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Enzymatic deacetylation of chitin for acetaminophen drug carrier administered in male mice (*Mus musculus L.*) albino swiss webster

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Abstract: A Fabrication and formulation of pharmaceutical preparations are currently being developed, this is due to the low effectiveness and bioavailability of the drug. therefore, the usage of drug carriers is a major factor for improving the bioavailability, reducing side effects and reducing drug waste. Chitosan is one of the potential biopolymers as a drug carrier because its cationic character could interact with anionic compounds through crosslinking links. The objective of this study is to create drug microspheres by using chitosan from enzymatic chitin deacetylation product as the drug carrier of acetaminophen in male *Mus musculus L.* Swiss Webster which will be carried out under in vivo process. Microsphere was fabricated as a yellow-brownish solid, the amount of acetaminophen per mg microsphere 1:1 and 1:2 was 0,007 mg and 0,0125 mg. Encapsulation efficiency was 0,7% and 1,25%. Acetaminophen percentage in the urine with control (0,5%), Microsphere 1:1 (6,4%) and microsphere 1:2 (19%), respectively.

1. Introduction

Chitosan can be easily derived from the partial deacetylation with the enzymatic process of a natural polymer called chitin (Figure 1) [4]. Some of the potential of chitosan can be utilized for various industrial and biomedical fields including biotechnology, agriculture, environment, food and nutrition, and medical [5]. In recent years, chitosan has been very attractive, especially in the biomedical field, one of which is the application in the field of pharmaceuticals and biomedicine used as drug carriers, anti-bacterial, anti-fungal, and anti-tumour [6]. The main factors that need to be considered in the preparation of drugs in the pharmaceutical field are creating effective pharmaceutical preparations and minimizing side effects [7]. However, some constraints in drug formulation are currently demonstrated by drug effectiveness and low bioavailability [8]. This is because the concentration of drug administration is still high but just in a small number of drugs that can reach the target site, caused toxic, low selectivity, and provide side effects [9]. The development and modification of drug administration and drug targeting systems are carried out to improve drug bioavailability, especially for oral drug formulations by using drug delivery (drug carrier) with the gastroretentive system, In order to, the drug can reach the target site [4].

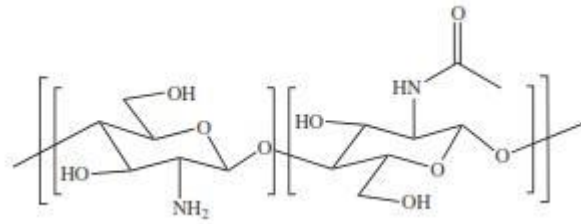


Figure 1. Chemical Structure of Chitosan

Drug carrier is a particle derived from a biological or synthetic component that can carry drugs either directly or through a spacer and can reach a specific target site [10]. The use of drug carriers also serves to reduce drug degradation, reduce drug waste and reduce side effects [11]. The characteristics of a good and safe drug carrier can increase the therapeutic effect, control the rate of drug release, and reduce the frequency of drug administration [12]. There are various types of drug carriers that have been applied in the pharmaceutical field such as microspheres, nanoparticles, liposomes, and lipoproteins [13]. Drug carriers can be classified into two types based on the biomaterials used namely natural polymer based such as (chitosan, pectin, alginate, carrageenan, gelatin, and cellulose) and synthesis of polylactic-co-glycolic acid (PLGA) polymers, poly anhydrides, and Polyethylene Glycol (PEG) [14, 15].

Chitosan can be used as drug carrier material and other biomedicine, such as drug carriers for anti-cancer drugs, anti-inflammatory drugs, antibiotics, vaccines, and gene therapy [16]. Acetaminophen is one of the non-steroidal anti-inflammatory drugs (NSAIDs) that has the efficacy of being anti-inflammatory, antipyretic and analgesic which is widely used by the public and has an oral bioavailability of 88% [17]. *M. musculus L. albino swiss webster* is a test animal that is often used for testing pharmaceutical preparations because handling maintenance is easy, resistant to disease, and has a long life span [18]. However, there is little research on acetaminophen using a carrier, especially a chitosan, which was applied *in vivo*. so it is necessary to test the potential of enzymatic deacetylation of chitin as a drug carrier of acetaminophen in *Mus musculus L.* The purpose of this study was to fabricate and test the enzymatic deacetylation potential of chitin as a drug carrier for acetaminophen and to improve the bioavailability of the drug in male (*M. musculus L.*) albino swiss webster mice.

2. Methods

2.1. Time and Place

This research was conducted in Nopember 2016 until July 2017 in the Microbiology and Biotechnology Laboratory, Department of Biology, FMIPA, ITS Surabaya.

2.2. Fabrication of Microsphere

Acetaminophen-loaded microsphere chitosan as a drug carrier were prepared following the methods used by Yu et al [19]. Acetaminophen is dissolved into 300 ml of 1% (v / v) acetic acid in the Beaker glass until it reaches a concentration of 0.5% (w/v), then powder-shaped chitosan is added and stirred using a stirrer with a speed of 8000 rpm for 24 hours without heating. After that, the crosslinker agent Sodium Tripolyphosphate (STPP) with a concentration of 1% (w / v) was added as much as 10 ml by dropping it periodically and continuously stirring using a stirrer with the same speed (8000 rpm) for 30 minutes. The mixture solution obtained was then dripped using a disposable syringe above a magnetic stirrer with a temperature of 100°C which was coated with aluminium foil to obtain the formation of chitosan microspheres. This microspheres preparation uses two different drug formulation ratios: chitosan polymers namely 1: 1 and 1: 2. So that it produces small droplets. The droplets are then in the oven at 50°C for 24 hours.

2.3. Confirmation of the Amount of Acetaminophen and Encapsulation Efficiency in the Chitosan Microspheres

20 mg chitosan microspheres were mashed, then put into the test tube and added 500 µl of FeCl₃ 1%, the mixture was then centrifuged with 1000 rpm for 1 minute. The supernatant was taken and analyzed using a spectrophotometer with a wavelength of 560 nm [17]. The absorbance value obtained is entered into $y = ax + b$ on the standard curve as the value of y , so that it can be determined the amount of drug content in 20 mg of chitosan microspheres. Furthermore according to [16], to obtain the encapsulation efficiency the value of the drug amount is entered into the formula below, with the theoretical concentration of the drug obtained from the number of chitosan microspheres taken (20 mg). Test the efficiency of the drug content calculated using the following equation:

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Calculated drug concentration}}{\text{Theoretical drug concentration}} \times 100$$

2.4. Characterization of Microspheres with Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a method used to identify functional groups of organic compounds [20]. Chitosan, acetaminophen and chitosan microspheres will be tested first in preparation by making pellets. Making pellets is done by taking a few milligrams of sample then mashed with mortar, and added KBr (Potassium Bromide). Powder that has been mashed is placed in a pellet press evenly, connected to a hydraulic compression pump, then pressed with a pressure of 10 t/in². The analysis was conducted to identify the emergence of changes and the presence of specific functional groups that play a role in the fabrication of microspheres [21].

2.5. Application of Chitosan Microspheres in M. musculus L. Mice Albino Swiss Webster

Standard acetaminophen dose for albino Swiss Webster (*M. musculus L.*) mice is 300 mg/kg, with the characteristics of albino *M. musculus L.* mice Swiss Webster male, old \pm 2.5 months and average body weight 25 grams [22]. Animals test for the application of drug carriers in the study were 15 animals, for 3 treatments and 5 replications placed in a modified cage tub [23]. Acetaminophen (control) and chitosan microspheres A (1: 1) and chitosan microspheres B (1: 2). The control dose, (A) microspheres and (B) microspheres are then dissolved into distilled water 1-2 ml until completely dissolved. then injected via oral using a cannula needle. Urine samples were collected from animals test for 24 hours after oral administration of acetaminophen and acetaminophen-chitosan microspheres

[24]. Taking urine samples was carried out by taking urine samples that were contained in the cage. Then \pm 1-2 ml urine samples obtained were placed into the test tube and stored at 4°C until this analysis was carried out.

2.6. Analysis of Acetaminophen Residues in Urine Samples with UV-Spectrophotometers

100 µl urine samples were analyzed using a single chromogenic reagent FeCl₃ 1% (w/v) of 400 µl. The mixture is then centrifuged for 1 minute with 1000 rpm. The supernatant was taken and analyzed using a spectrophotometer with a wavelength of 560 nm [17]. So that the amount of drug in the urine sample can be determined.

3. Results and Discussions

3.1. Fabrication of Microsphere

The Microspheres produced by chitosan as drug carrier acetaminophen was carried out using a droplet method that utilizes sodium tripolyphosphate as a crosslinker agent. Visualization of microspherical fabrication forms is shown in Figure 2.



Figure 2. Visualization of Microsphere

In Figure 2. it can be seen that the droplet microspheres are solid, yellow- brownish in colour and each droplet microsphere formation has a size of ± 1 mm which is thought to be formed because of the interaction between chitosan and acetaminophen. Previous research stated that the interaction between chitosan and acetaminophen caused the group (N-H) in chitosan to bind to the group (C=O). The more groups (N-H), the more group formation (O-H) indicates the formation of hydrogen bonds between chitosan and acetaminophen [25].

3.2. Confirmation of the Amount of Acetaminophen and Encapsulation Efficiency in the Chitosan Microspheres

The droplet microsphere that forms uses for confirmed the amount and the encapsulation efficiency of acetaminophen contained in chitosan microspheres are shown in Table 1. to determine the amount of bound acetaminophen.

Table 1. The Amount of Acetaminophen and Encapsulation Efficiency in the Chitosan Microspheres

Microsphere	The Amount of Acetaminophen per 20 mg Microsphere (mg)	The Amount of Acetaminophen per mg Microsphere (mg)	Encapsulation Efficiency (%)
A	0,14	0,007	0,7
B	0,25	0,0125	1,25

Based on Table 1, it can be seen the results of the confirmation of the amount of acetaminophen bound every 20 mg of the microspheres. In microsphere B, there is more content of bound acetaminophen, which is equal to 0.25 mg of acetaminophen. Whereas in microspheres A only has a bound acetaminophen content of 0.14 mg of acetaminophen. This is due to the higher number of chitosan. Thus indicating the number of groups (N-H), which can bind to the group (C=O) of acetaminophen. In the study [25] it was stated that the interaction between groups (N-H) on chitosan and group (C=O) on acetaminophen caused group formation (O-H). In addition, by knowing the amount of acetaminophen per mg of microspheres, So it can be known the percentage which the encapsulation efficiency for microspheres (B) have a greater encapsulation of 1.25% while the encapsulation efficiency of microspheres (A) is only 0.07%. This percentage is directly proportional to the amount of acetaminophen bound to each mg of chitosan microspheres.

3.3. FTIR Analysis of Chitosan, Acetaminophen and Microspheres

Characterization of FTIR spectra showed the presence of functional groups that interacted with acetaminophen and microspheres shown in Table 2.

Table 2. FTIR Analysis of Chitosan, Acetaminophen and Microspheres

Sample	Wavelength of Functional Group	Wavelength (cm)	Functional Group
Chitosan		3254.24 ; 1623.62	N-H and C=O
Acetaminophen	3100-3500 (N-H)	3321.05 ; 3160.19; 1651.49	N-H and C=O
Microsphere A	1650-1690 (C=O) 2500-3400 (O-H)	3256.03 ; 2921.80 ; 1618.14	N-H and O-H
Microsphere B		3255.00 ; 2918.54 ; 1654.28	N-H and O-H

The range of chitosan absorbance is in FTIR spectra at wavelengths of 4,000-500 cm^{-1} . The range 3.100 – 3.500 shows stretch vibrations on the amine group (NH) which are indicated by the presence of absorption bands in the absorbance range. The stretch of an amine group (NH) on chitosan before becoming a drug carrier has a higher absorption intensity at 3254.24 cm^{-1} . whereas in acetaminophen it only has a vibrational range stretch of an amine group (NH) at wavelengths of 3160.19 cm^{-1} and 3321.05 cm^{-1} . In addition to acetaminophen, there is also an absorption band at wavelength 1651.49 cm^{-1} which indicates that there is a carbonyl group (C=O) which is in the absorbance range of 1650-1690 [26]. The following is the peak of FTIR analysis results shown in Figure 3.

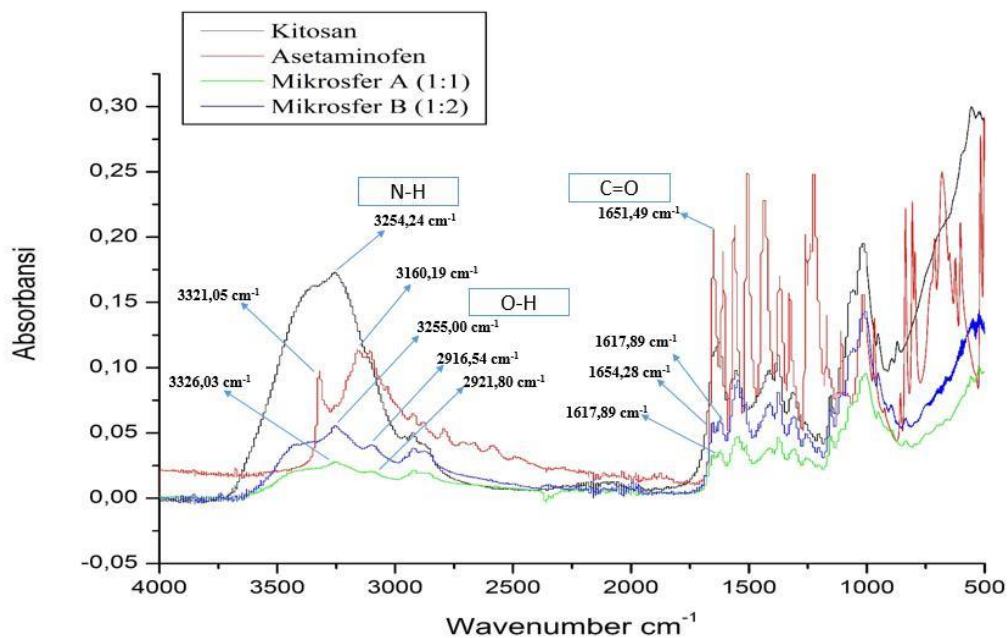


Figure 3. Peak of FTIR Analysis of Chitosan (Black), Acetaminophen (Red), Microspheres A (Green), and Microspher B (Blue).

Based on Figure 3, there are drug microspheres which are produced by binding between amine groups found in chitosan biopolymers as drug carriers with carbonyl groups found in acetaminophen, so hydrogen (O-H) bonds are formed with an absorbance range of 2500 - 3400 [20]. This is indicated by the presence of hydrogen bonds formed on microspheres with a ratio of

1: 1 and 1: 2, on microspheres (A) having absorption at wavelengths of 2921.80 cm^{-1} and 3256.03 cm^{-1} while microspheres (B) has the absorption intensity is at a wavelength of 2918.54 cm^{-1} and 3255.00 cm^{-1} , in which the microspheres (B) shows the absorbance of absorption is stronger than the microspheres (A), it indicates that the microspheres (B) form more hydrogen (OH) bonds.

3.4. Analysis of Acetaminophen Residues in Urine Samples

The results of the analysis of acetaminophen residues in urine samples of male Swiss Webster albino mice (*Mus musculus L.*) were carried out using the UV-Spectrophotometer analysis method shown in Table 3. Mice were used as animals test is male mice, due in male mice is not affected by reproductive hormones, such as the female mice were feared to undergo estrus phase, as well as another, reproduce hormone that affects metabolism.

Tabel 3. Acetaminophen residue in urine sample

Samples	Amount of Acetaminophen	Percentage of Acetaminophen
	Residue in Urine Sample (mg)	Residue in Urine Sample (%)
Kontrol	0,005	0,5
A	0,064	6,4
B	0,19	19

Based on Table 3. shows that the control of the amount of acetaminophen found in urine was 0.5%, while for acetaminophen with the addition of chitosan found in microspheres A and microspheres B were 6.4% and 19%, respectively. The amount of acetaminophen residue in the urine indicates the amount of the drug that is not absorbed. This is because each drug that enters will undergo a process of absorption, distribution, metabolism and excretion. So that it can be said that the acetaminophen found in urine has been absorbed and this is drug waste. In the results of this in vivo study, the drug carrier acetaminophen in the form of chitosan has a greater residue than the control (acetaminophen without chitosan). In control, the drug will enter the body orally, then it will experience absorption in the small intestine and bind to receptors so that it can enter the bloodstream and directly adapt distributed throughout the body and target site, However, some drugs can be directly excreted through the feces without undergoing metabolism (biotransformation) in advance, so that the drug contained in the urine as the amount of drug absorbed less. As stated that each drug that enters will experience the process of absorption, distribution, metabolism and excretion [27].

In A and B microspheres, each drug that is not absorbed is 6.4% and 19%, respectively. In A and B microspheres, the amount of drug found in urine samples is greater than the control. The high drug residue that is not absorbed in the microsphere compared to the control can be caused by the ineffectiveness of the role of chitosan as a drug carrier that can be influenced by several factors including low deacetylation levels and requires enzymatic deacetylation optimization and microsphere fabrication with manual methods. The results of this study are inversely proportional to the literature which states that the presence of chitosan in the form of microspheres as drug carriers that have mucoadhesive properties, so as to increase the retention time for drug absorption, as in the study [28]. It was revealed that the small intestine is a place for absorption of various types of drugs, therefore the presence of mucoadhesive polymers can protect or cover the drug from the influence of enzyme degradation, high displacement activity, and relatively short transit times. The presence of these polymers can prolong the time of contact with the membrane so that it can increase absorption. In addition [29] states that several mucoadhesive systems such as particles, granules or pellets can increase retention of absorption time in rat animals test.

4. Conclusions

This study concluded that the chitosan microspheres produced are solid and brownish yellow in colour and have a size of ± 1 μ m in each droplet formation. The amount of drug in each mg of A and B microspheres is 0.007 mg and, 0.125 mg, with an encapsulation efficiency of 0.7% and 1.25%. The results of the analysis through the amount of acetaminophen in the urine sample were equal to 0.5% control, microspheres (A) 6.4%, and microspheres (B) 19%. Chitosan resulting from enzymatic deacetylation of chitin has the potential as a drug carrier. However, it is necessary to optimize enzymatic deacetylation of chitin to increase chitosan as a drug carrier and require improved fabrication and analysis methods with better methods such as using Spray Drying and HPLC for analysis of urine samples.

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