CHAPTER IV
RESULTS AND DISCUSSION

4.1 Chitosan beads containing chloramphenicol

The physical insertion studies can be done by various types of sample preparations. Most studies were achieved by preparing as a membrane, film, fiber owing to unique properties of chitosan (Kanke, 1989). The present work is mainly focused on the controlled release system applied to a model drug, thus, it is our interest to prepare chitosan as a bead to approach this studies.

Chitosan beads containing chloramphenicol can be prepared as mentioned in 3.3.1. The size of the beads prepared was approximately 1 mm in diameter. The appearance was pale-yellow and there was some white powder stick outside the beads. This white powder could be dissolved when soaking in the buffer solution. Comparing to the controlled beads or the blank system, it could be concluded that the excess amount of chloramphenicol might be coated and formed as a white powder outside the beads. Thus, all beads were prewashed thoroughly in buffer before the release studies.

4.2 Release studies of chloramphenicol from chitosan beads

The release amount of chloramphenicol was studied by measuring the concentrations of chloramphenicol in buffer solution. The prepared beads were immersed in the buffer and the quantitative analysis of chloramphenicol was

done by measuring the concentrations of chloramphenicol as a function of time.

According to the structure of chloramphenicol, there is a benzene ring chromophore which is sensitive to UV. Thus, UV-VIS spectrophotometric technique is applicable for measuring the concentration of chloramphenicol. The release profile of chloramphenicol from chitosan beads in various buffer solutions is shown in Figure 4.1.

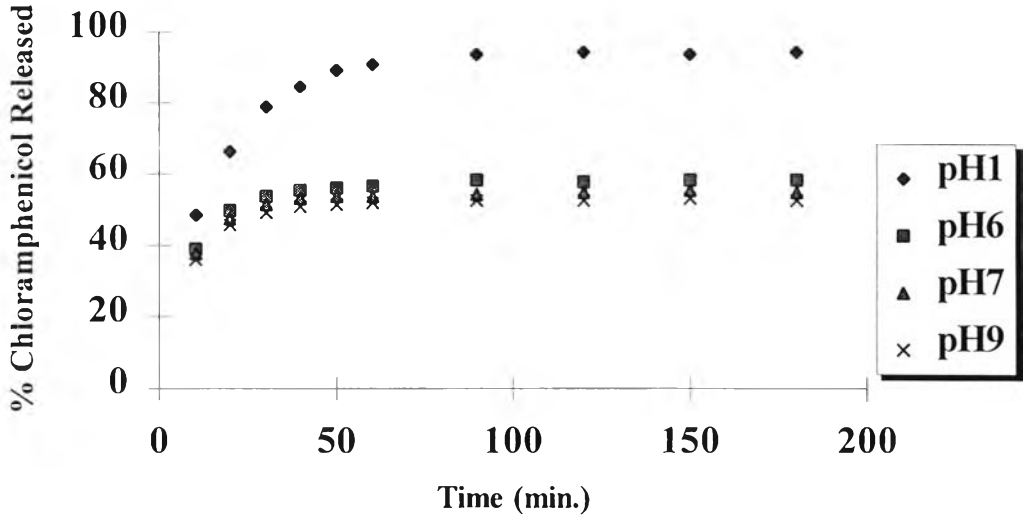

Figure 4.1 Release profile of chloramphenicol from chitosan beads in 0.1N HCl (pH 1) and Kolthoff's buffer solution pH 6, 7 and 9 at room temperature.

As shown in Figure 4.1, the release of chloramphenicol is found to be dependent with pH. In the acidic condition (low pH), the release of chloramphenicol is increased significantly in the first 30 minutes. Then, the release is gradually decreased and reached the equilibrium state at 60 minutes. The total amount of the release is nearly 100%. In contrast, when the solution media become neutral or basic, the release amount of chloramphenicol is less. The amount of chloramphenicol is released only 50% to 60% at equilibrium state.

Bodmeier (1989) and Yao (1994) proposed that amino groups of ionic polysaccharide chitosan can form hydrogen bond in base condition. The

change of pH level can control the interaction between matrix due to the hydrogen bonding and leads to the change of the structure, especially, the swelling. On this viewpoint, it can be discussed as follows (Figure 4.2).

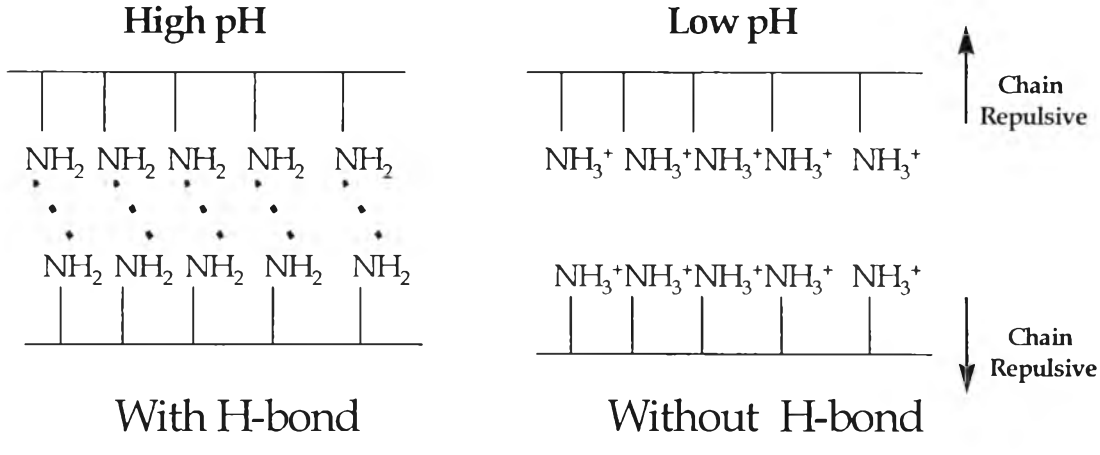

Figure 4.2 Schematic representation of chitosan chain in acidic and basic condition.

In neutral and base condition, where the pH is more than 6, hydrogen bond between amino groups is easily formed. The structure of chitosan matrix should be a close system due to the hydrogen bonding between chains. This makes the chloramphenicol molecules or penetrants difficult to diffuse out of the chitosan matrix.

However, in acidic condition, this hydrogen bond will be dissociated due to the protonation at amino groups. This causes the repulsive between chitosan chains which leads to the opened structure of chitosan matrix or swelling. Thus, chloramphenicol can easily diffuse out of the chitosan matrix compared to that in the basic condition.

It should be noted that the fast release is achieved for the acidic condition showing nearly the total amount of chloramphenicol released. In the case of neutral or base condition, the slow release is satisfactory. However, the total amount released is required the improvement. In order to increase to

release amount for 100%, other factors for controlled system should be considered; for example, temperature, additional crosslinking group (Shiraishi, 1993). In the case of increasing temperature, it can be expected that the chain expanded due to higher kinetic energy and the release amount will be significant.

4.3 Preparation of chitosan acetate

Many methods were known to protect the amino groups such as a selective *N*-acylation (Hirano, 1976), salt formation (Horton, 1973), etc. However, the easiest and most inexpensive procedure is the salt formation. By this method, the decreasing in molecular weight due to the loss of repeating unit in polymer chain can be neglected.

This reaction was used in order to form an acetate salt at the amino group. The IR-spectrum is shown in Figure 4.3. Compared to the IR spectrum of chitosan, there is no significant difference between those two spectra since the absorbance of C=O from acetate salt is occured near C=O of acetamide group in chitosan itself.

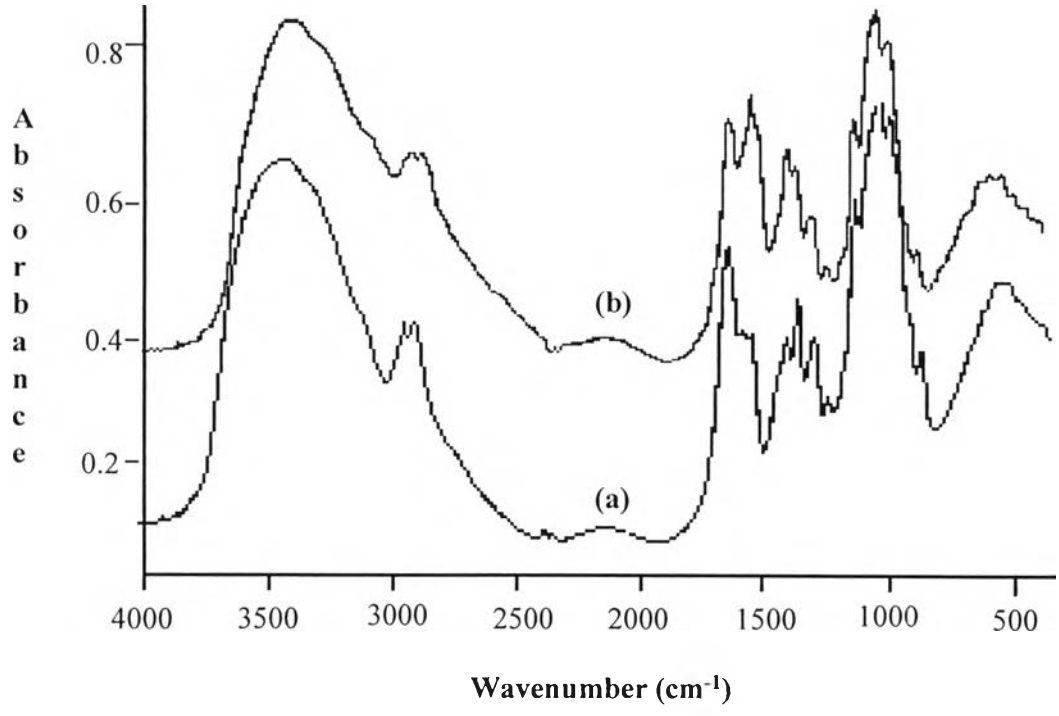

Figure 4.3 Compared FTIR spectra of (a) chitosan; (b) chitosan acetate

FTIR (KBr, cm^{-1}) :- 3400 (O-H stretch.); 2900 (C-H stretch.);
1654,1560 (C=O amide stretch);
~1100 (pyranose ring).

Generally, the reaction with *N,N'*-carbonyl diimidazole is progressed by the nucleophilic substitution mechanism in which both hydoxyl and amino can act as a nucleophile. The objective of this reaction is to protect the amino group by forming acetate salts to get the further reaction occur selectively at the hydroxyl group of the C-6 position.

4.4 Synthesis of chitosan precursors

The chemical conjugation of chloramphenicol onto chitosan is focused on chemical bonding via the active spacer group in chitosan precursor. In chitosan, two different functional groups, i.e., the hydroxyl and the amino groups, are practical for the reaction. This present work was emphasized on hydroxyl group at C-6 position since the amount of amino groups depends on the degree of deacetylation. Thus, the conjugation of model drug is limited to the chitosan starting material.

By considering at C-6 position, one of the interesting reaction is esterification, that is introducing an ester bond between chloramphenicol and chitosan. Here, it can be expected that the release from the obtained product will be controlled by hydrolysis reaction. This present work is focused on two different types of modification at C-6. One is to form an activated ester using coupling agents. The other is to oxidize this hydroxyl group to be a carboxylic acid.

4.4.1 Synthesis of β-(1→4)-2-amino-2-deoxy-β-D-glucopyranuronan perchlorate (6-*O*-carboxyl chitosan perchlorate)

The modification of the primary alcohol of chitosan at C-6 by oxidation reaction is an attractive approach since it can be expected to obtain a reactive precursor for further reaction with a model drug having alcohol or amine moiety to form an ester or amide compound. Another advantage also can be mentioned as an introduction of the spacer, such as, acid chloride to get more reactive chitosan.

To oxidize the hydroxyl group at C-6 of chitosan, many kinds of oxidants were reported; for example, dinitrogen tetraoxide, oxygen-platinum (Whistler, 1971) which showed the low degree of substitution of carboxylic

acid in the obtained product. Chromic acid was originally studied by Horton (1973). The achieved product gave high degree of substituion and no degradation of amino group was occurred. Hence, this present work is focused on the use of chromium (VI) oxide in acetic acid which will be formed a chromic acid as oxidant.

The oxidation reaction of chitosan was done by chromium (VI) oxide. The IR spectrum of the product is shown in Figure 4.4.

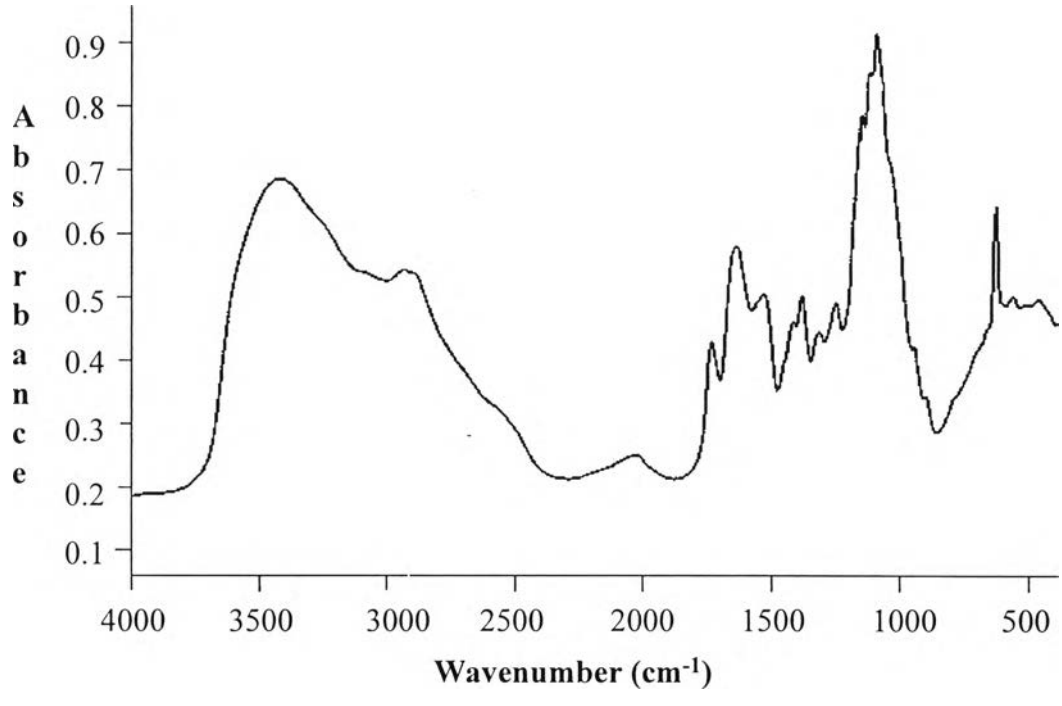

Figure 4.4 FTIR spectrum of β-(1→4)-2-amino-2-deoxy-β-D-glucopyranuronan perchlorate (KBr pellet).

FTIR (KBr, cm^{-1}) :- 3400 (O-H stretch.); 2900 (C-H stretch); 1730 (C=O acid); 1634,1520 (C=Oamide); ~1100 (pyranose ring).

The obtained product shows the C=O stretching of carboxylic acid at 1731 cm^{-1} (Figure 4.4). Comparing to chitosan, the carboxyl peak of the

product is obvious which can be concluded as a carboxylic group on C-6 position.

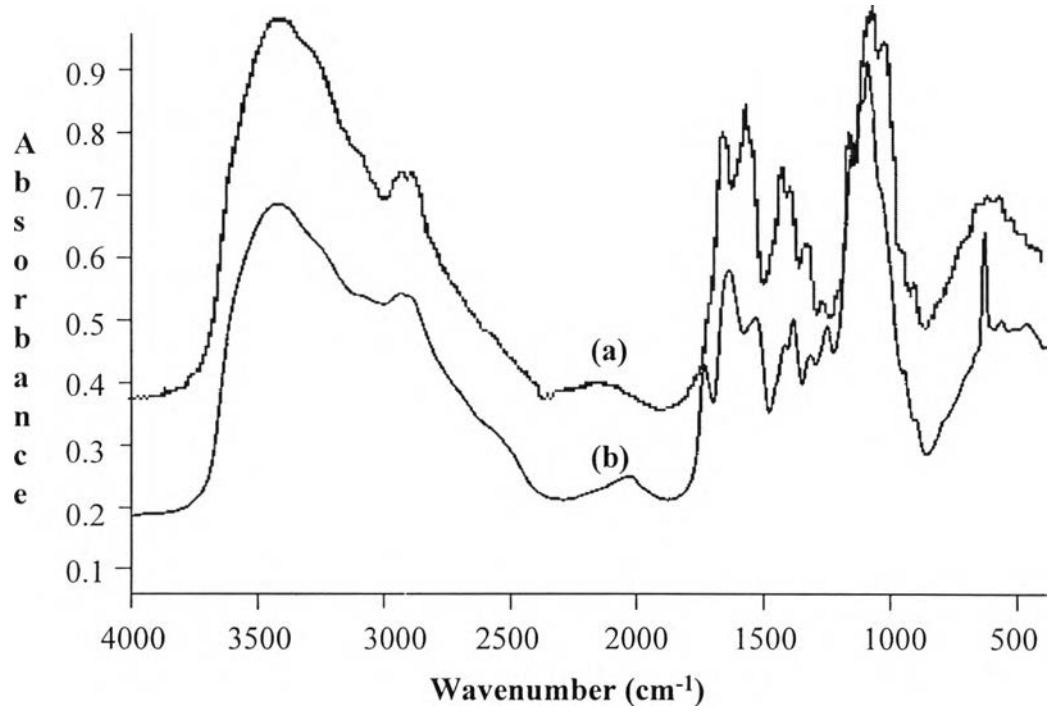

Figure 4.5 Compared FTIR spectra of (a) chitosan acetate; (b) β-(1→4)-2-amino-2-deoxy-β-D-glucopyranuronan perchlorate.

Horton *et al.*(1973) reported the preparation of 6-*O*-carboxyl chitosan as a precursor for synthesis of 2-sulfoamino derivative which can show the blood anticoagulant property. However, the purification procedure is unclear on the elimination of oxidant entrapped in chitosan after the oxidation process.

It should be noted that the obtained product shows green color powder. This implied that some chromium ions from the oxidant are still entrapped in the structure owing to the ion interaction property of chitosan itself. In this present work, the obtained product needs further purification since it may be in the perchlorate salt form.

Considering the carboxyl chitosan, it should be noted that it is an effective method since the reaction is not required the expensive reagent. With

this modification, carboxyl chitosan can not only be applied as a precursor for coupling with drug, but also a reactive precursor for further modification. However, the derivatization at C-6 to carboxylic acid still has the problem on the complex formation between metal oxidant and chitosan which results in the difficult purification process.

4.4.2 Reaction of chitosan acetate and *N,N'*-carbonyl diimidazole (CDI)

Focused on the activated ester formation, many coupling agents are known; for example, dicyclohexylcarbodiimide (DCC) which was used by Simionescu *et al.*(1985) to introduce chloramphenicol -- a bioactive molecule, into Biozan R® to apply this product for drug delivery system. This studies showed that introducing spacer would improve for not only the stabilization but also the solubility. Thus, the use of coupling agent is one of the interesting approach for polymer-drug conjugation.

In the present work, owing to its high reactivity with alcohols, carboxylic acids, amines, etc., *N,N'*-carbonyl diimidazole (CDI) was chosen as a coupling agent to form an activated ester at hydroxyl group of C-6 and further reacted with model drug at this position. Hence, protecting amino group from this reaction is essential.

Thus, the present work emphasized on the application of CDI onto primary alcohol at C-6 of chitosan acetate salt and the introduction of chloramphenicol at this position (see 4.5). The release of chloramphenicol from the obtained product was further studied as a term of pH dependence and the release mechanism (see 4.6).

According to the high reactivity of CDI with water, the reaction condition should be non-aqueous in order to decrease the CDI degradation. *N,N*-dimethylformamide (DMF), *N,N*-dimethylacetamide (DMAc), chloroform and methylene chloride are considered to be a suitable solvent for this reaction.

However, due to the low solubility of chitosan in these organic solvents, those conditions will make the reaction be heterogeneous. It must be noted that the reaction progresses in heterogeneous have the weak point in the reactivity. To compensate this point, the amount of CDI used was two mole equivalent larger than chitosan. The reaction with CDI was done in severe condition, i.e., at 120°C in vacuo for 1 hour and then reduced to 60°C at atmospheric pressure overnight. The magnesium methoxide catalyst is also needed to have CDI substitution onto chitosan.

After the product of chitosan acetate was obtained, the reaction with CDI was next proceeded. The IR spectrum of the obtained product was shown in Figure 4.6.

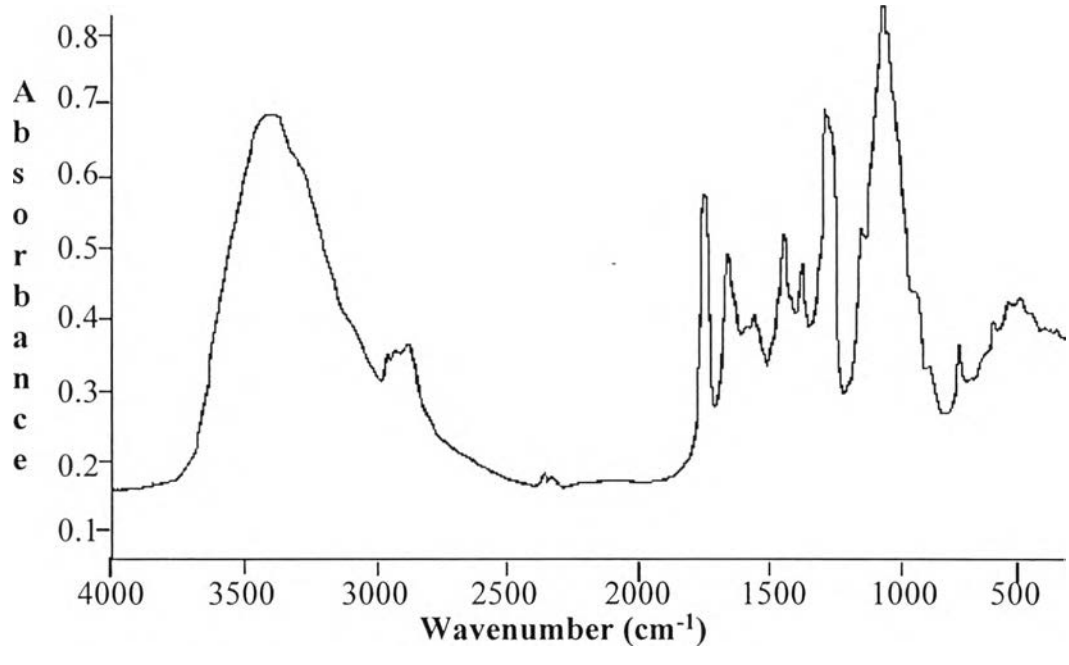

Figure 4.6 FTIR spectrum of chitosan acetate after reaction with CDI to be chitosan-carbonyl imidazolide.

FTIR (KBr, cm^{-1}) :- 3400 (O-H stretch.); 2900 (C-H stretch.);
1750 (C=O imidazolide stretch.);
1654,1560 (C=O amide);
~1100 (pyranose ring).

The obtained product showed the absorbance at 1750 cm^{-1} which is the characteristic peak of an active ester carbonyl of imidazolide. The reaction was found to be succeeded in severe condition (120°C in vacuo for 1 hour and at 60°C overnight) and essentially having a catalytic amount of magnesium methoxide.

This reaction mechanism was proposed to be the nucleophilic substitution at the carbonyl carbon or tetrahedral mechanism as shown in Figure 4.7.

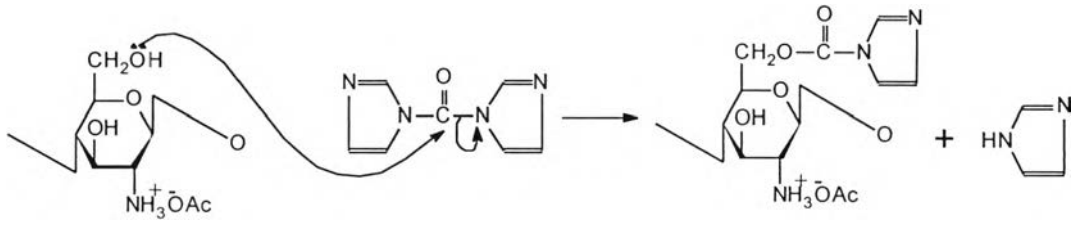

Figure 4.7 Reaction mechanism between chitosan acetate and CDI.

In this reaction, by-products were obtained as an imidazole. The purification process is necessary by thoroughly washing the obtained product with water and chloroform several times to eliminate imidazole and remove the excess CDI. The purified product was qualitatively confirmed with FTIR to confirm the absorbance of carbonyl peak of CDI, insisting the stability of the precursor.

4.5 Coupling reaction of chloramphenicol

By considering the appropriate model drug for controlled release system, the molecule should have a bioactive property and have some moieties for further modification. Moreover, it should be easily detected by any common

analytical techniques. In the present work, chloramphenicol was chosen as a model drug according to its antibiotic property, low cost and easy availability. Nowadays, chloramphenicol has been used in opthalmic therapy. For the modification aspect, this model drug also has a hydroxyl group in the structure which is suitable for further modification with chitosan having an active ester from CDI. In addition, a chromophore of benzene ring in the structure can be easily characterized by UV-VIS spectroscopic method. Precisely, this model drug shows the toxicity in high concentration and easy deactivated in the gastrointestinal tracts.

The coupling reaction between chloramphenicol and chitosan carbonyl imidazolide was found to progress in mild condition at 40^{o}C for overnight. As a result, the degradation of chloramphenicol can be avoided. Similar to the coupling reaction of CDI onto chitosan, the reaction proceeded heterogeneously and magnesium methoxide was used as a catalyst. However, the amount of chloramphenicol was two equivalent mole larger than in the case of chitosan carbonyl imidazolide.

The product from 4.3.3 was then reacted with chloramphenicol to obtain the pale yellow powder product. The IR spectrum of the obtained product is shown in Figure 4.8.

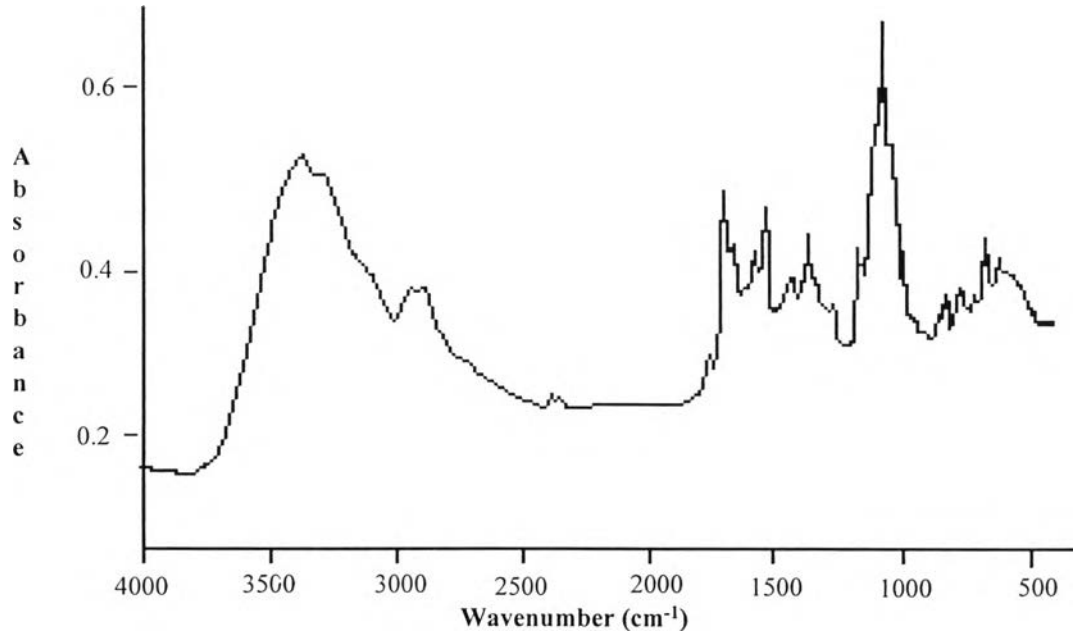

Figure 4.8 FTIR spectrum of chitosan-chloramphenicol conjugate.

FTIR (KBr, cm^{-1}) :- 3400 (O-H stretch.); 2900 (C-H stretch.);
1750 (C=O imidazole stretch.);
1690 (C=O chloramphenicol);
1654,1560 (C=Oamide);
1520 (Characteristic of Chloramphenicol);
~1100 (pyranose).

Comparing to the IR spectra of chloramphenicol and chitosan, this conjugate shows both characteristic peaks of chloramphenicol (~1686 and 1520 cm^{-1}) and chitosan (~1100 cm^{-1}). Moreover, the peak at 1750 cm^{-1}, which is the absorption of carbonyl of imidazolide, is reduced significantly (Figure 4.9). This result can be concluded that the chloramphenicol is chemically bound to the chitosan.

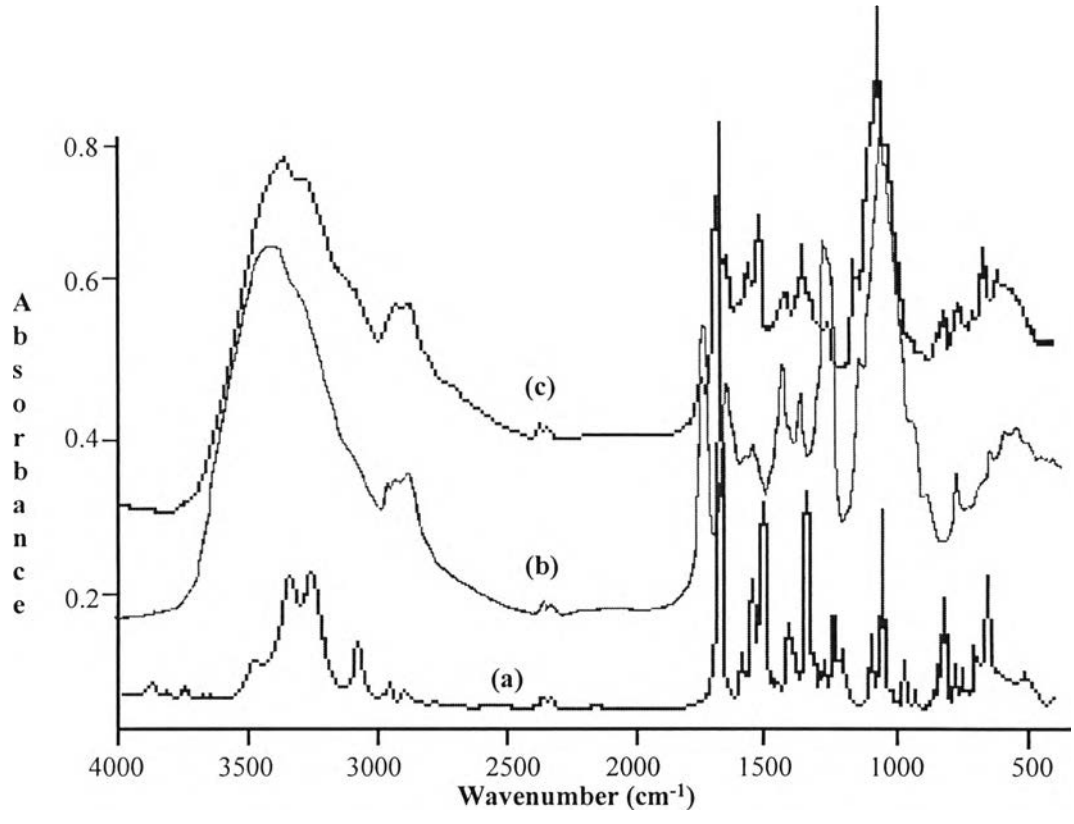

Figure 4.9 Compared FTIR spectra of (a) chloramphenicol; (b) chitosan having carbonyl imidazolide; (c) chitosan-chloramphenicol conjugate.

Dumitriu (1989) studied the coupling between chloramphenicol and ampicillin onto xanthan by activation using DCC. The present work is originally used CDI as a coupling agent instead of DCC to eliminate the limitation from DCC such as a *N*-acyl urea formation as by-product.

4.6 Release studies from chitosan-chloramphenicol conjugate

In order to study the release of model drug comparing to the physical insertion system (see 4.2), the chemical conjugation also should be studied as a bead type. However, during bead preparation, the alkali-ethanol solution has to be used and it may cause the release of chloramphenicol owing to the hydrolysis of ester bond as concerned to the conjugate structure. Thus, in the present studies, the release of chloramphenicol was studied directly from the obtained powder of chitosan-chloramphenicol conjugate.

The release profile of the chitosan-chloramphenicol conjugate in various pH is shown in Figure 4.10.

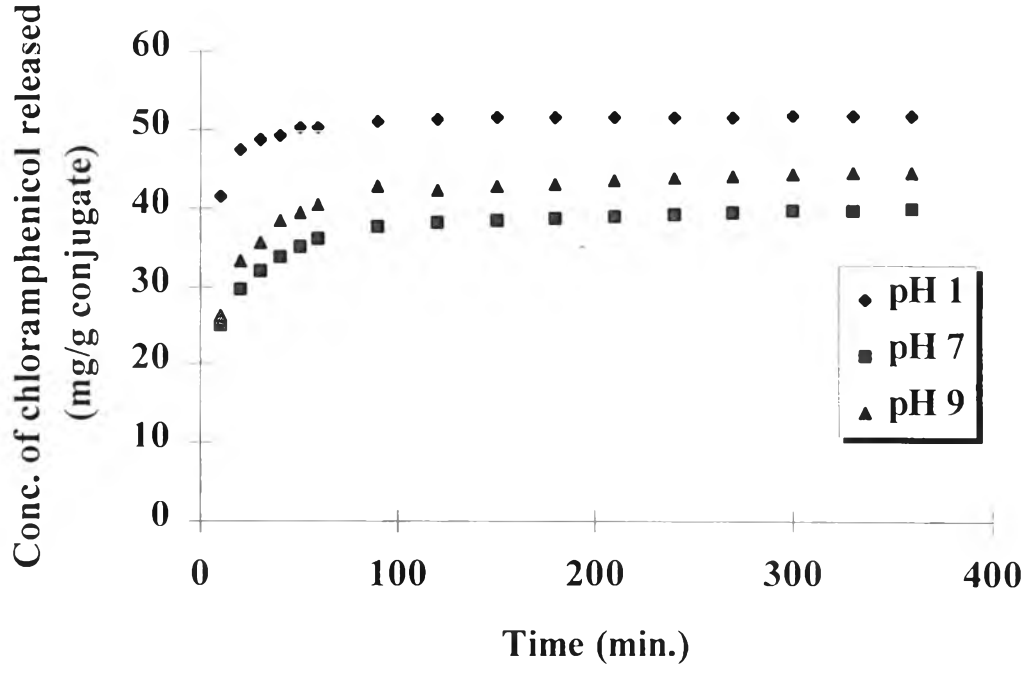

Figure 4.10 Release profile of chitosan-chloramphenicol conjugate in pH 1, 7 and 9 solution at room temperature.

As shown in Figure 4.10, the release of chloramphenicol from the conjugate is found to be significant in the order of acid, base and neutral condition respectively. In acid condition, the release of chloramphenicol is increased rapidly at initial twenty minutes and reaches to approximately 50 mg/g conjugate within 100 minutes. For base and neutral condition, the release is achieved for 40 mg/g conjugate in the first 60 minutes with the less release rate comparing to that of acid condition. It should be noted that after 100 minutes, the release cannot be observed.

It is our interest to consider the release owing to the differences of mechanisms referred to the pH. Based on the hydrolysis mechanisms, ester compounds can be easily hydrolyzed both in acid or base condition since the ester hydrolysis is usually catalyzed by acids or bases (March, 1992). When base catalyzes the reaction, the attacking species is more powerful nucleophile, OH^-. For acid, it catalyzes the reaction by making the carbonyl carbon more positive via protonation and therefore more susceptible to attack by the nucleophile, which is the water molecule. Moreover, the ease of ester hydrolysis also depends on the steric effect.

Since the chitosan-chloramphenicol conjugate is an ester compound of carbonic acid. The ester hydrolysis should be expected and the released amount of chloramphenicol depends on the reaction mechanism. The release mechanism of the conjugates can be proposed as shown in Figure 4.11.

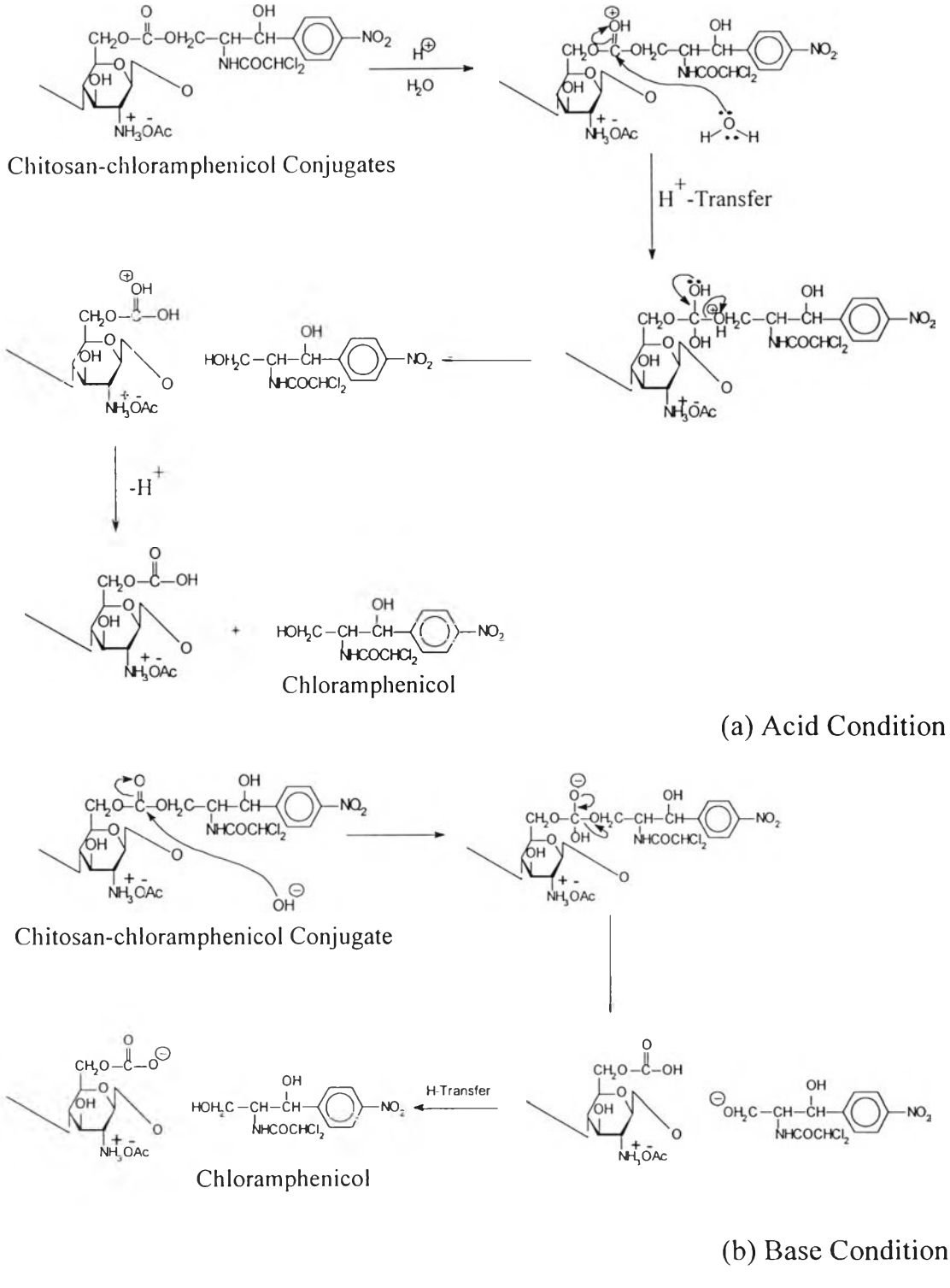

Figure 4.11 Release mechanism of chitosan-chloramphenicol conjugate via the hydrolysis reaction for both (a) acid; (b) base condition.

Both acid and base in the media will act as the catalyst for the hydrolysis reaction. This caused the more release of chloramphenicol from conjugate comparing to the neutral condition.

As mentioned above, the ester hydrolysis does not depend only on the acid or base catalyst, but also the steric hindrance. It should be considered that for acidic condition, the swelling of conjugates was found in the release process. This may be due to the ease occuring of ester hydrolysis because the steric hindrance from hydrogen bonding between chain is insignificant. As a result, the conjugate was swelled and tended to deform after the release process for the initial step. In contrast, for base condition, there was no swelling of conjugates. Thus, the more release of chloramphenicol in acidic condition may be due to the fact that the conjugates is more swell which leads to the ease of hydrolysis reaction to occur. It should be noted that after the release, the conjugates were found to be swollen and formed as a gel. In the case of base condition, the gel forming could not be obsesrved.