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Synthesis of Thiolated Chitosan as Matrix for the Preparation of Metformin Hydrochloride Microparticles

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Thiolated chitosan is synthesized by reacting chitosan and cysteine as the carriers of thiol groups with 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC) as catalyst. The properties of thiolated chitosan was then characterized, and being formulated with metformin HCl and sodium tripolyphosphate (STPP) to form microparticles. Metformin HCl microparticles tested for drug release use type II dissolution tester (paddle). Statistical data analysis performed using one-way ANOVA test followed by t-test significance level of at least P <0.05. The results showed that thiolated chitosan formed a slightly yellowish white solid, distinctive smells, looks fibrous, soluble in acid, in water, and mixed with water to form transparent gels with high viscosity. Chitosan-cysteine conjugates synthesized by the addition of 50 mM EDAC has the highest content of thiol groups of 265,169 µmol per 50 mg of conjugates. Mucoadhesive test in vitro using rotating cylinder showed that thiolated chitosan with the addition of 50 mM EDAC was able to adhere to the fresh cow's intestine for more than 10 hours. Release profiles of Metformin HCl microparticles showed that the chitosan-cysteine conjugates can be used as a matrix for the controlled release dosage forms. The lowest rate of drug release was found in microparticles using thiolated chitosan with the addition of 50 mM EDAC, seen at minute 360, a drug that released of 26,256%. The release of metformin HCl from microparticles are more likely to follow the kinetics of drug release according to Higuchi equation.

Key words: thiolated chitosan, metformin HCl, microparticles, drug release

The presence of polymers in sustained release drug delivery systems is important, because almost all of the system using the polymer as a carrier. Some time ago, polymers are divided into three major groups that is soluble polymers, biodegradable polymers or bioerodible, and mucoadhesive polymer (Agoes, 2008). Over time, the presence of polymers today are quite varied, even leading to multifunctional polymers, which can be as mucoadhesive, enzyme-inhibitor, permeation-enhancers, and efflux pump-inhibitor (Vigl, 2009).

One of the polymers included in the multifunctional polymer is chitosan. Chitosan has mucoadhesive properties, permeation-enhancers, and enzyme-inhibitor (Vigl, 2009). Chitosan obtained from chitin deacetylation resulting the free amino group that can make it be policationic (Khan et al., 2002). Chitosan has been shown to have mucoadhesive properties due to electrostatic interactions between positively charged chitosan and negatively charged mucosal surface. Chitosan has one primary amino group and two free

hydroxyl groups for each monomer. Free amino group in chitosan is positively charged subsequently react with the surface/mucus are negatively charged (Bernkop-Schnurch et al., 2004).

Various modifications have been made to the existing mucoadhesive polymer resulting in a better mucoadhesvie properties. One modification is done is with the immobilization of thiol groups to mucoadhesive polymer so as to form disulfide bonds with cysteine-rich subdomains of mucus glycoproteins. Unlike the first generation mucoadhesive polymers attached to the mucus gel layer through noncovalent bonding, the new generation of mucoadhesive polymers capable of forming covalent bonds to the layer of mucus (Bernkop-Schnurch et al., 2004).

Modification of the thiol group attachment has also been made to the chitosan. This modification is based on the immobilization of thiol bearing movement on chitosan backbone, thus known as thiolated chitosan. This modification was developed to improve the solubility of chitosan, mucoadhesive property, and/or property of permeation (Bernkop-Schnurch, 2005). Improved properties of mucoadhesive thiolated chitosan expected to increase the contact time of the drug in the gastrointestinal tract that it can increase the bioavailability of the drug.

One drug that has less maximum bioavailability is metformin, a type of blood glucose-lowering biguanide's compound. Metformin is widely used for the management of type 2 diabetes therapy. Although the mechanism of action of metformin is not yet fully understood, but these compounds are known to reduce hepatic glucose output, reducing the rate of intestinal glucose absorption and increasing glucose uptake by muscle cells (McEvoy, 2004). Chronic therapy with metformin hydrochloride (HCl) can cause problems, the most notable being the high dose (1.5 to 2.0 g/day), low bioavailability (60%) and the high incidence of gastrointestinal side effects (30% of cases).

Therefore, continued efforts to increase the dosage forms formulation of metformin HCl in order to achieve optimal therapy, especially focusing on the sustained release drug. The formula of this dosage form should be made a long stay in the stomach, the drug release slowly in order to be absorbed gradually in the intestine. The release of drugs that slow but complete in the stomach is expected to increase the bioavailability of drugs, lower doses, and reduce side effects in the gastrointestinal tract. Multi-unit formulations such as microparticles for oral use is considered to release the drug at a controlled rate and stay in the stomach for a long time, so to minimize the occurrence of dose dumping (Patel et al., 2006).

Materials and methods

Materials

1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC), cysteine, chitosan manufactured by the Laboratory of Marine Biotechnology, Bogor Agricultural University (IPB), Ellman's reagent [*5,5'-dithiobis (2-nitrobenzoic acid)*], metformin hydrochloride, potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), hydrochloride acid (HCl), sodium hydroxide (NaOH), Tris powder, sodium acetic (CH_3COONa), acetic acid (CH_3COOH), aquades, aqua bidestilata, fresh cow's intestine, sodium tripolyphosphate (STPP).

Synthesis of chitosan-cysteine conjugates

Chitosan-cysteine conjugates are made by: prepared beaker glass capacity 500 mL of 4 pieces. Made of 1% acetic acid solution of 250 mL in each beaker. Then into each beaker glass was dissolved 5 g of chitosan. After all the soluble chitosan, into each beaker glass is added 2,5 g of cysteine until dissolved. Then added with a concentration 0 mM EDAC, 30

mM, 40 mM, and 50 mM in each solution. pH value adjusted to 5 with the addition of NaOH 1 N. The mixture was incubated for 3 hours with continous stirring. The solution was dialyzed twice in a dialysis tubing (MW cut-off 12-14 kDa) protected from light at 10^0C with 1 mM HCl containing 1% NaCl for 1 day and then with 1 mM HCl for 2 days to eliminate EDAC and cysteine of the conjugate solution. The solution is lyophilized and conjugates (dry conditions) maintained at 4^0C until ready for use (Roldo et al., 2004).

Characterization of thiolated chitosan.

Characterization on thiolated chitosan that has been formed, among others:

a. Organoleptic characterization

   Organoleptic characterization is carried out by physical observation the synthesis results of chitosan-cysteine conjugates. The observations include: shape, color, odor, solubility, and mixed with water.

b. Determination of the content of thiol groups with Ellman's reagent method.

   Content of free thiol groups immobilized on chitosan can be determined by spectrophotometry using Ellman reagent [5.5 '-dithiobis (2-nitrobenzoic acid)] (DTNB) (Kafedjiiski et al., 2005).

   Before determining the content of thiol groups in the sample, must be made the solution of cysteine standard curve. Solution of cysteine standard curve prepared by the procedure: DTNB stock solution containing 50 mM sodium acetate and 2 mM DTNB prepared using molecular biology degree water (aqua bidestilata). Tris solution was then made with a final concentration of 1 M and its pH adjusted to 8.0. A series of cysteine standard solution was made starting at a concentration of 10 µM. Amount of 10 µL standard solution of cysteine inserted into the measuring flask, then added 50 µL DTNB solution, 100 µL solution of Tris, and aqua bidestilata until the final volume of 1000 µL. Solution was mixed well and incubated at room temperature for 5 minutes, then absorbance was measured at a wavelength of 412 nm (Bulaj et al., 1998 and Van Horn et al., 2001). Absorbance obtained and graphed against concentration to obtain the regression equation.

   Sample solution prepared by dissolving 50 mg of chitosan-cysteine conjugate in 25 mL of aqua bidestilata. Amount of 10 µL of sample solution is inserted into a measuring flask, then added 50 µL DTNB solution, 100 µL solution of Tris, and aqua bidestilata until the final volume of 1000 µL. Solution was mixed well and incubated at room temperature for 5 minutes, then absorbance was measured at a wavelength of 412 nm. Absorbance obtained subsequently incorporated into the regression equation of standard solution of cysteine thus obtained concentration of the sample being examined.

c. Mucoadhesive test with rotating cylinder method

   50 mg of chitosan-cysteine conjugate liophilized compressed to be tablets (disc) diameter of 5 mm. Compression pressure is maintained constant during the preparation of all tablets. Tablet attached to a pressure of 500 Pa in the fresh cow's intestinal mucosa, which has been placed on a stainless steel cylinder (diameter 4.4 cm, height 5.1 cm, USP XXVI) using cyanoacrylate adhesive. The cylinder is placed in the instrument of dissolution according to USP, entirely submerged in the dissolution medium 100 mM phosphate buffer pH 6.8, at 37ºC and stirred with 100 rpm. Testing tools can be seen in Figure 1. Regardless of test tablets, disintegration, and/or erosion was observed during a period of 10 hours (Bernkop-Schnurch, 2002).

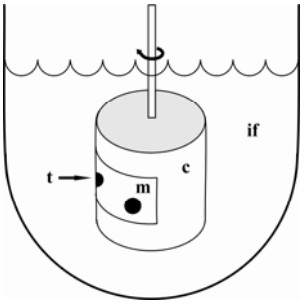

Figure 1. Scheme of the testing system used to evaluate the properties of mucoadhesive tablets based on kinds of polymers. c: cylinder, if: intestinal fluid, m: mucosa bovine intestinal, t: tablets (Bernkop-Schnurch, 2002).

d. Swelling behavior test with gravimetric method
50 mg of chitosan-cysteine conjugate liophilized compressed to be tablets (disc) diameter of 5 mm. Test tablet is immersed in a beaker containing 10 mL of 0.1M phosphate buffer pH 6.8 with 1% NaCl, at 37ºC. Every 5 minutes until the 25th minute, expands tablets taken from the incubation medium, excess water is removed, and the amount of water uptake is determined by gravimetric (Kafedjiiski et al., 2007). The swelling ratio is calculated according to the following equation:

$$swelling\ ratio = \frac{Wut}{W0}$$

information: W_{ut} is the weight of water taken at time t and W_0 is the initial weight of dry tablet.

Preparation microparticles of metformin HCl

Microparticles prepared by ionotropic gelation method using cross-link agent sodium tripolyphosphate (STPP). Complexation mechanism is cross-linked ionotropic or interpolimer complex. Chitosan-cysteine conjugates (40 mg) was dissolved in 2 mL of 1% acetic acid. Furthermore, metformin HCl (40 mg) was dissolved and dispersed in a solution of chitosan-cysteine conjugates. Solution of metformin hydrochloride-chitosan-cysteine was dripped slowly using a syringe to a solution of 1% STPP (pH adjusted to 6 to 6.5) with magnetic stirring at room temperature. Stirring was continued for 20 minutes and then dried in a freeze dryer.

Evaluation of metformin HCl microparticles

Characterization is carried out on metformin HCl microparticles are:

a. Microparticles morphology with scanning electron microscope (SEM)
Samples placed on carbon tape and fine gold sputtering was applied to the high pressure evaporator. Acceleration voltage is regulated during the scanning to get the desired microphotograph. Microphotograph taken at optimal magnification to obtain the most obvious surface morphology (Ghodake et al., 2010).

b. Determination of drug content (% b/b), drug entrapment (%), and yields of microparticles
The first step taken is to weigh the total mass of the dry microparticles obtained. Then calculated recovery (yield) microparticles by using the following equation:

$$yields\ (\%) = \left(\frac{mass\ obtained\ microparticles}{total\ mass\ used\ in\ the\ formula}\right) x100$$

The next step is determines the amount of the drug metformin HCl is incorporated in the

microparticles. Amount of 5 mg microparticles crushed and dissolved in distilled water in 10 mL measuring flask. The remaining insoluble microparticles were separated by filtration, the filtrate obtained absorbance was measured with UV-vis spectrophotometer at a wavelength of 233 nm maximum. Furthermore, the amount of metformin HCl in microparticles can be calculated using the regression equation obtained from a standard curve of metformin HCl.

After known amount of drug in the microparticles, the drug content (% w/w) and the drug entrapment (%) can be calculated by the following equation:

$$drug\ content\ (\%) - \frac{mass\ of\ drug\ in\ the\ microparticles}{mass\ obtained\ microparticles} x100$$

$$drug\ entrapment\ (\%) = \frac{mass\ of\ drug\ in\ the\ microparticles}{total\ mass\ used\ in\ the\ formula} x100$$

c. Drug release test in vitro with dissolution test method

The rate of drug release from microparticles performed using a dissolution of type II (paddle). Amount of microparticles equivalent to 5 mg of the drug is dispersed in 900 mL of phosphate buffer pH 6.8 as dissolution medium, maintained at 37 ± 0.5^0C and stirred at 100 rpm. One mL samples were taken after minute 10, 20, 30, 40, 50, and 60, then 2, 3, 4, 5 and 6 hours and the same volume of dissolution medium incorporated into the flask after every taking aliquots to maintain sink conditions. The collected samples were analyzed by spectrophotometry at a wavelength of 233 nm to determine the concentration of drug in dissolution medium (Ghodake et al., 2010). Testing of drug release in vitro was performed in triplicate for each sample.

Data analysis

Statistical data analysis performed using one-way ANOVA test followed by t-test significance level of at least P <0,05.

Results and Discussion

Chitosan-cysteine conjugates liophilized slightly yellowish white solid, distinctive smell, and fibrous. The conjugate is soluble in acid solution and water, and if the conjugate is mixed with water to form a high viscosity gel is transparent.

Cysteine is one of the reagent used for immobilization thiol group on primary amino groups of chitosan. Carboxylic acid groups of cysteine activated by EDAC to form an O-asilurea derivative as intermediate product which then reacts with primary amino groups of chitosan. Cysteine covalently attached on primary amino groups of chitosan via amide bond formation.

Primary amino group at position 2 of subunit-glucosamine chitosan is a prime target for the immobilization of thiol groups. Formation of an amide bond between carboxylic acid groups of cysteine with chitosan ligands, mediated by carbodiimide soluble in water, the EDAC.

Formation of disulfide bonds by air oxidation during synthesis avoided by performing the process of preparation at the conditions below pH 5. In this pH range the concentration of thiolate anion, which is a form of reactive for the oxidation of thiol groups, are in low numbers, and the formation of disulfide bonds can not be expected to occur (Bernkop-Schnurch, 2002).

Dialysis process performed in the synthesis chitosan-cysteine conjugates aims to eliminate EDAC and cysteine remaining after the synthesis process.

Contents of thiol groups in the chitosan-cysteine conjugate is calculated by inserting the sample absorbance of chitosan-cysteine conjugates were read in the uv-vis

spectrophotometer into the regression equation curve standard solution of cysteine, i.e y = 0,089 x + 0,017; R^2 = 0,982. Absorbance data and concentration of free thiol groups in the chitosan-cysteine conjugates can be seen in Table 1.

Table 1. Absorbance and content of free thiol groups in the 50 mg of chitosan-cycteine conjugate

No.	Sample	Absorbance (409,2 nm)	Concentration (μmol)
1	Chitosan-cysteine A (without EDAC)	0,019	0,562
2	Chitosan-cysteine B (with EDAC 30 mM)	0,751	206,180
3	Chitosan-cysteine C (with EDAC 40 mM)	0,853	234,831
4	Chitosan-cysteine D (with EDAC 50 mM)	0,961	265,169

The results showed that there are free thiol formed. Chitosan-cysteine conjugates by the addition of EDAC 50 mM has the highest content of thiol groups of 265,169 μmol per 50 mg of chitosan-cysteine conjugates.

This study varying the amount of the addition of EDAC which acts as a catalyst in the manufacture of chitosan-cysteine conjugates. The results showed that chitosan-cysteine conjugates have a thiol group, it became evident that the amide bond formed between chitosan and cysteine.

Mucoadhesive study carried out by apparatus of dissolution according to USP in combination with a standard steel cylinder and fresh cow intestine mucosa. Mucoadhesive test in vitro results with rotating cylinder method can be seen in Table 2.

Table 2. The results of mucoadhesive test in vitro

No.	Sample	Average (n=3)	SD
1	Chitosan-cysteine A (without EDAC)	1487,33	522,66
2	Chitosan-cysteine B (with EDAC 30 mM)	3039,00	1495,98
3	Chitosan-cysteine C (with EDAC 40 mM)	9811,33	3071,69
4	Chitosan-cysteine D (with EDAC 50 mM)	> 36000	

The contact time of chitosan-cysteine conjugates in the mucosal tissue increased with increasing content of thiol groups covalently attached to the polymer by more than 10 hours. Tests with this rotating cylinder method, rotation speed of 100 rpm, played for 10 hours. After more than 10 hours, the dissolution tool is turned off but the tablet/disc chitosan-cysteine conjugates D which have not been separated from bovine intestinal mucosa and observed the tablet is allowed to escape from the mucosa after settling more than 3 days.

Improved mucoadhesive properties of chitosan-cysteine conjugates is caused by the formation of disulfide bonds between the conjugate with the mucus gel layer. The formation of disulfide bond between chitosan-cysteine conjugates with mucus gel layer takes place either through an exchange reaction thiol/disulfide or through a simple oxidation process of free thiol groups.

Swelling behaviour of the four chitosan-cysteine conjugates in aqueous solution did not different significantly, because the significance value of 0,923 which is greater than 0,05 ($p < 0,05$). Profile of swelling behaviour thiolated chitosan can be seen in Figure 2.

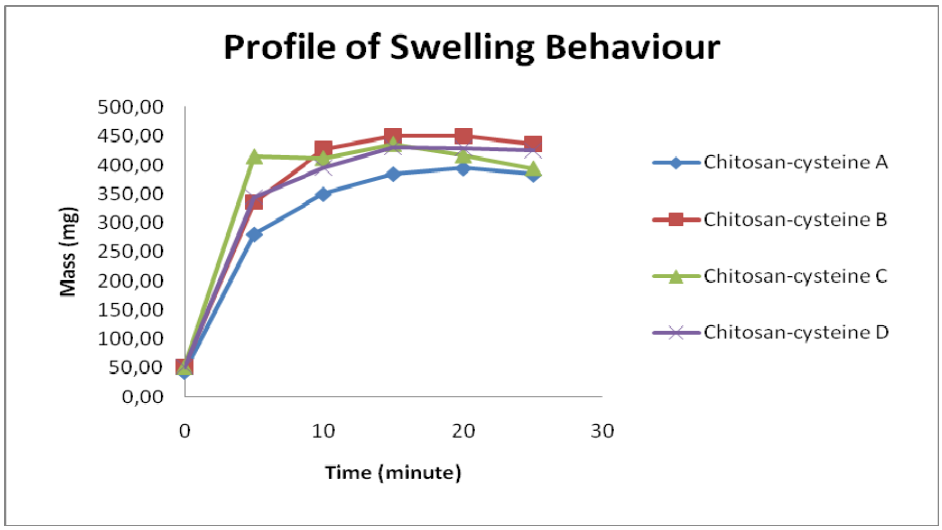

Figure 2. Profile of swelling behaviour thiolated chitosan

Observed differences are not statistically indicating that the covalent attachment of cysteine has no effect on the swelling behaviour of chitosan. Therefore, the swelling behaviour do not affect the improved mucoadhesive properties of the conjugates (Kafedjiiski et al., 2005).

Photos of the scanning electron microscope (SEM) of metformin HCl microparticles can be seen in Figure 3.

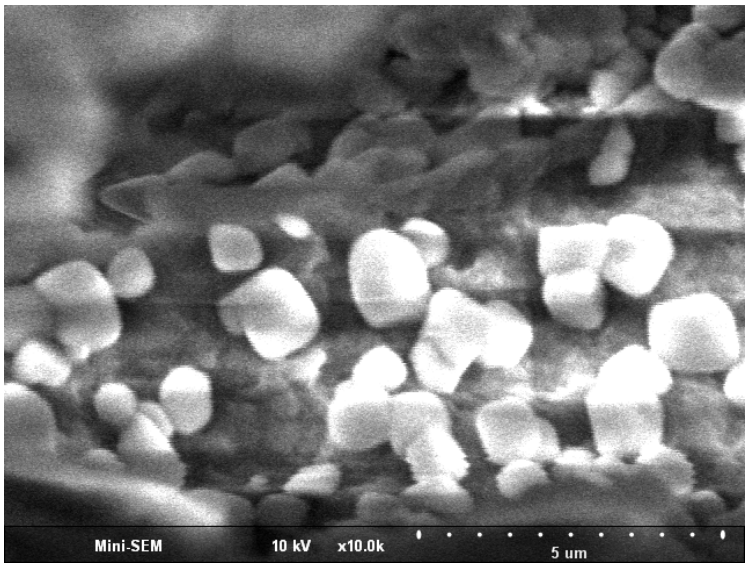

Figure 3. Photos of the scanning electron microscope (SEM) of metformin HCl microparticlses-chitosan-cysteine conjugates D 10 magnification 10.000x.

The observations with scanning electron microscope (SEM) obtained particle sizes ranged from 0,5 to 2,0 µm with a less uniform particle shape, there are spherical and cube less regular.

The results of the calculation of the efficiency of drug incorporation can be seen in Table 3.

Table 3. Data of drug content (% b/b) and drug entrapment (%)

Microparticles	Total mass in the formula (mg)	Drug content (%)	Drug entrapment (%)	Yields (%)
Metformin HCl-chitosan-cysteine A	130	39,628	43,096	33,462
Metformin HCl-chitosan-cysteine B	130	44,684	49,488	34,077
Metformin HCl-chitosan-cysteine C	130	50,409	58,600	35,769
Metformin HCl-chitosan-cysteine D	130	54,349	64,268	36,385

The results of the calculation of the efficiency of drug incorporation in microparticles shows that the higher the concentration of EDAC is added, the percentage yield, drug content, and drug entrapment also higher. This indicates that the higher the concentration of EDAC were added to make the structure of thiolated chitosan more compact.

The results of in vitro drug release test can be seen in Figure 4.

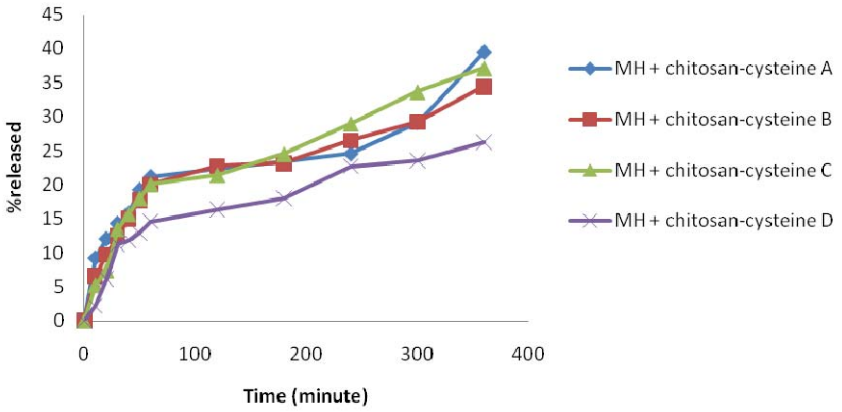

Figure 4. Profiles of drug release in vitro

Release profiles of metformin HCl showed that the chitosan-cysteine conjugates can be used as a matrix for the controlled release dosage forms. The lowest rate of drug release was found in microparticles with thiolated chitosan with the addition of 50 mM EDAC concentration, seen at minute 360, a drug that released of 26,256%. This indicates that the polymer can form a compact wall and show that they have sustained drug release for an extended period.

Comparison of metformin HCl release rate from microparticles based on thiolated chitosan with EDAC concentration variations showed that the release profile did not different significantly, because the significance value of 0,526 which is greater than 0,05 (p <0,05). This means that the thiol groups present in chitosan-cysteine conjugates are more dominant on the effect of increasing mukoadhesive properties.

Kinetics of drug release can be known through calculation according to zero order, first order, and Higuchi equation. Zero-order kinetics describe the drug release takes place at a constant rate, first-order kinetics describe the rate of drug release decreases exponentially proportional to the amount of drug remaining, while the kinetics according to the Higuchi equation illustrates that the rate of drug release occurs by passive diffusion dependent root of time according to Ficks law, i.e ($Q_t/Q_0 = k_H t^{1/2}$) (Macheras and Iliadis, 2006).

Kinetics of the release of metformin HCl from microparticles are more likely to follow Higuchi kinetics, seen from the regression coefficient (r value) that comes closest to 1 (Table 4). The curves of the drug release according to Higuchi equation can be seen in Figure 5.

Table 4. Data regression coefficient (r value) of the release profiles of metformin HCl by zero-order kinetics, first order, and Higuchi equation

Microparticles	r value C vs t graphic	r value log C vs t graphic	r value C vs √t graphic
Metformin HCl-chitosan-cysteine A	0,892	0,481	0,948
Metformin HCl-chitosan-cysteine B	0,903	0,502	0,972
Metformin HCl-chitosan-cysteine C	0,927	0,530	0,980
Metformin HCl-chitosan-cysteine D	0,908	0,540	0,972

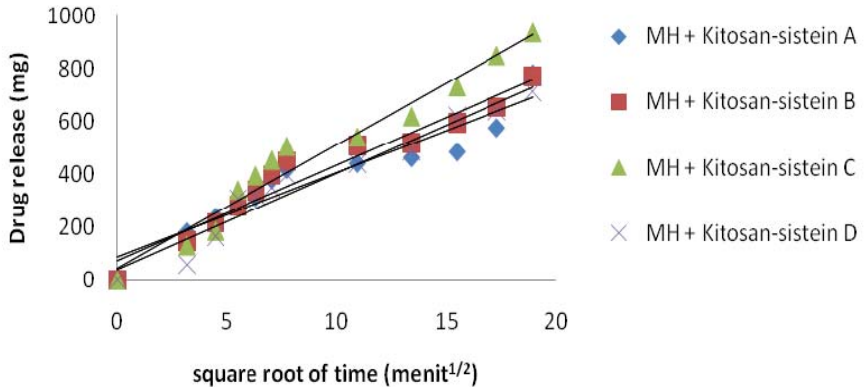

Figure 5. Curves of drug released per square root of time is calculated according to Higuchi equation

Conclusion

Thiolated chitosan (chitosan-cysteine conjugates) can be prepared by reacting chitosan and cysteine with EDAC catalyst. Thiolated chitosan characteristics include: the form of slightly yellowish white solid, distinctive smells, fibrous, soluble in acid solution and water, and when mixed with water will form a high viscosity gel is transparent. Chitosan-cysteine conjugates by the addition of 50 mM EDAC has the highest thiol groups, i.e 265,169 µmol per 50 mg of conjugates. Chitosan-cysteine conjugates by the addition of 50 mM EDAC also has mucoadhesive properties most powerful, that can be attached to the fresh intestinal mucosa of beef for more than 10 hours.

Metformin HCl release profiles showed that the chitosan-cysteine conjugates can be used as a matrix for the controlled release dosage forms. The lowest rate of drug release was found in microparticles with thiolated chitosan with the addition of 50 mM EDAC concentration, seen at minute 360, a drug that released of 26,256%. The release of metformin HCl from microparticles are more likely to follow the kinetics of drug release according to Higuchi equation.

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