot water (90$^{o}$C) under continuous stirring to obtain homogeneous dispersion and then left overnight to dissolve in refrigerator at 5$^{o}$C. Poloxomer 407 was added to the water for gelation at room temperature and left overnight to dissolve in refrigerator at 5$^{o}$C.

Diltiazem hydrochloride-entrapped in microsponge formulation (F 9)-loaded 2% gels (*micro-l-diltiazem hydrochloride* 2% gels) were prepared as follows. Microsponges were sieved through pore size stainless steel sieve (d=250 μm) and an appropriate amount of microsponges, corresponding to 0.2 g diltiazem hydrochloride, was added to each of the gel bases (up to 10 g) and homogenized. In such a way, both F9 loaded Mc-based *micro-l*-diltiazem hydrochloride 2% gel and F9 loaded Px-based *micro-l*-diltiazem hydrochloride were prepared using Methylcellulose (Mc) and Poloxamer 407 (Px) gel bases, respectively.

Conventional diltiazem hydrochloride 2% (*c*-diltiazem hydrochloride 2%) gels were prepared as follows. At first, diltiazem hydrochloride (0.2 g) was dissolved in a sufficient amount of water for gelation. The remaining amount of water for gelation was used for the preparation of each of the gel bases. Diltiazem hydrochloride solution was added in small aliquots via slow manual stirring to the corresponding gel base to obtain 10 g gel. Thus, both Mc-based *c*-diltiazem hydrochloride and Px-based *c*-diltiazem hydrochloride were prepared using Methylcellulose (Mc) and Poloxamer 407 (Px) gel bases, respectively.

### 2.7. *In vitro drug release from gels*

Drug release studies from the above mentioned gels were carried out on Franz diffusion cell trough cellulose acetate membrane. The cell possessed exposed membrane surface area of 0.98 $cm^2$ and receptor volume of 8 mL. Receptor volume was filled almost to the top with PBS pH 7.4 and the cell was set at 37±0.5°C with the aid of circulating thermostatic bath. About 400 mg of each test formulation (corresponding to 8 mg diltiazem hydrochloride) were carefully applied on the surface of the cellulose membrane through the open cell cap, adhesion to the membrane was ensured with the aid of a tiny piston, after which the open cell cap was covered with Parafilm® to prevent evaporation from the dosage form and release medium during the test. Receptor volume was adjusted to the top and stirring was set at 300 rpm. Sufficient volumes (from 0.1 to 0.5 mL) of release medium were withdrawn at selected time-point, analyzed spectrophotometrically for drug content and replaced with fresh buffer medium. Data for cumulative drug release were obtained for the period of 12 h for *c*-diltiazem hydrochloride 2% and 24 h for *micro-l*-diltiazem hydrochloride 2%.

### *2.8. Drug permeation through porcine rectal mucosa of diltiazem hydrochloride from rectal gels*

#### *2.8.1. Mucosal tissue preparation*

A fresh specimen of a pig rectum received from a local slaughterhouse of 10 cm in length was harvested, cut longitudinally to open the lumen and observed for any abnormalities, thoroughly washed with PBS pH 7.4 and frozen at -80 C°. Following a 24 h freezing time at -80C°, the rectum was left to defrost at room temperature and placed in cooled PBS pH 7.4 (15 C°).

The specimen was manually dissected under a stereomicroscope with a magnification of 15 x with cold steel instruments. The instruments used were a sharp tipped dissection forceps, for grasping the *lamina propria*, *tela submuscosa*, *tela muscularis* and *the adventitia* and a De Bakey forceps for grasping and non-traumatic mechanical peeling of the mucosa. After the dissection, the separated mucosa was further observed under the same magnification of the stereomicroscope for removal of any attached blood vessels left and remaining non-mucosal tissue. The separated mucosa was measured to be 0.080±0.005 cm thick. After being thoroughly washed in PBS pH 7.4, the mucosal tissue was cut in sections measuring 2 x 2 cm with a cold steel scalpel. The prepared sections were placed in cooled PBS pH 7.4 (Amores et al., 2014).

#### *2.8.2. Procedure*

*Ex vivo* permeation study was performed on Franz diffusion cell following the same procedure as described above (Section 2.7), using mucosa, isolated from pig rectum, as membrane. Mucosa was placed so that the smooth mucosal surface acts as the donor compartment. The percentage of cumulative drug permeated was acquired at 2, 4, 8, 12 and 24 h of the experiment.

#### *2.8.3. Data analysis*

To evaluate the results from *ex vivo* permeation studies, the linear region of each permeation profile (representing the steady state diffusion) was used by applying the Fick's first law for steady state

diffusion ($J_{ss}$= $K_p$·ΔC, where $J_{ss}$ is the steady state flux, $K_p$ is the permeability coefficient and ΔC is the concentration gradient representing the difference between the drug concentration in the donor compartment ($C_d$) and that in the receptor compartment ($C_r$). Permeation parameters were obtained from the cumulative drug permeated versus time plots. The steady state flux ($J_{ss}$) was calculated from the slope of the linear tract of permeation curve and presented as mg·cm$^{-2}$ h$^{-1}$. The permeability coefficient ($K_p$) was calculated as $K_p = J_{ss}/ \Delta C$, assuming that the concentration gradient ($\Delta C = C_d - C_r$) is constant and thus ΔC can be replaced with initial drug concentration in the dosage form Cd) [mg.cm$^{-3}$] ($\Delta C \approx Cd$, and $K_p \approx J_{ss}/C_d$). The lag time ($T_L$) represents the time necessary to establish steady state diffusion after the initial contact of the dosage form with the mucosal tissue. TL was calculated for y = 0 using the linear regression of the flux (Brodin, et al., 2009; Khan, et al., 2012; Herkenne, et al., 2007). These experiments were carried out in duplicates.

### 2.9. *Permeation through porcine rectal mucosa or dialysis membrane of diltiazem hydrochloride from diltiazem hydrochloride 2% aqueous solutions*

To estimate the impact of the membrane on drug permeation, *in vitro* permeation study was performed on diltiazem hydrochloride 2% aqueous solution through porcine rectal mucosa or dialysis membrane using a Franz diffusion cell. A stock solution of diltiazem hydrochloride was prepared at concentration 2% in distilled water corresponding to the concentration used in pharmaceutical gels. Samples from the release media (PBS 7.4, volume – 8 mL) were withdrawn and analyzed spectrophotometrically at scheduled times. Cumulative graphs, describing the permeation profile of diltiazem hydrochloride through both types of membrane were built.

### *2.10. Drug deposition of diltiazem hydrochloride after application on porcine rectal mucosa*

Deposition of diltiazem hydrochloride on the mucosal tissue 24 h after application was determined by two methods:

Method I: At the end of the drug permeation assay, mucosal tissue was carefully detached from the receptor compartment's surface, washed thoroughly with PBS pH 7.4, poured in absolute ethanol (10 mL) and shattered in small pieces with the aid of scissors. The so prepared mucosa was kept in ethanol at room temperature, protected from the light, for 24 h. Ethanol was then decanted in 25–mL volumetric flask and replaced with fresh portion solvent (4-5 mL) every 2 h. This procedure was repeated several times until final volume of 25 mL was obtained in the volumetric flask. The ethanol extract was filtered through 0.22 µm pore size PES syringe filter and analyzed spectrophotometrically for diltiazem hydrochloride content.

Method II: At the end of the drug permeation study, all cell parts having had contact with the test dosage form, including the mucosal tissue, were thoroughly washed with absolute ethanol in a 250 mL beaker and then removed from the glass. The mixture was well homogenized as remaining gel parts were visible and then transferred to 25 mL volumetric flask. Volume was adjusted up to the mark with absolute ethanol, filtered as above and analyzed spectrophotometrically for drug remained unreleased. Drug deposition in mucosa was calculated as (Equation 4):

$$Deposition\ in\ mucosa = total\ amount\ of\ drug\ applied\ - drug\ unreleased - drug\ released\ in\ receptor\ compartment \quad \text{Eq. 4}$$

For both methods drug deposition was presented as $mg \cdot cm^{-3}$, considering the mucosal tissue volume of 0.078 $cm^3$ (exposed area 0.98 $cm^2$ x thickness 0.08 cm = 0.078 $cm^3$ volume). These experiments were carried out in duplicates.

### *2.11. Statistical analysis*

The results are shown as means ±standard deviation (SD). Data were statistically analyzed by one-way analysis of variance (ANOVA) and the Bonferroni's "post hoc" test for multiple comparison using GraphPad Prism v.4 software. Differences were considered statistically significant when $p<0.05$.

## 3. Results and Discussion

The aim of the present work was to evaluate gels containing diltiazem hydrochloride-loaded microsponges as a modified delivery system of this calcium channel blocker in an attempt to overcome some drawbacks showed by the use of conventional topical diltiazem hydrochloride formulations at the concentration of 2%. for the treatment of chronic anal fissures. Such microsponges were based on Eudragit RS 100 and prepared by the quasi emulsion solvent diffusion method (Srivastava and Pathak, 2012; Kumari et al., 2016) which involves formation of an emulsion whose internal phase is constituted by a drug/polymer solution in volatile organic solvent (DCM) and the external one by an aqueous solution of surfactant (PVA) (Figure 1S). Moreover, since several factors may influence the results in the development of microsponges as drug carriers (Crcarevska et al., 2015), the most satisfactory combination of factor levels has been investigated using a $2^3$ full factorial screening design. It must be noted that, in the search of an optimized formulation, in any case we used diltiazem base applying the quasi emulsion solvent diffusion method to prepare the Eudragit RS 100 based microsponges. In fact, being diltiazem hydrochloride freely water soluble (453 mg/mL in distilled water 37°C or 58.23 mg/mL in PBS pH 7.4 37°C), a considerable diffusion in water of the salt should take place and, consequently, a limited encapsulation efficiency should result, a limitation that cannot be presented by the poorly water soluble diltiazem base.

### *3.1. Factorial design*

Three formulation parameters (*i.e.,* Drug:polymer weight:weight ratio ($X_1$), DCM volume ($X_2$) and PVA conc ($X_3$)) were evaluated for assessing their effects on Production Yield ($Y_1$), Entrapment Efficiency ($Y_2$) and Particle size ($Y_3$) using a $2^3$ full factorial screening experimental design where $X_1$-$X_3$ constitute the independent variables and $Y_1$-$Y_3$ the dependent ones. The goals to be achieved are maximization of $Y_1$ and $Y_2$ and minimization of $Y_3$. From the design polynomial equations were generated which relate the independent variables with each of the dependent ones. The data obtained were statistically examined to gain information on the optimum values of the independent variables to accomplish the goals of the study. Table 2 shows the results of regression analysis for $Y_1$-$Y_3$ and the full model polynomial equations are presented in Eq 5-7, respectively.

$Y_1$ (P.Y. %) = 70.41 – 2.43 $X_1$+ 0.79 $X_2$ – 39.05 $X_3$ – 0.12 $X_1$ $X_2$ + 4.18 $X_1$ $X_3$ – 0.06 $X_2$ $X_3$ Eq. 5

$Y_2$ (E.E.%) = 79.09 + 3.58 $X_1$ – 0.16 $X_2$ + 41.82 $X_3$ + 0.017 $X_1$ $X_2$ – 15.66 $X_1$ $X_3$ – 0.41 $X_2$ $X_3$ Eq. 6

$Y_3$ (P.S.) = -14.67 – 0.82 $X_1$+ 23.36 $X_2$ – 153.86 $X_3$ - 6.82 $X_1$ $X_2$ + 125.46 $X_1$ $X_3$ – 20.63 $X_2$ $X_3$ Eq. 7

[Insert Table 2]

The goodness of fit is substantiated by the correlation coefficient $R^2$ values and even more by the adjusted $R^2$ (Adj $R^2$) values which, if are near to 1, indicate an optimal-excellent correlation between the independent variables and the selected response. As reported in Table 2, the $R^2$ and Adj $R^2$ values for P.Y. and E.E. are greater than 0.99 and it means that the corresponding models are highly significant and explain more than the 99% of variation around the mean value. In contrast, the $R^2$ and Adj $R^2$ values for P.S. are lower than 0.99 suggesting that the significance of the corresponding model is worse compared to the previous ones. The coefficients of the equations evaluate the effect of a particular independent variable from a quantitative point of view and the statistical significance of the coefficients is assessed by the *p*-value (Table 2) or graphically by the Pareto charts and response surface plots.

*3.1.1. Effect of independent variables on production yield (P.Y.)*

P.Y.% of the particles studied in the factorial design varied in the range from 58.30% to 76.80%. The effect of independent variables on P.Y. is shown by the polynomial Eq. 5 and data reported in Table 2 as well as graphically by Figure 1. Based on these findings, it is evident that decrease of drug:polymer ratio or PVA concentration led to a significant increase in P.Y.% of particles prepared using the quasi emulsion solvent diffusion method (Srivastava and Pathak, 2012; Kumari et al., 2016). In contrast, an increase of DCM volume brought about a less significant increase in P.Y.%. All these effects of independent variables on P.Y.% have been already described in literature and possible rationalizations have been presented (Srivastava and Pathak, 2012; Jain and Singh, 2010). Further information which can be deduced from the reported findings is that the interaction terms *(i.e.,* the effect of two factors simultaneously) did not have significant influence and it has been not previously described in the literature.

[Insert Figure 1]

*3.1.2. Effect of independent variables on entrapment efficiency (E.E.)*

E.E.% of microsponges ranged from 79.63% to 88.95% and again the effect of independent variables on E.E.% can be deduced from the polynomial equation (*i.e.*, Eq. 6) data reported in Table 2 and from Figure 2. All the results clearly show that decrease of drug:polymer ratio or PVA concentration caused a significant increase in E.E.% and once again possible explanations have been reported (Srivastava and Pathak, 2012; Jain and Singh, 2010). However, the most significant effect on E.E.% was shown by the interaction between drug:polymer ratio and PVA concentration ($X_1X_3$) and, more specifically, decrease of this interaction term brought about a significant increase in E.E.%. To the

best of our knowledge, the effect of this interaction term has been not previously shown in the literature.

[Insert Figure 2]

*3.1.3. Effect of independent variables on particle size (P.S.)*

As above mentioned, no independent variables or their interactions had significant effect on particle size ($p > 0.05$) of the microsponges prepared. This insignificant effect of the independent variables chosen, in our opinion should be due to the wide standard deviation observed in the size measure of the particles studied in the factorial design by SEM analysis (Table 1). However, it can be useful to evaluate from a qualitative point of view the corresponding polynomial equation (*i.e.*, Eq. 7), data reported in Table 2 and Figure 3S. Focusing our attention to the main independent variables of the model, it can be deduced that increasing the drug:polymer ratio results in small particles as reported in literature (Cetin et al., 2012). A similar effect should be played by the interaction between drug:polymer ratio and DCM volume ($X_1X_2$).

By using the "response optimization" option of the Statgraphics Centurion XVI.II software, it was calculated the optimum desirability in order to identify an optimized formulation which accomplishes an optimum combination of factor levels. It was found that a total optimum desirability value of 72.66% corresponds to the following selection of factor levels: drug:polymer ratio $X_1 = 3$, DCM volume $X_2 = 12$; PVA concentration $X_3 = 0.05$. Using this combination of factor levels, it results a predicted P.Y. % of 66.83 (confidence interval 60.94% - 72.71%) and a predicted E.E.% of 87.98% (confidence interval 84.91% - 91.05%). However, we noted that, in general, using high drug/polymer mass ratio $X_1 \geq 3$ the work-up and isolation procedure resulted somewhat difficult and substantial losses of final product were observed. Therefore, further experimental runs were carried out in order to find possible additional experimental conditions with good optimum desirability value and the amount of each component used in these supplementary formulations together with the corresponding responses are reported in detail in Table 1. As seen, spherical microsponges were obtained in high yield (63.10% - 86.25 %) and high entrapment efficiency (79.63% - 91.50%) as well as with mean diameters ranging from 33 μm to 239 μm. In particular, using a drug:polymer ratio of 0.33, a DCM volume of 8 mL and a PVA concentration of 0.15 % w/v, spherical microsponges endowed with P.Y. % of 82.90% and E.E.% greater than 90% were obtained (F 9, Table 1) and such experimental conditions seem a good combination of factor levels for a satisfactory optimized formulation. Under such circumstances spherical microsponges were obtained while in other cases, irregular spherical microsponges or even rods were observed (Table 1).

*3.2. Physicochemical characterization of microsponges*

The DSC thermogram of diltiazem base showed a sharp endothermic peak at 106°C which corresponds to the melting of the drug in crystalline form, while the thermogram of the polymer alone displayed a less intense sharp endothermic peak at 198°C (Figure 3). Blank microsponges showed the endothermic peak attributable to the polymer, although at lower temperature (*i.e.*, at 173°C) while, in the case of F 9, the endothermic peak of the polymer occurs at very low intensity and at intermediate temperature (*i.e.*, at 182°C) without the characteristic endothermic peak due to the drug melting. This

finding may suggest a change in the drug crystallinity in the Eudragit RS 100 matrix of the optimized formulation involving the presence of the drug in an amorphous state or disordered crystalline phase (Ahmed and Aljaed, 2017; Sun et al., 2015).

The FT-IR spectrum of pure diltiazem base showed a characteristic peak of the acetate stretching at 1743 $cm^{-1}$ as well as the C=O stretching of the amide group at 1680 $cm^{-1}$(Figure 3). The spectrum of blank microsponges displayed a large peak centered at 1732 $cm^{-1}$ due to the C=O stretching of the carbonyl groups of the Eudragit RS 100 matrix, while in the F 9 the characteristic drug stretching of the amide group at 1680 $cm^{-1}$ can be detected besides to the large peak centered at 1732 $cm^{-1}$ of the polymeric matrix. Such result should suggest that no interaction between drug and polymer occurs and, hence, they should be compatible in the formulations studied.

.

[Insert Figure 3]

The particle size of the microspheres was determined by SEM analysis and it resulted below enough 250 μm. Furthermore, SEM analysis allowed to evaluate the surface and the whole morphology of the particles (Figure 4 and Figure 4S) which resulted porous enough and predominantly spherical, although in some cases irregular spherical microsponges or even rods were obtained. It has been proposed that the pores are brought about by diffusion of solvent from surface of microsponges during their preparative procedure (Srivastava and Pathak, 2012). Morever, using the Energy Dispersive X-ray Analysis (EDX) option of the SEM-EDX microscope, it was found that the particles were constituted by a very low amount of chlorine atom on the surface (Figure 4) suggesting so that DCM may be essentially removed by the experimental work-up followed by us. Therefore, it can be concluded that the quasi-emulsion solvent method we used allows the preparation of microsponges without residual traces of organic solvents and, consequently, without concerns from a toxicological point of view. Moreover, SEM analysis allowed us to gain also information on the surface chemical nature of the microparticles since elemental analysis of the surface components can be deduced. Appreciable sulfur content is indicative of surface location. The sulfur content of the unloaded microsponges reflected the amount of this element as impurity of the polymer used.
At this regard, EDX analysis showed that the diltiazem hydrochloride-loaded microparticles were constituted by an amount of sulfur atom on the surface greater than that found for blank microsponges (*i.e.*, 0.21±0.07 weight percent) and it suggests that diltiazem hydrochloride may be in part present on the surface of the drug-loaded particles. Thus, for instance, in the case of F 9, it was found a surface sulfur content of 1.68±0.99 weight percent, different enough from that occurring for blank microsponges (Figure 4).

[Insert Figure 4]

*3.3. In vitro drug release studies on microsponges from Formulation 9*

To evaluate the potential of the studied MDS for sustained delivery of diltiazem, the release kinetic of selected formulations was evaluated in PBS at pH 7.4 and pH 5.5. It should be noted that the colonic pH has been reported to range by values fluctuating from pH 5 to pH 8 depending on the transit times and composition of food intake (Koziolek et al., 2014; Marques et al., 2011). Of course,

such features are highly important for the understanding of drug release from dosage forms containing ionizable drugs as diltiazem hydrochloride. The selected pH values employed in drug release studies in this paper can be considered near to the two above mentioned limits of the pH range accepted for colon (*i.e*., pH 7.4 and pH 5.5) and the obtained results for the representative Formulation 9 (F 9) are shown in Figure 5.

[Insert Figure 5]

Results showed that after 6 h at pH 5.5 about 72% of diltiazem was released from F 9. Instead, the same formulation at pH 7.4 only after 18 h released about 61% of the calcium channel blocker drug, demonstrating that under the latter conditions a sustained release of the occurs.

*3.4. Ex vivo permeability experiments through porcine rectal mucosa*

As above mentioned, prior to proceed with release studies from hydrogels as well as with those of *ex vivo* permeability through and drug deposition on porcine mucosa, the entrapped diltiazem base of F 9 was converted to the corresponding diltiazem hydrochloride by using the HCl generating chamber (Supplementary Material). This treatment on microsponges from F 9 was made before their dispersion in hydrogel bases and it was necessary to increase the therapeutic potential of diltiazem base which should be limited due to the poor water solubility of this compound (0.202 mg/mL in PBS pH 7.4, 37°C). Such conversion method is unprecedented and non-destructive and it can be envisaged as an approach to incorporate water soluble substances as hydrochloride salts in microsponges through the quasi emulsion solvent diffusion method.

The two gel bases were used to prepare the *micro-l*-diltiazem hydrochloride at 2% and they are well-known for their peculiar features as the temperature-sensitive properties of Poloxamer 407 and the mucoadhesive ones of Methylcellulose (Singh et al., 2009).

As shown in Figure 6 A, the drug release from conventional *c*-diltiazem hydrochloride 2% gels resulted practically constant after 6-12 h, whereas *micro-l*-diltiazem hydrochloride 2% gels exhibited a prolonged release up to 24 h. In addition, it was established that the use of microsponges in diltiazem hydrochloride hydrogels reduces the initial “burst effect”. Using methylcellulose as gelling agent, for both *c*-diltiazem hydrochloride 2% and *micro-l*-diltiazem hydrochloride 2% gels, a faster and more complete diltiazem hydrochloride release compared to Poloxamer 407 occurred. It seems that these results can be probably related to different viscosity between the two gel types.

The permeability profiles through porcine rectal mucosal are reported in Figure 6 B and the corresponding permeation parameters are shown in Table 3. The highest permeation rate of diltiazem hydrochloride was observed from Mc-based *c*-diltiazem hydrochloride, whereas the lowest from F9 loaded Mc-based *micro-l*-diltiazem hydrochloride. A possible explanation accounting for the greater permeation rate of Methylcellulose gels compared to the Polaxamer 407 ones might be related to better mucoadhesive properties of the former polymer. An almost bimodal trend in the permeation profiles was observed for most formulations, in particular, changes in the permeation course occurred between 1-2 h for conventional gels and between 3-6 h for microsponge loaded gels. This phenomenon is likely observed in the time required for the permeation of diltiazem hydrochloride to establish an equilibrium between the dosage form and the mucosal tissue. As a consequence of these

bimodal trends, in the calculation of steady state diffusion flux ($J_{ss}$) values, low regression coefficients were obtained (Table 3). As can be seen in Table 3, the flux values observed for the conventional formulations were greater than those entrapping diltiazem hydrochloride in microsponges, as expected taking into account that the latter formulations constitute sustained delivery systems of the calcium channel blocker drug. Next, our attention was focused on the negative values of the lag time ($T_L$). From the plots in Figure 6 B it is evident that a substantial "delay" in the steady-state permeation establishment after application of the dosage form does not occur for none of the formulations tested. However, such negative values are also probably related to the aforementioned deviations from linearity observed in these permeation curves (Table 3).

An *in vitro* permeation study through rectal mucosa or synthetic membrane using diltiazem hydrochloride 2% solution, evidenced the marked impact of the former membrane in drug permeation. When artificial cellulose acetate membrane was used, indeed, equilibrium in drug concentration between the donor and receptor compartment of Franz cell was established within 2.0-2.5 h with a drug permeation of about 70% after 5h (Figure 6 C). In contrast, using rectal mucosa a slower transport resulted with a drug permeation of 39% after 24 h. This result should be likewise ascribed to the fact that equilibrium between three compartments (*i.e.,* donor- membrane- receptor-compartment) of rectal mucosa occurs much more slower than that through cellulose acetate membrane. Moreover, the solubility of diltiazem hydrochloride decreases substantially under the slightly alkaline physiological pH in the rectum (pH 7.4) due to a partial conversion to diltiazem base.

[Insert Figure 6]

The drug deposition on porcine rectal mucosa is an important indication of the local chronic anal fissures therapy efficacy. In this regard, we used two experimental procedures referred to as Method I and Method II, respectively (Section 2.9), both characterized by some limitations which should be taken into account. Thus, a shortcoming of Method I is that the complete drug extraction from the mucosa is not feasible and the interference on the assay caused by lipids extracted from the mucosa may occurs. On the other hand, a drawback of Method II is represented by a higher risk of error. However, the results from both Methods clearly showed that *micro-l*-diltiazem hydrochloride 2% gels provide a diltiazem hydrochloride concentration twofold higher than that displayed by conventional formulations (Table 3 and Figure 7). Similarly, the percentages of drug deposited on rectal mucosa resulted more than twofold higher than those of conventional formulations (Figure 7). Altogether, these results confirmed our expectations that a modified-release diltiazem hydrochloride semi-solid dosage form should be advantageous compared to the conventional one by lowering the release and permeation rate. *Micro-l*-diltiazem hydrochloride 2% gels showed 15.39% and 17.95% higher diltiazem hydrochloride retention on mucosa for Poloxamer 407 and Methycellulose gels, respectively.

[Insert Table 3 and Figure 7]

**Conclusions**

In this work, gels containing diltiazem hydrochloride-loaded microsponges were evaluated as an approach to overcome some drawbacks showed by the conventional topical diltiazem hydrochloride formulations for the treatment of chronic anal fissures. Such microsponges were based on Eudragit RS 100 and prepared by the quasi emulsion solvent diffusion method. The optimized microsponge formulation used for these studies was selected on the basis of a $2^3$ full factorial screening design. The *in vitro* drug release resulted faster for conventional formulations and essentially complete within 6-12 h, while the microsponges loaded gels showed a prolonged release up to 24 h. *Ex vivo* permeability studies demonstrated that the highest drug permeation rate was observed for the conventional diltiazem hydrochloride 2% (*c*-diltiazem hydrochloride 2%) formulations, whereas the lowest occurred from Poloxamer 407 20% diltiazem hydrochloride loaded microsponges 2% gel (F9 Px-diltiazem hydrochloride). From drug deposition after application on porcine rectal mucosa studies, it was observed that *micro-l*-diltiazem hydrochloride 2% gels provide a diltiazem hydrochloride concentration twofold higher than that displayed by conventional formulations. Overall, the results obtained lend support for the hypothesis that a sustained delivery system of diltiazem hydrochloride may be helpful to enhance patient compliance and improve the results of the treatment of chronic anal fissures using diltiazem hydrochloride based medical approach. In particular, the use of microsponge loaded formulations, characterized by a lower permeation rate combined with a higher deposition on mucosa compared to the conventional ones, may be considered as a promising approach to overcome the side effects and patient non-compliance showed by the conventional diltiazem hydrochloride formulations employed in the treatment of chronic anal fissures. In this context, the performance of the Poloxamer 407 20% microsponge loaded- diltiazem hydrochloride 2% gel (F9 Px-diltiazem hydrochloride) appears particularly interesting and its further development with *in vivo* studies is worthy to be carried out.

**Declarations of interest**

None.

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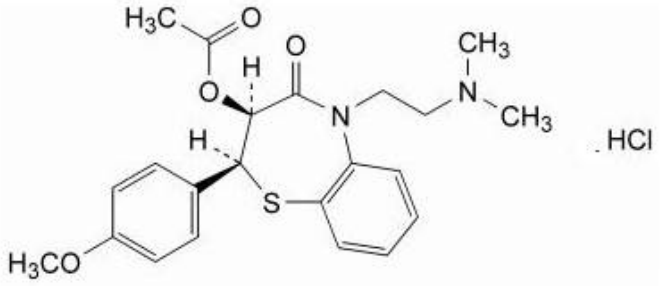


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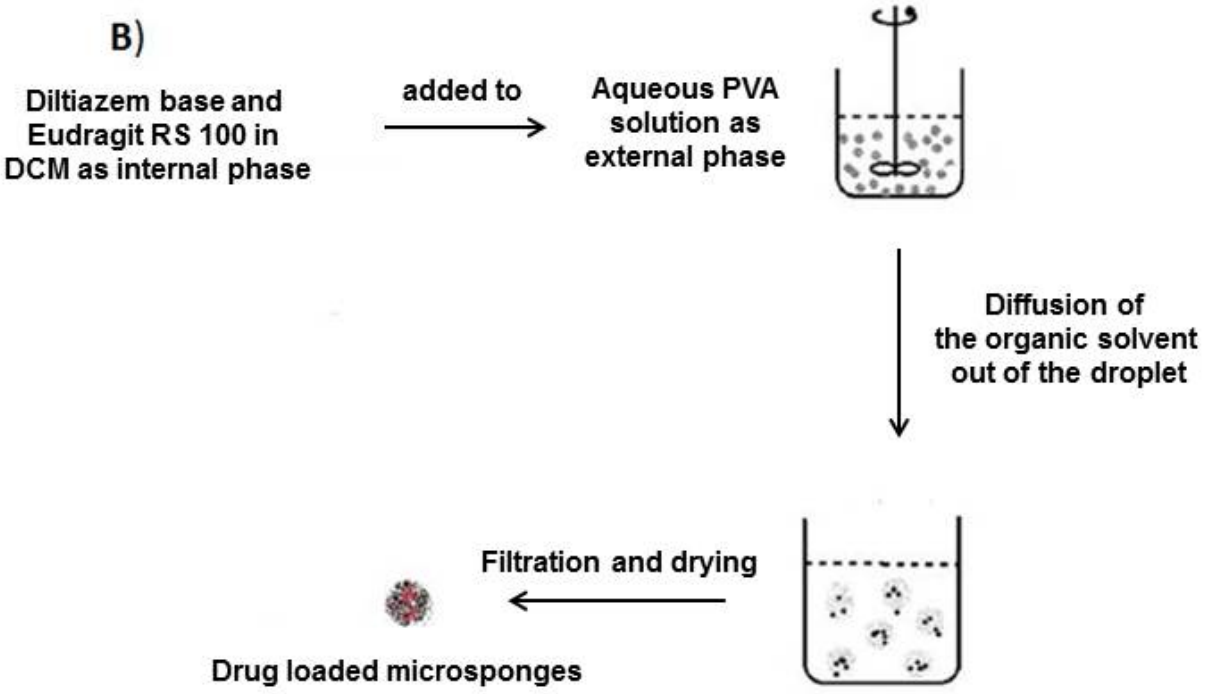

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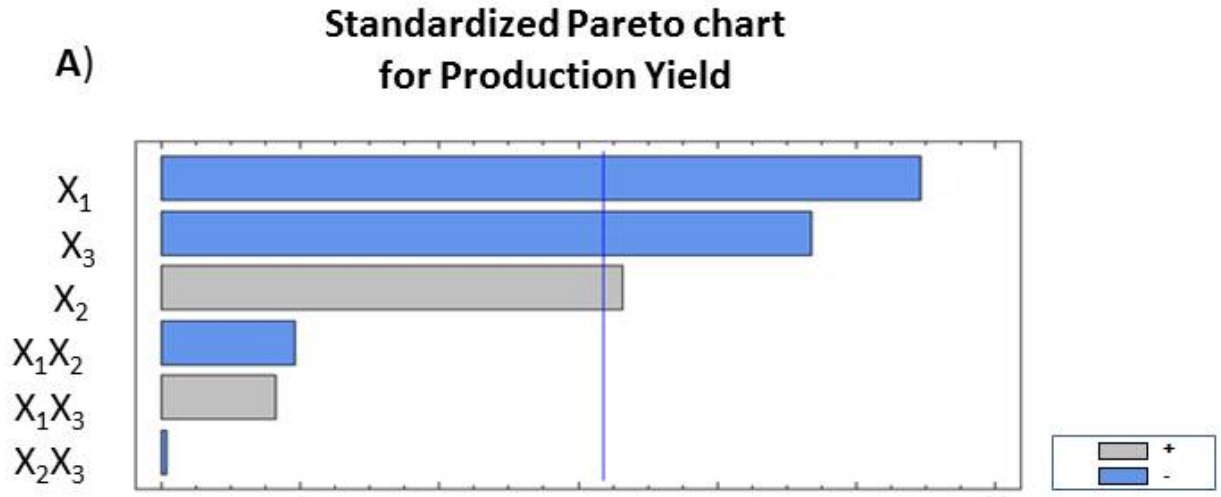
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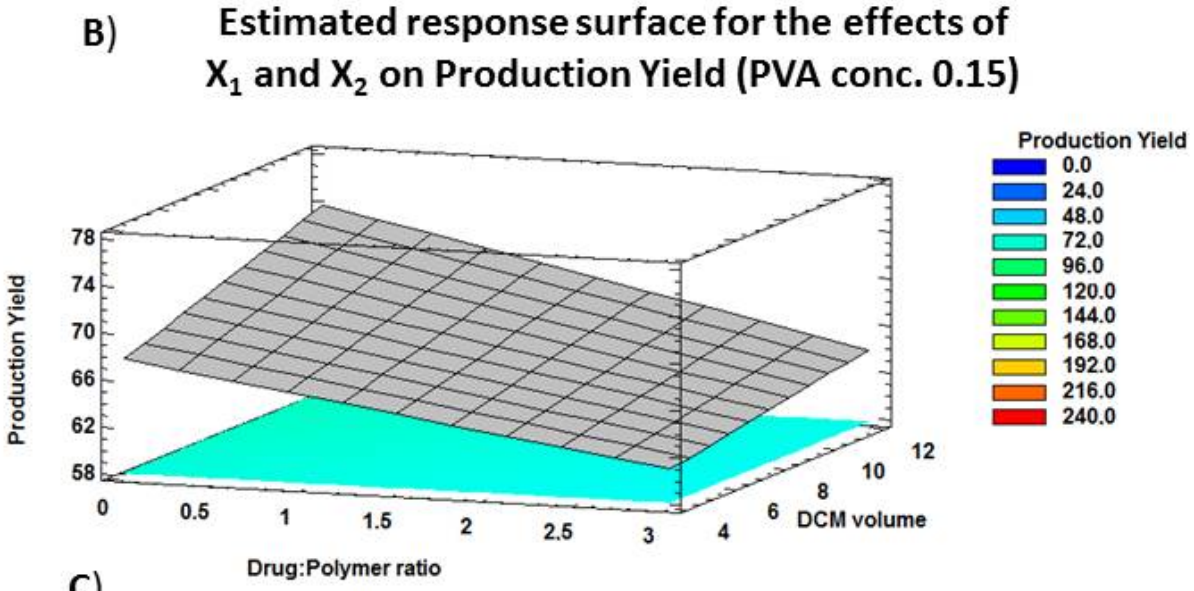
B)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X1 and X2 on Production Yield (PVA conc. 0.15)
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24.0
48.0
72.0
96.0
120.0
144.0
168.0
192.0
216.0
240.0
Production Yield
78
74
70
66
62
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Drug:Polymer ratio
4
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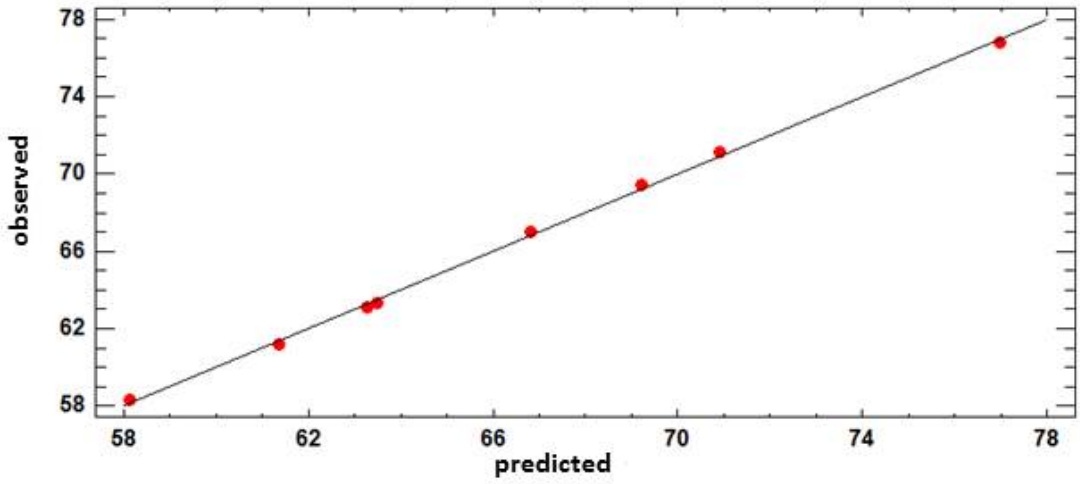
DCM volume

C)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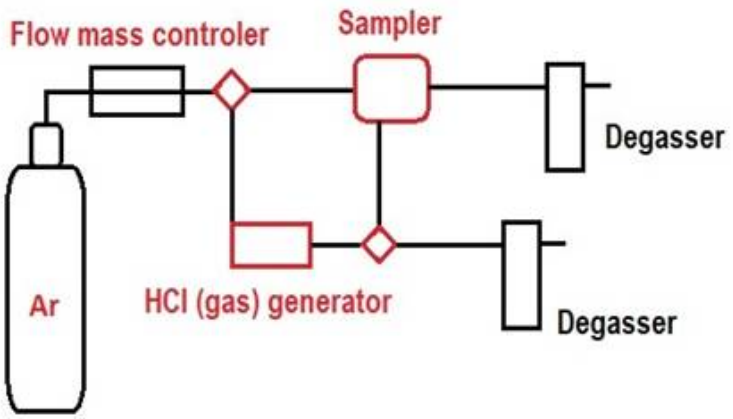
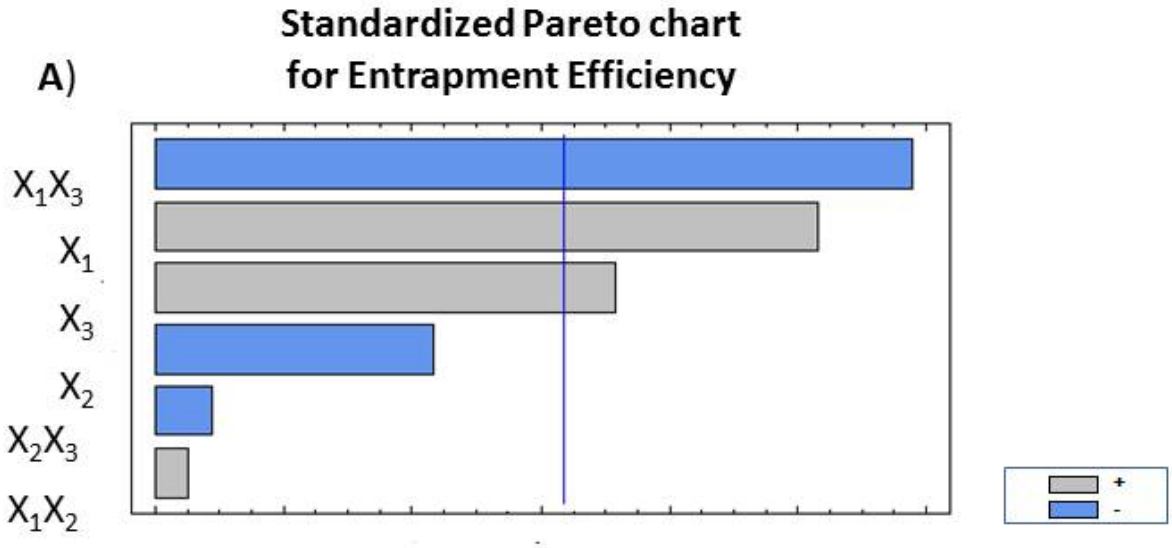
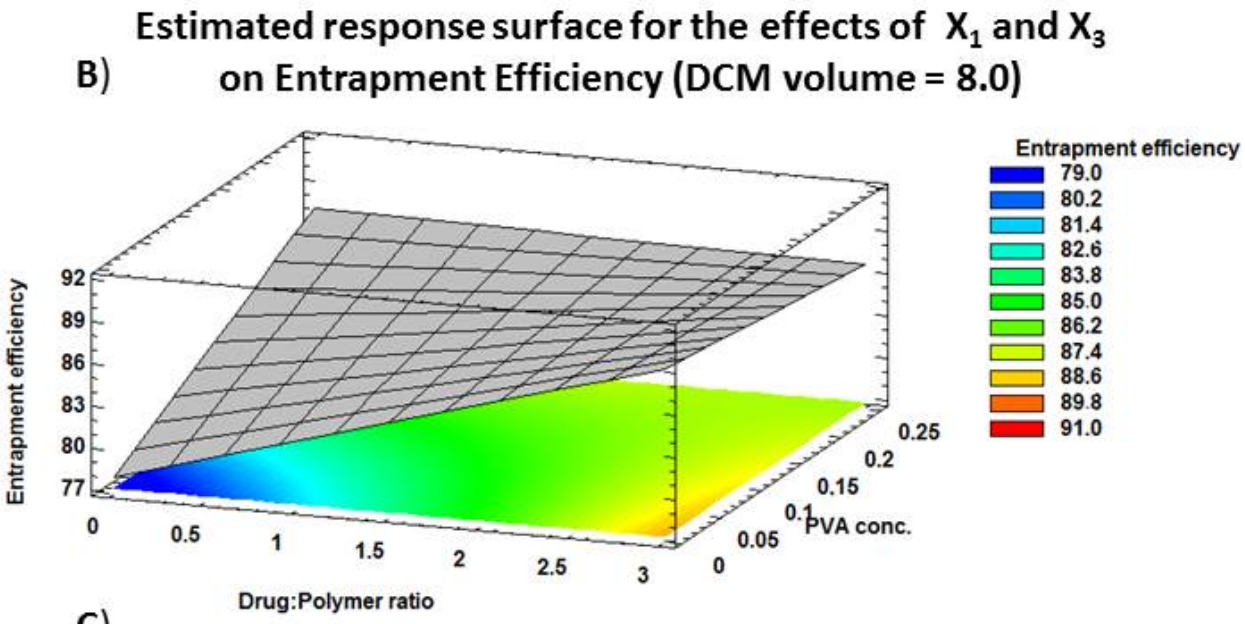


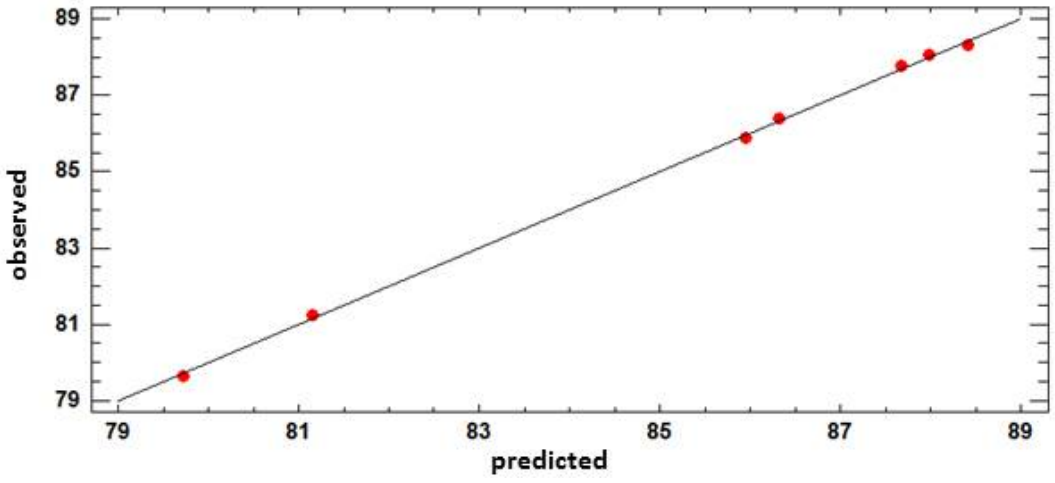
A)


Standardized Pareto chart
for Entrapment Efficiency
X1X3
X1
X3
X2
X2X3
X1X2
+
-

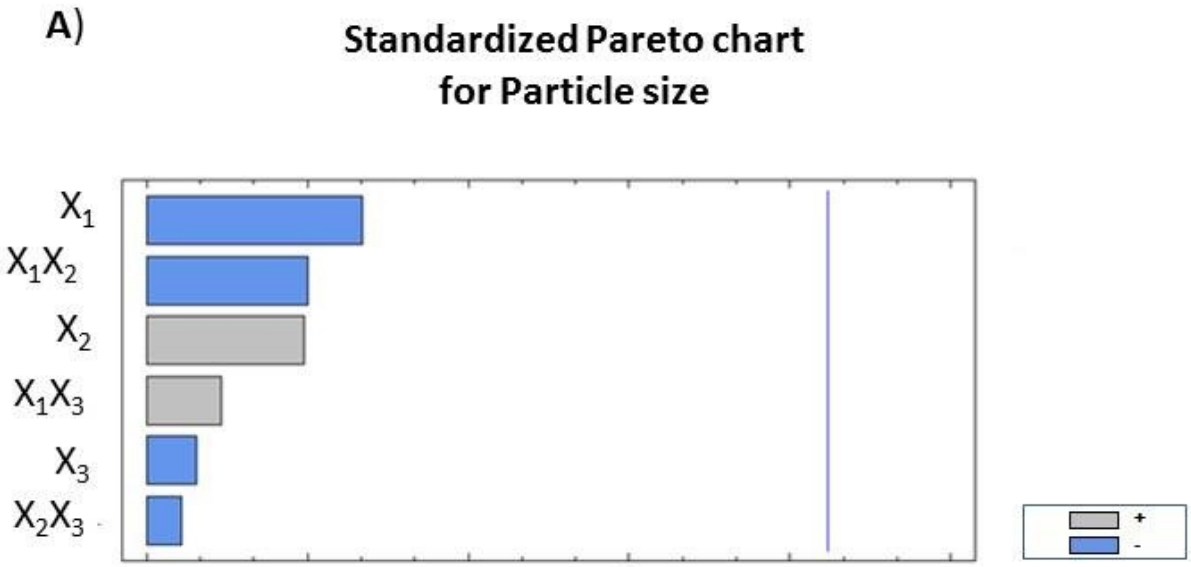
B)


Estimated response surface for the effects of X1 and X3
on Entrapment Efficiency (DCM volume = 8.0)
Entrapment efficiency
79.0
80.2
81.4
82.6
83.8
85.0
86.2
87.4
88.6
89.8
91.0
Entrapment efficiency
92
89
86
83
80
77
0
0.5
1
1.5
2
2.5
3
Drug:Polymer ratio
0
0.05
0.1
0.15
0.2
0.25
PVA conc.

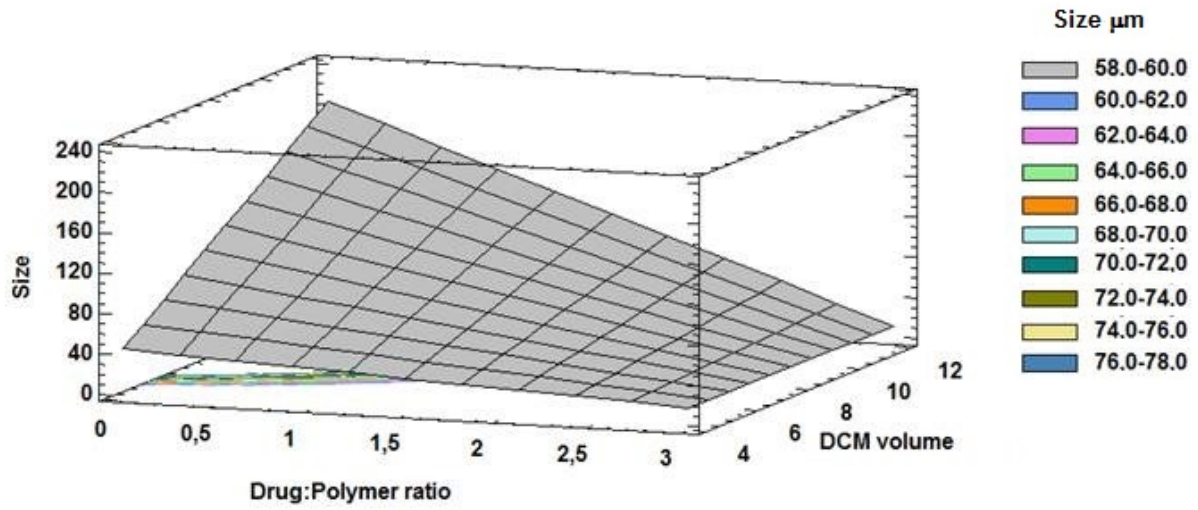
C)

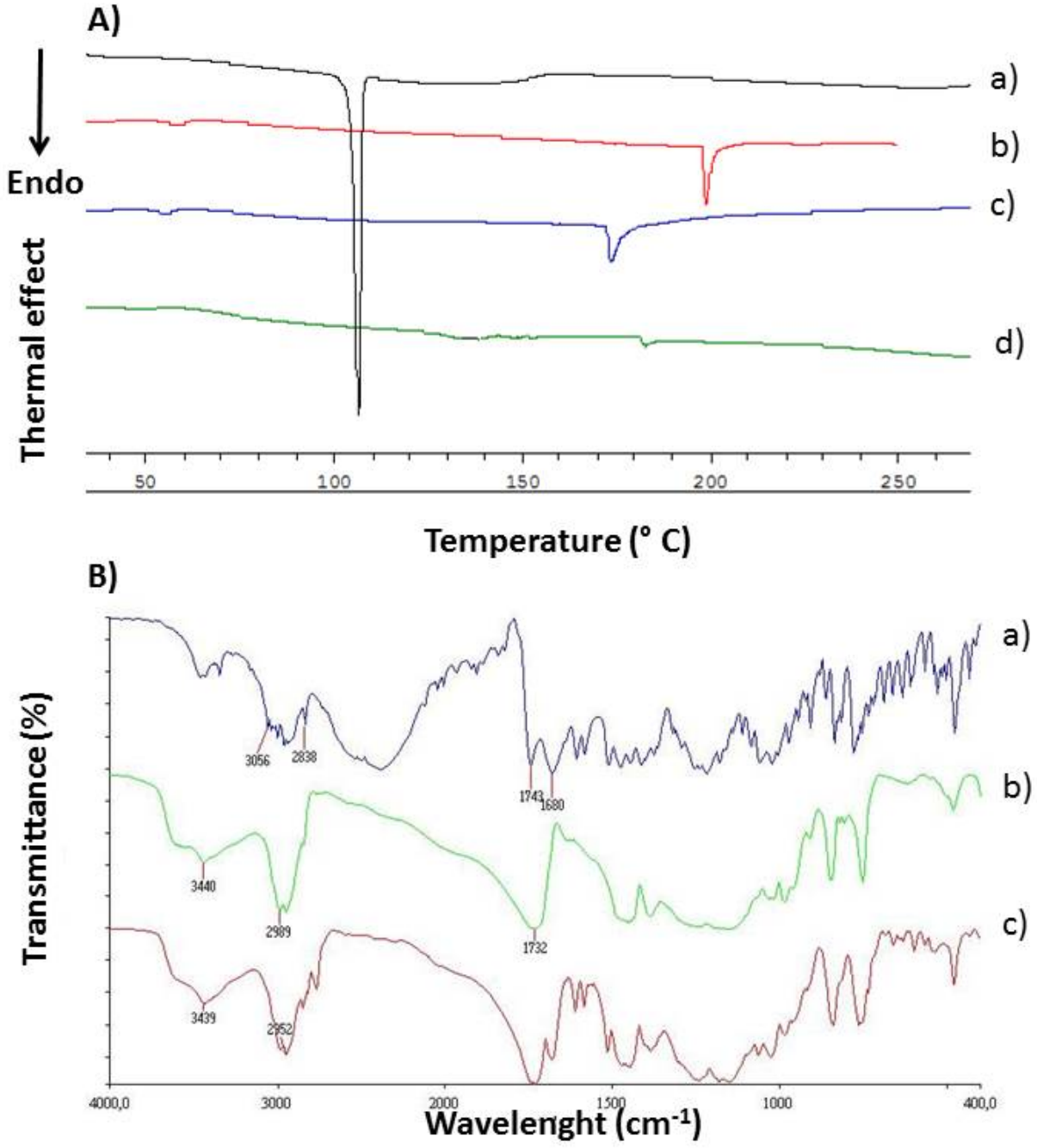

observed
89
87
85
83
81
79
79
81
83
85
87
89
predicted

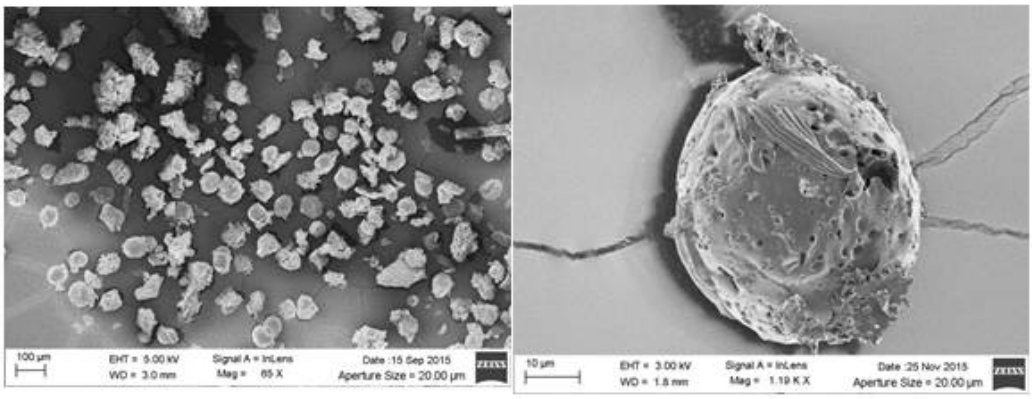
A)



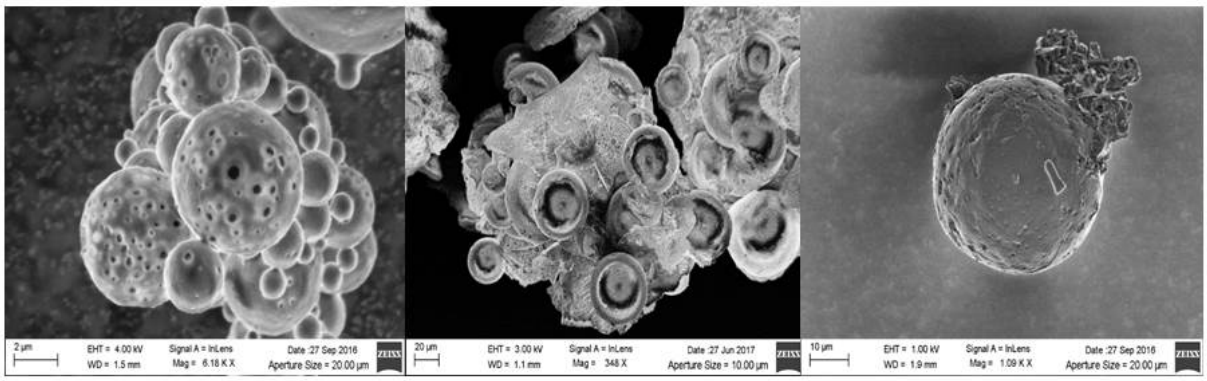

B) Estimated response surface for the effects of $X_1$ and $X_2$ on Particle size (PVA conc = 0.15)




A)
Endo
Thermal effect
a)
b)
c)
d)
50
100
150
200
250
Temperature (° C)
B)
Transmittance (%)
3056
2838
1743
1680
3440
2989
1732
3439
2952
a)
b)
c)
4000,0
3000
2000
1500
1000
400,0
Wavelenght (cm-1)



A) B) C)



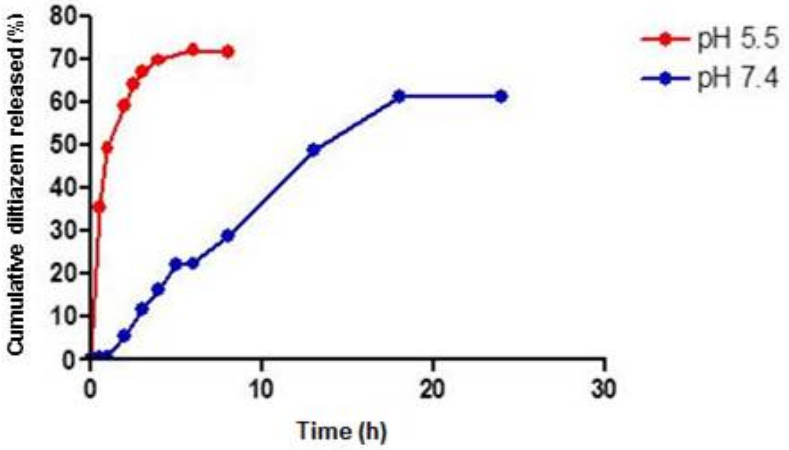

## EDX Elemental Analysis

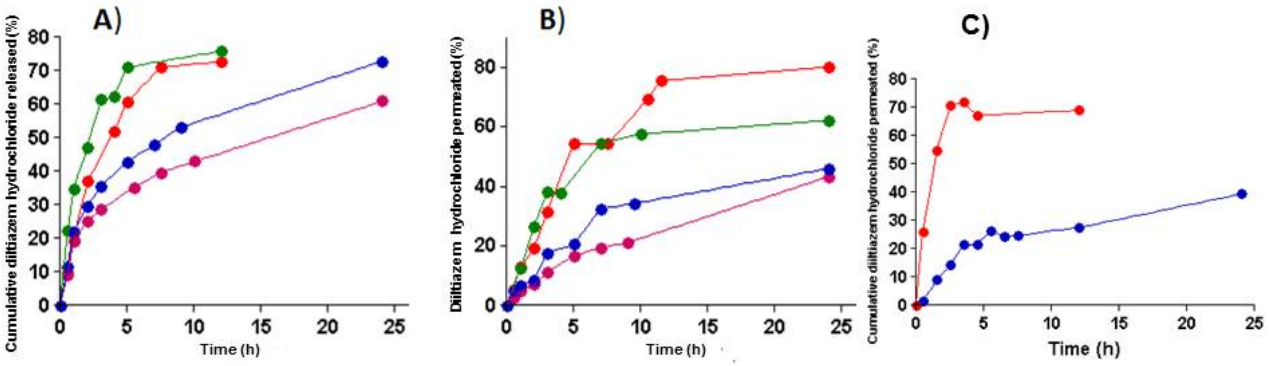
| Blank | Weight % |
|---|---|

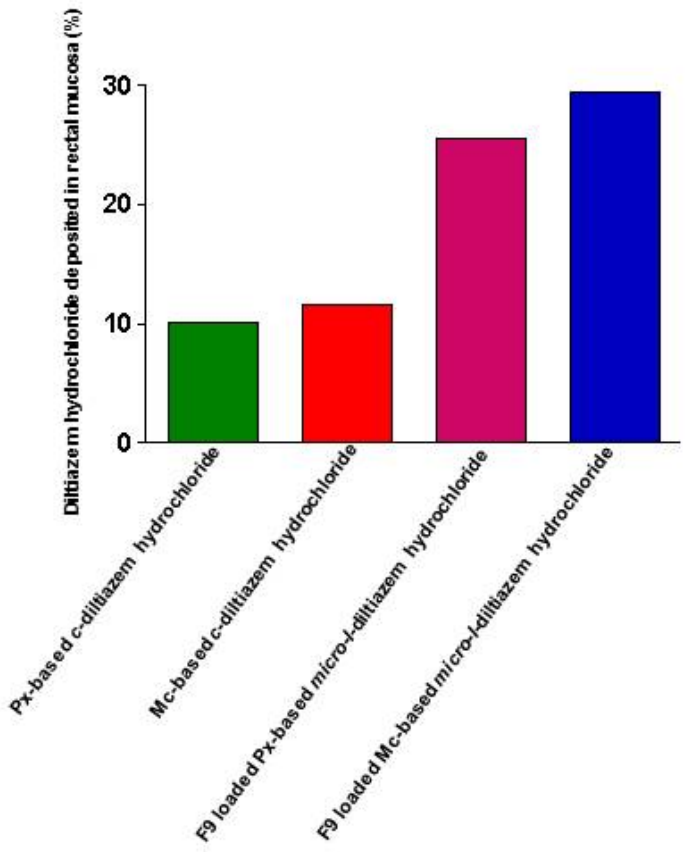
| Statistics | C | N | O | Na | S | Cl |
|---|---|---|---|---|---|---|
| Max | 90.98 | 0.00 | 13.72 | 0.21 | 0.27 | 0.31 |
| Min | 85.85 | 0.00 | 8.75 | 0.21 | 0.12 | 0.31 |
| Average | 88.98 | 0.00 | 10.68 | | 0.21 | |
| Standard Deviation | 2.20 | 0.00 | 2.14 | | 0.07 | |

| F 9 | Weight % |
|---|---|

| Statistics | C | N | O | F | Si | S | Cl | Mo |
|---|---|---|---|---|---|---|---|---|
| Max | 92.01 | 2.17 | 14.67 | 1.46 | 0.16 | 3.17 | 0.29 | 0.61 |
| Min | 82.59 | 0.00 | 4.82 | 1.46 | 0.16 | 0.56 | 0.29 | 0.61 |
| Average | 87.08 | 0.27 | 10.65 | | | 1.68 | | |
| Standard Deviation | 3.20 | 0.77 | 3.33 | | | 0.99 | | |








**Captions to Figures**

**Figure 1.** A) Standardized Pareto chart for the independent variable Production Yield ($Y_1$); B) 3D response surface plot for the effect of the independent variables $X_1$ and $X_2$ on Y1; C) Predicted *vs* observed plot for Production Yield ($Y_1$).

**Figure 2.** A) Standardized Pareto chart for the independent variable Entrapment Efficiency ($Y_2$); B) 3D response surface plot for the effect of the independent variables $X_1$ and $X_3$ on $Y_2$; C) Predicted *vs* observed plot for Entrapment Efficiency ($Y_2$).

**Figure 3.** A) DSC thermograms of pure Diltiazem-base a), Pure Eudragit RS 100 b), Blank microsponges c) and Formulation 9 d); B) FTIR spectroscopy for pure Diltiazem-base a), Blank microsponges b) and Formulation 9 c).

**Figure 4.** SEM microphotographs of A) Blank microsponges; B) Formulation FD 8; C) Formulation F 9. Elemental analyses of the surface components of blank microsponges and formulation F 9 obtained by EDX option of SEM analysis (Lower panel).

**Figure 5.** Drug release profiles in PBS pH 5.5 (red) and pH 7.4 (blu) of diltiazem from F 9 formulation.

**Figure 6**. A) *In vitro* drug release from *c*-diltiazem hydrochloride [Mc-based *c*-diltiazem hydrochloride (red) and Px-based *c*-diltiazem hydrochloride (green)] 2% gels and *micro-l*-diltiazem hydrochloride [F9 loaded Mc-based *micro-l*-diltiazem hydrochloride (blu) and Px-based *micro-l*-diltiazem hydrochloride (magenta)] 2% gels. B) *Ex vivo* permeation of diltiazem hydrochloride from *c*-diltiazem hydrochloride [Mc-based *c*-diltiazem hydrochloride (red) and Px-based *c*-diltiazem hydrochloride (green)] and *micro-l*-diltiazem hydrochloride [F9 loaded Mc-based *micro-l*-diltiazem hydrochloride (blu) and Px-based *micro-l*-diltiazem hydrochloride (magenta)] 2% gels through porcine rectal mucosa. C) Diltiazem hydrochloride permeated from a 2% aqueous solution through artificial cellulose acetate membrane (red) and porcine rectal mucosa (blu) each set between the donor and receptor compartment of a Franz diffusion cell.

**Figure 7.** Diltiazem hydrochloride percentages deposited on porcine rectal mucosa by *c*-diltiazem hydrochloride [Mc-based *c*-diltiazem hydrochloride (red) and Px-based *c*-diltiazem hydrochloride (green)] 2% gels and *micro-l*-diltiazem hydrochloride [F9 loaded Mc-based *micro-l*-diltiazem hydrochloride (blu) and Px-based *micro-l*-diltiazem hydrochloride (magenta)] 2% gels.

**Table 1.** Independent and dependent variables and experimental runs used in the $2^3$ full factorial screening design for diltiazem-loaded microsponges prepared by the quasi emulsion solvent diffusion method.

| **Independent variables** | **Levels** | |
|---|---|---|
| | **Low** | **High** |
| Drug:polymer weight:weight ratio ($X_1$) | 0.25 (-1) | 3 (+1) |
| DCM volume ($X_2$) | 4 (-1) | 12 (+1) |
| PVA conc ($X_3$) | 0.05 (-1) | 0.25 (+1) |

| **Dependent variables** | **Goal** |
|---|---|
| Production Yield ($Y_1$, %) | Maximize |
| Entrapment Efficiency ($Y_2$, %) | Maximize |
| Particle size ($Y_3$, μm) | Minimize |

| | **Drug:Polymer mass ratio** | **DCM volume** (mL) | **PVA conc** (% w/V) | **Production Yield** (%)[b] | **Entrapment Efficiency** (%)[b] | **Particle size**±SD (μm) | **Morphology**[d] |
|---|---|---|---|---|---|---|---|
| **Experimental runs studied in the $2^3$ full** | | | | | | | |

| **factorial screening design and observed responses**[a] | | | | | | | |
|---|---|---|---|---|---|---|---|
| FD1 | 3(+1) | 4 (-1) | 0.25 (+1) | 58.30 | 87.77 | 17±7 | No SM |
| FD2 | 3 (+1) | 12 (+1) | 0.05 (-1) | 67.00 | 88.07 | 4±2 | No SM |
| FD3 | 3 (+1) | 12 (+1) | 0.25 (+1) | 61.20 | 85.87 | 24±5 | No SM |
| FD4 | 3 (+1) | 4 (-1) | 0.05 (-1) | 63.30 | 88.95 | 14±12 [c] | No SM |
| FD5 | 0.25 (-1) | 12 (+1) | 0.25 (+1) | 69.40 | 86.41 | 140±55 | SM |
| FD6 | 0.25 (-1) | 12 (+1) | 0.05 (-1) | 76.80 | 79.63 | 239±105 | SM |
| FD7 | 0.25 (-1) | 4 (-1) | 0.05 (-1) | 71.10 | 81.25 | 49±18 | SM |
| FD8 | 0.25 (-1) | 4 (-1) | 0.25 (+1) | 63.10 | 88.32 | 33±12 | SM |

[a]Formulation code Factorial Design (FD). [b]Standard Deviation < 10%. [c] Very few particles completely covered by micrometer sized structures; the covering structures don't allow to measure precisely the particle diameters. [d]SM = Spherical microsponges; No SM = Morphology different from the spherical one, e.g., rods or irregular spherical microsponges.

**Table 1** (continued). Further experimental runs

| **Formulation code** | **Drug:Polymer mass ratio** | **DCM volume** (mL) | **PVA conc** (% w/V) | **Production Yield** (%)[b] | **Entrapment Efficiency** (%)[b] | **Particle size**±SD (μm) | **Morphology**[c] |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| F 9 (Formulation 9) | 0.33 | 8 | 0.15 | 82.90 | 91.50 | 59.09±13.00 | SM. |
| F 10 (Formulation 10) | 3 | 8 | 0.25 | 65.50 | 86.26 | 0.78±0.60 | No SM |
| F 11(Formulation 11) | 0.33 | 8 | 0.25 | 79.90 | 86.85 | 1.56±5 | SM. |
| F 12 (Formulation 12) | 0.33 | 8 | 0.05 | 86.25 | 80.54 | 58.76±16.41 [c] | SM |
| F 13 (Formulation 13) | 1 | 8 | 0.25 | 70.90 | 86.96 | 140±55 | No SM |
| Blank sample (without diltiazem) | - | 8 | 0.15 | 83.30 | - | 2.40±1.31 | SM |

[a] All the formulations including FD1-FD8 and Formulation 9-Formulation 13 were prepared staring from 200 mg of DTZ. [b]Standard Deviation < 10%. [c]SM = Spherical microsponges; No SM = Morphology different from the spherical one, e.g., rods or irregular spherical microsponges.

**Table 2.** Results of regression analysis of $2^3$ full factorial screening design for diltiazem microsponges

| Response | Production Yield (%) | | Entrapment Efficiency (%) | | Particle size (μm) | |
|---|---|---|---|---|---|---|
| | X coefficient | *p*-value | X coefficient | *p*-value | X coefficient | *p*-value |
| Constant ($b_0$) | 70.41 | | 79.09 | | -14.67 | |
| $X_1(b_1)$ | -2.43 | 0.0291 | 3.58 | 0.0309 | -0.82 | 0.1552 |
| $X_2$ ($b_2$) | 0.79 | 0.0478 | -0.16 | 0.0733 | 23.36 | 0.2087 |
| $X_3$ ($b_3$) | -39.05 | 0.0340 | 41.82 | 0.0443 | -153.86 | 0.5265 |
| $X_1$ $X_2(b_{12})$ | -0.12 | 0.1615 | 0.017 | 0.4914 | -6.82 | 0.2048 |
| $X_1$ $X_3(b_{13})$ | 4.18 | 0.1881 | -15.66 | 0.0270 | 125.46 | 0.3992 |
| $X_2$ $X_3(b_{22})$ | -0.06 | 0.9097 | -0.41 | 0.3237 | -20.63 | 0.6286 |
| $R^2$ | 0.9990 | | 0.9992 | | 0.9737 | |
| Adj $R^2$ | 0.9932 | | 0.9945 | | 0.8157 | |

**Table 3.** Permeation parameters and diltiazem hydrochloride deposition on porcine rectal mucosa

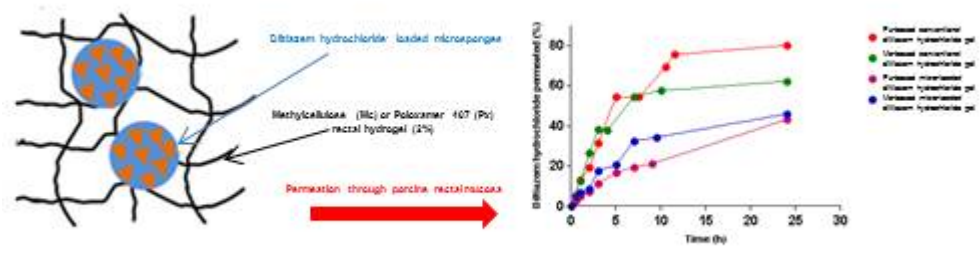
| Formulation (gel) | Flux | | Kp $10^2$ (cm·h$^{-1}$)±SD | P·D[b]·$10^2$ (cm$^2$·h$^{-1}$)±SD | $T_L$[c] (h)±SD | Drug deposition on mucosa, mg·cm$^{-3}$ ±SD | |
|---|---|---|---|---|---|---|---|
| | Jss[a] (mg·cm$^{-2}$·h$^{-1}$) ± SD | $R^2$ | | | | Method I | Method II |
| Px-based *c*-diltiazem hydrochloride | 0.59±0.02 | 0.937 | 2.94±0.10 | 0.24±0.008 | -1.01±0,14 | 12.33±1.70 | 20.20±5.30 |
| Mc-based *c*-diltiazem hydrochloride | 0.66±0.03 | 0.914 | 3.30±0.14 | 0.26±0.01 | -0.66±0.10 | 12.78±1.09 | 14.49±4.00 |
| F9 loaded Px-based *micro-l*-diltiazem hydrochloride | 0.22±0.02 | 0.969 | 1.08±0.12 | 0.09±0.009 | -0.56±0.43 | 26.16±2.71 | 23.19±5.47 |
| F9 loaded Mc-based *micro-l*-diltiazem hydrochloride | 0.36±0.03 | 0.963 | 1.81±0.16 | 0.14±0.01 | -0.35±0.25 | 32.57±3.32 | 35.27±8.71 |

[a] $J_{ss} = K_p \Delta C$; $\Delta C = C_d - C_r$, in steady state $\Delta C \approx C_d$, thus $K_p \approx J_{ss}/C_d$; $K_p = P \cdot D/h$, where $J_{ss}$ is steady state flux, $K_p$ – permeability coefficient, P – partition coefficient, D – diffusion constant, $\Delta C$ – concentration gradient, $C_d$ - initial drug concentration in gel, $C_r$ – concentration in receptor media;

[b] $P \cdot D = K_p \cdot h$

[c] $T_L$ = lag time





# *In vitro* and *ex vivo* studies on Diltiazem hydrochloride-loaded microsponges in rectal gels for chronic anal fissures treatment

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**Declarations of interest**

None.