

# SYNTHESIS OF THIOLATED CHITOSANS: PROMISING POLYMERS FOR PROLONGED MUCOADHESIVE DRUG DELIVERY

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**ABSTRACT:** The successful development of multifunctional drug delivery systems is mainly based on the development and evaluation of new mucoadhesive polymers. Chitosan is a natural polycationic copolymer consisting of glucosamine and N-acetylglucosamine units. The polymer has valuable properties as a biomaterial because it is considered to be biocompatible, biodegradable and non-toxic. The derivatization of the primary amino groups of chitosan with coupling reagents bearing thiol functions leads to the formation of thiolated chitosans. Various properties of chitosan are improved by this immobilization of thiol groups. Due to the formation of disulfide bonds with mucus glycoproteins, the mucoadhesiveness is augmented. The permeation of paracellular markers through mucosa can be enhanced utilizing thiolated instead of unmodified chitosan. Moreover, thiolated chitosans display *in situ* gelling features, due to the pH dependent formation of inter- as well as intramolecular disulfide bonds. This latter process provides a strong cohesion and stability of carrier matrices being based on thiolated chitosans and guarantees a prolonged controlled release of embedded therapeutic ingredients.

**KEY WORDS:** Thiomers, derivatization, mucoadhesion, cohesiveness, *in vivo*, disulfide bonds.

## INTRODUCTION

Mucoadhesion can be defined as the ability of synthetic or biological macromolecules to adhere to mucosal tissues. Since the early 1980s the concept of mucoadhesion has gained considerable interest in pharmaceutical technology. If this concept can gain its full potential, it might open the door for novel, highly efficient dosage forms especially in oral drug delivery. Mucoadhesive drug delivery systems promise several advantages that arise from the localization at a given target site (I), a prolonged residence time at the site of drug absorption (II), and an intensified contact with the mucosa increasing the drug concentration gradient (III).<sup>1</sup> Hence, the uptake and subsequently the bioavailability of the drug may be increased, the frequency of dosing should be reduced, and as a result the patient compliance improved. Accordingly, various natural and synthetic polymers have been discovered as mucoadhesive excipients so far. Their mucoadhesive properties can be

explained due to the interaction with the glycoproteins of the mucus mainly based on non-covalent bonds such as ionic interactions, hydrogen bonds and van der Waal's forces.<sup>2</sup>

The biopolymer chitosan is obtained by alkaline deacetylation of chitin, which is one of the most abundant polysaccharides in nature. Shell wastes of shrimp, lobster and crab are the main industrial sources of chitin.<sup>3</sup> Chitosan is a polysaccharide consisting of copolymers of glucosamine and N-acetylglucosamine. The primary amino group accounts for the possibility of relatively easy chemical modification of chitosan and salt formation with acids. At acidic pH the amino groups are protonated, which promotes solubility, whereas chitosan is insoluble at alkaline and neutral pH.<sup>4</sup> Because of its favorable properties, such as enzymatic biodegradability, non-toxicity and biocompatibility,<sup>3</sup> chitosan has received considerable attention as a novel excipient in drug delivery systems, and has been included in the European Pharmacopoeia since 2002. So far, chitosan has been

utilized in various fields of pharmaceutical technology, including the formulation of controlled release dosage forms, such as tablets, gels and microspheres, as mucoadhesive and/or permeation enhancing excipient for oral, nasal, ocular and buccal drug delivery<sup>5-8</sup> and in non-viral gene delivery.<sup>9,10</sup>

Recently it could be shown that polymers with thiol groups provide much higher adhesive properties than polymers generally considered to be mucoadhesive. The enhancement of mucoadhesion can be explained by the formation of covalent bonds between the polymer and the mucus layer which are stronger than non-covalent bonds. These thiolated polymers, or the so-called *thiomers*, are supposed to interact with cysteine-rich subdomains of mucus glycoproteins via disulfide exchange reactions<sup>11</sup> or via simple oxidation process as shown in Fig. 1.

To further enhance the solubility of chitosan and to improve its mucoadhesive and/or permeation enhancing properties, various derivatives such as trimethylated chitosan,<sup>12</sup> mono-N-carboxymethyl chitosan,<sup>13</sup> N-sulfochitosan<sup>14</sup> and chitosan-EDTA conjugates<sup>15</sup> were developed. A further modification is based on the immobilization of thiol bearing moieties on the polymeric backbone of chitosan. To date, four different thiolated chitosan derivatives have been synthesized: chitosan-thioglycolic acid conjugates,<sup>16,17</sup> chitosan-cysteine conjugates,<sup>18</sup> chitosan-4-thio-butyl-amidine (chitosan- TBA)<sup>19</sup> and chitosan-thio-ethyl-amidine (chitosan- TEA) conjugates.<sup>20,21</sup>

These thiolated chitosans have numerous advantageous features in comparison to unmodified chitosan, such as significantly improved mucoadhesive and permeation enhancing properties.<sup>18-23</sup> The strong cohesive properties of thiolated chitosans make them highly suitable excipients for prolonged controlled drug release dosage forms.<sup>19,24</sup> Moreover, solutions of thiolated chitosans display *in situ* gelling properties at physiological pH values.<sup>17</sup>

The aim of this article is to provide synthesis of different thiolated chitosans that can be used for the formulation of mucoadhesive dosage forms which increases the mucoadhesiveness, cohesiveness and swelling power of the parent polymer chitosan, significantly with proof utilizing various *in vitro* and *in vivo* test systems.

## SYNTHESIS OF THIOLATED CHITOSANS

The primary amino group at the 2-position of the glucosamine subunits of chitosan is the main target for the immobilization of thiol groups. Sulfhydryl bearing agents can be covalently attached to this primary amino group via the formation of amide or amidine bonds. In case of the formation of amide bonds the carboxylic acid group of the ligands cysteine and thioglycolic acid reacts with the primary amino group of chitosan.<sup>2</sup> The formation of disulfide bonds by air oxidation during the

synthesis is avoided by performing the process at a pH below 5. At this pH-range the concentration of thiolate anions, representing the reactive form for oxidation of thiol groups, is low, and the formation of disulfide bonds can be almost excluded. Alternatively, the coupling reaction can be performed under inert conditions.

In the case of the formation of amidine bonds 2-iminothiolane and thio ethyl amidine are used as a coupling reagent.<sup>19</sup> These offers the advantage of a simple one step coupling reaction. In addition, the thiol group of the reagent is protected towards oxidation because of the chemical structure of the reagent. Orientating studies with all these thiolated chitosans showed that a degree of modification of 25–250 mmol thiol groups per gram chitosan leads to the highest improvement in the mucoadhesive and permeation enhancing properties. The amount of immobilized thiol groups in reduced and oxidized form can be determined via Ellman's reagent<sup>17</sup> with and without previous quantitative reduction of disulfide bonds with borohydride.<sup>23</sup> The simple reaction scheme for all the four thiolated chitosan preparation are shown in Fig.2.

## PROPERTIES OF THIOLATED CHITOSANS MUCOADHESIVE PROPERTIES

The improved mucoadhesive properties of thiolated chitosans are explained by the formation of covalent bonds between thiol groups of the polymer and cysteine rich subdomains of glycoproteins in the mucus layer.<sup>24</sup> These covalent bonds are supposedly stronger than noncovalent bonds, such as ionic interactions of chitosan with anionic substructures of the mucus layer. This theory was supported by the results of tensile studies with tablets of thiolated chitosans, which demonstrated a positive correlation between the degree of modification with thiol bearing moieties and the adhesive properties of the polymer.<sup>2,20</sup> These findings were confirmed by another *in vitro* mucoadhesion test system, where the time of adhesion of tablets on intestinal mucosa is determined. The contact time of the thiolated chitosan derivatives increased with increasing amounts of immobilized thiol groups.<sup>2,19</sup>

With chitosan-thioglycolic acid conjugates a 5–10-fold increase in mucoadhesion in comparison to unmodified chitosan was achieved. The mucoadhesive properties of chitosan-TBA (chitosan-4-thio-butyl-amidine) conjugates were even further improved. One explanation for this phenomenon can be given by the theory that chitosan-TBA conjugates have additionally increased mucoadhesive properties due to improved ionic interactions between the additional cationic amidine substructure of the conjugate and anionic substructures within the mucus layer. Tensile studies with chitosan-TBA conjugates of low, medium and high molecular mass (150, 400 and 600 kDa) furthermore indicated that medium molecular mass thiolated chitosans display the relatively, the highest mucoadhesiveness. Utilizing a

medium molecular mass chitosan-TBA conjugate displaying 264 mM thiol groups per gram polymer consequently led to a more than 100-fold improvement in mucoadhesion in comparison to unmodified chitosan. This represents the greatest so far made progress in the development of mucoadhesive polymers.<sup>20</sup>

### PERMEATION ENHANCING EFFECT

In 1994 Illum *et al.* showed the permeation enhancing capabilities of chitosan for the first time.<sup>6</sup> Chitosan is able to enhance the paracellular route of absorption, which is important for the transport of hydrophilic compounds such as therapeutic peptides and antisense oligonucleotides across the membrane. Various studies carried out on Caco-2 cell monolayers demonstrated a significant decrease in the transepithelial electrical resistance after the addition of chitosan.<sup>25-27</sup> The mechanism underlying this permeation enhancing effect seems to be based on the positive charges of the polymer, which interact with the cell membrane resulting in a structural reorganization of tight junction-associated proteins.<sup>28</sup> In the presence of the mucus layer, however, this permeation enhancing effect is comparatively lower, as chitosan cannot reach the epithelium because of size limited diffusion and/or competitive charge interactions with mucins.<sup>29</sup> Nevertheless, these results obtained on Caco-2 cell monolayers could be confirmed by *in vivo* studies, showing an enhanced intestinal absorption of the peptide drug busserelin in rats due to the co-administration of chitosan hydrochloride.<sup>30</sup>

The permeation enhancing effect of chitosan can be strongly improved by the immobilization of thiol groups. This effect of thiolated chitosans could meanwhile be shown in various permeation studies in Ussing<sup>18</sup> type chambers using freshly excised intestinal mucosa. The uptake of fluorescence labeled bacitracin, for instance, was improved 1.6-fold utilizing 0.5% of chitosan-cysteine conjugate instead of unmodified chitosan.<sup>18</sup> In another study the permeation enhancing effect of chitosan-TBA in comparison to the permeation enhancing effect of unmodified chitosan was shown. The uptake of the cationic marker compound rhodamine 123 was 3-fold higher in the presence of thiolated chitosan versus unmodified chitosans.<sup>21</sup>

Chitosan-TEA improves mucoadhesive properties along with a controlled release out of the polymer matrix have rendered chitosan-TEA as a promising tool, which increases drug bioavailability by adhering to mucosal tissues. It has been shown that the presystemic metabolism of orally given peptide drugs can be reduced, if the delivery system provides an intimate contact with the intestinal mucosa. For instance, *in vivo* studies has shown that the use of a chitosan-TEA carrier matrix provides a significantly enhanced oral bioavailability of several model drugs.<sup>20,21</sup>

The likely mechanism responsible for this improved permeation enhancement has been attributed to the inhibition of protein tyrosine phosphatase. This enzyme seems to be involved in the opening and closing process of the tight junctions. Protein tyrosine phosphatase is responsible for the dephosphorylation of tyrosine subunits of occludin, representing an essential transmembrane protein of the tight junctions. When these tyrosine subunits of occludin are dephosphorylated, the tight junctions are closed. In contrast, when these tyrosine subunits are phosphorylated, the tight junctions are opened. The inhibition of protein tyrosine phosphatase by compounds such as phenylarsine oxide, pervanadate or reduced glutathione leads consequently to a phosphorylation and opening of the tight junctions.<sup>31-33</sup> In contrast to the stable but toxic protein tyrosine phosphatase inhibitors phenylarsine oxide and pervanadate, the inhibitory effect of glutathione is lower as it is rapidly oxidized on the cell surface losing its inhibitory activity.<sup>34</sup> Due to the combination of reduced glutathione with thiolated chitosans, however, this oxidation of the inhibitor on the membrane can be restricted, as thiomers are capable of reducing oxidized glutathione.<sup>31</sup>

### THIOLATED CHITOSANS AS MATRICES FOR CONTROLLED DRUG RELEASE

Chitosan represents, primarily due to its mucoadhesive properties, a valuable tool for non-invasive drug delivery.<sup>5</sup> The longer residence time of formulations based on mucoadhesive polymers at the absorption site is believed to contribute to an increased absorption rate of the incorporated drug. However, such an enhanced bioavailability can be achieved only if a controlled release of the active agent out of the formulation is provided.

Thiolated chitosans also display, beside their strong mucoadhesive and permeation enhancing properties, excellent cohesive properties. The reduced thiol functions on the chitosan backbone enable thiolated chitosans not only to form disulfide bonds with mucus glycoproteins, but also to form inter- as well as intra-molecular disulfide bonds. Such a crosslinking of the polymeric chains results in a high stability of drug carrier systems based on thiolated chitosans.<sup>20,21</sup>

The cohesion and stability of a drug delivery system over the intended duration of drug liberation is often a substantial requirement for a controlled release. The usefulness of thiolated chitosans as carrier matrices for controlled drug release was demonstrated by means of model drugs, like clotrimazole<sup>19-22</sup> or salmon calcitonin.<sup>36-37</sup>

Clotrimazole is well-established as an antimycotic drug in the treatment of vaginal infections. In order to improve its therapeutic efficacy, a sustained release of the drug over a period of several days might be highly beneficial. The release of clotrimazole out of

matrix tablets based on either chitosan-thioglycolic acid conjugate or chitosan-TBA conjugate was quantified. Both thiolated chitosan tablets remained stable during the whole experiment (6 hours) and no disintegration could be observed. However, only the chitosan-TBA conjugate was able to guarantee a significant delay in the drug release in comparison to unmodified chitosan, leading to a sustained release over a much longer time period.<sup>19-22</sup>

The release profile of salmon calcitonin out of matrix tablets based on the chitosan-TBA conjugate was determined. A pseudo zero order release profile of salmon calcitonin over the first 8 hours was observed in an simulated intestinal fluid. During the experiment the tablets swelled continuously, maintaining a good cohesiveness and releasing the active agent via a controlled diffusion process.

These release studies, in which a peptide drug was liberated from a thiolated chitosan matrix system permit information concerning the chemical events within the formulation to be gained. Strong unintended interactions between the polymeric matrix system and the peptide drug could be excluded according to this controlled and sustained release profile.<sup>37</sup>

The study design of authors by formulating cefuroxime axetil mucoadhesive matrix tablets and microspheres exhibit prolonged controlled drug release. The cumulative release of cefuroxime axetil matrix tablets released 89.93% at the end of 72 hours and the microspheres released 96.15% at the end of 72 hours shown in the Fig. 3 and Fig. 4 respectively. Both studies confirm that a controlled drug release out of thiolated chitosan drug carrier system can be achieved.

### **IN SITU GELLING PROPERTIES**

Rapid clearance from the site of drug action is one important factor that limits the efficacy of drugs administered to the ocular, nasal and vaginal mucosa. It is widely accepted that limiting the clearance by increasing the viscosity of a drug formulation will result in an increased bioavailability of these drugs. A very promising strategy to obtain drug formulations of sufficient viscosity is based on *in situ* gel formation. The formation of a gel at the site of drug delivery combines the advantages of a solution, which can be easily administered, with the favorable viscoelastic properties of a gel, providing a prolonged residence time of the formulation. The sol-gel transition occurs in the physiological environment as a result of physicochemical changes, such as changes in the pH,<sup>38</sup> in temperature<sup>38,39</sup> or in electrolyte concentration.<sup>40,41</sup> Thiolated chitosans display *in situ* gelling properties due to the oxidation of thiol groups at physiological pH-values, which results in the formation of inter- and intramolecular disulfide bonds. This cross-linking process can be observed within a pH range of 5-6.8.<sup>2</sup>

The *in situ* gelling behavior of thiolated chitosans was characterized *in vitro* by rheological measurements. The sol-gel transition of thiolated chitosans at pH 5.5 was completed after 2 hours, when highly cross-linked gels were formed. In parallel, a significant decrease in the thiol group content of the polymers was observed, indicating the formation of disulfide bonds.<sup>17,19</sup> The rheological properties of unmodified chitosan remained constant over the whole observation period. Rheological investigation of thiolated chitosans furthermore demonstrated a clear correlation between the total amount of polymer-linked thiol groups and the increase in elasticity of the formed gel. The more thiol groups were immobilized on chitosan, the higher was the increase in elastic modulus in solutions of thiolated chitosan.<sup>17,19</sup>

Thiolated chitosan derivatives therefore seem to be promising new excipients for liquid or semisolid formulations, which should stabilize themselves once applied on the site of drug delivery. The *in situ* gel formation within a pH range from 5 to 6.8 makes the application of thiolated chitosans on vaginal, nasal and ocular mucosa possible.

### **IN VIVO STUDIES: PROOF OF CONCEPT**

The potential of thiolated chitosans for the oral administration of hydrophilic macromolecules could meanwhile be shown by various *in vivo* studies.<sup>35,36</sup> As model drug, for instance, salmon calcitonin was utilized, which is a peptide drug of cationic net charge and a molecular mass of 3.2 kDa. Salmon calcitonin is used for the treatment of chronic bone diseases.<sup>37,42</sup> It is currently marketed in nasal spray and injectable forms, both having the drawback of a low patient acceptance. A higher patient compliance should be achieved by the application of an oral delivery system for this drug. However, the oral bioavailability thus far obtained is too low to permit therapeutic employment.<sup>43</sup> Therefore; this peptide was regarded as a challenging model drug for testing the potential of thiolated chitosans.

Different drug carrier matrices, comprising chitosan-TBA conjugate as substantial polymeric excipient and containing equal amounts of salmon calcitonin and optionally the permeation mediator reduced glutathione, were developed. In order to avoid an enzymatic degradation of the peptide drug in the gastrointestinal tract chitosan-enzyme inhibitor conjugates were added. All compounds were homogenized and directly compressed to tablets. To enteric coated tablets targeted to the small intestine, a chitosan-BBI conjugate (Bowman-Birk inhibitor)<sup>44</sup> and a chitosan-elastatinal conjugate<sup>45</sup> were added. Furthermore, an alternative strategy was evaluated, focusing on a targeted drug release and absorption in the stomach. Tablets targeted to the stomach contained a chitosan-pepstatin A conjugate,<sup>36</sup> which should avoid pepsinic

igestion of salmon calcitonin. In order to prevent mucoadhesion in the oral cavity and oesophagus these tablets were coated with a triglyceride.

The different tablets were orally given to rats and the plasma calcium level was monitored as a function of time. Pharmacological efficacy was calculated on the basis of the area under the reduction in plasma calcium levels of the oral matrix tablets versus intra venous injection.

The main biofeedback parameters after application of the drug carrier matrices for the oral delivery of salmon calcitonin are shown in Table 1. *In vivo* studies showed no statistically significant ( $P < 0.05$ ) reduction of the plasma calcium level caused by salmon calcitonin, which was orally given in solution. Furthermore, no significant effect was observed after oral administration of tablets comprising the peptide drug and unmodified chitosan, although the native polymer is reported to be mucoadhesive and to exhibit a permeation enhancing effect for hydrophilic macromolecules<sup>46</sup> (see Table 1).

Table 1 shows that the presence of the chitosan-TBA conjugate is essential for calcitonin absorption, since only tablets being based on the thiolated chitosan caused a decrease of plasma calcium level of more than 5% for several hours. The increased absorption of the peptide, when embedded in a thiolated chitosan matrix, occurs due to the properties of the polymer derivative: the high stability and cohesiveness can provide a sustained release of the peptide,<sup>47</sup> while the mucoadhesive features should lead to a prolonged residence time of the dosage form on the site of absorption. Moreover, the combination of thiolated chitosan with the permeation mediator, reduced glutathione, seems to have an impact on the bioresponse of orally given calcitonin. The significantly higher pharmacological efficacy of thiolated chitosan tablets containing glutathione in comparison to corresponding tablets without glutathione (see Table 1) indicates that glutathione contributes to the drug absorption process. These results are in good agreement

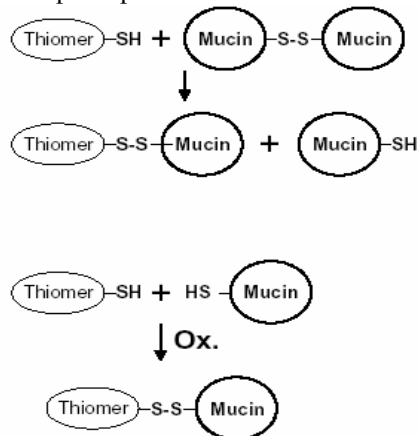
with *in vitro* results demonstrating that thiomers for each show a strong permeation enhancing effect, which can be further improved by the addition of glutathione.<sup>31</sup> Therefore, the high *in vivo* efficacy of thiolated chitosans can be additionally raised by the use of glutathione.

Among all thiolated chitosan formulations, stomach targeted tablets based on chitosan-TBA conjugate with the addition of both glutathione and chitosan-pepstatin A conjugate showed the strongest effect. They led to a decrease of the plasma calcium level of more than 10% for at least 12 hours, thus demonstrating the validity of the systemic peptide delivery via the stomach. Moreover, a faster and more reproducible onset of action was obtained by this novel approach.<sup>35,36</sup>

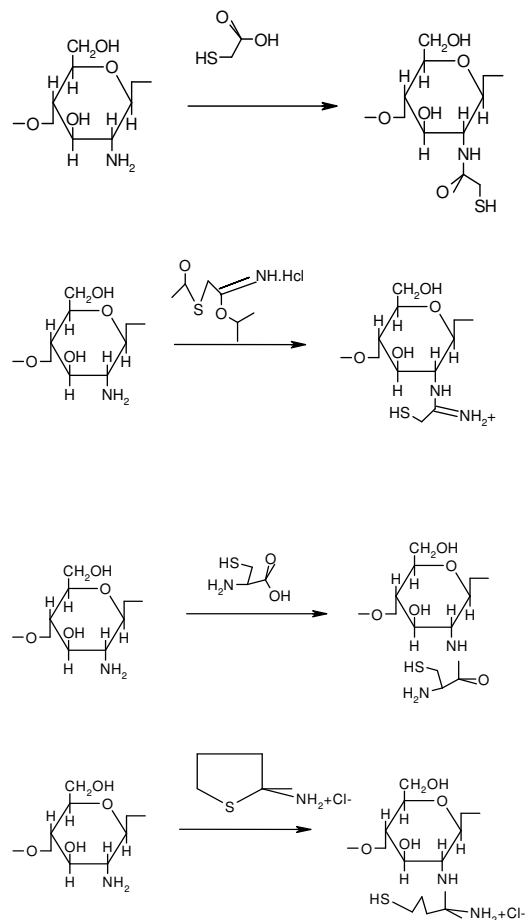
According to these results the applicability of thiolated chitosans for the oral administration of other peptide drugs seems also likely and is the subject of ongoing studies.

## CONCLUSION

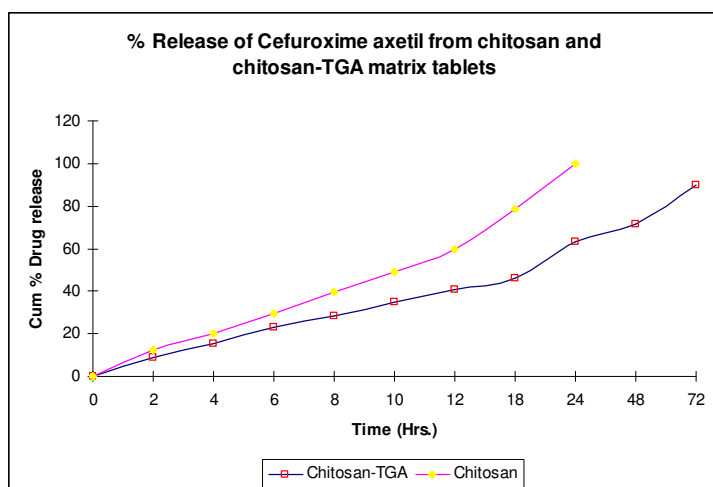
The chemical modification of chitosan via derivatization with various reagents bearing sulfhydryl functions causes a dramatic change in the polymer's properties. Mucoadhesiveness and cohesiveness are strongly improved. In view of drug delivery, these improved mucoadhesive properties along with a controlled release out of the thiolated polymer matrix should render these thiolated chitosans as promising tool, which might increase drug bioavailability by adhering to mucosal tissues. Furthermore, thiolated chitosans display *in situ*-gelling features and facilitate a controlled drug release. Due to these advantageous features thiolated chitosans have been successfully used for peroral administration of peptide drugs and other therapeutic agents. They seem to represent a promising new generation of polymeric excipients in particular for the non-invasive administration of hydrophilic macromolecular drugs.



**Fig.1: Mechanism of disulfide bond formation between thiomers and mucus glycoproteins (mucins).**



**Fig. 2: Synthetic pathway for the preparation of thiolated chitosans (Chitosan-Thioglycolic acid, Chitosan-4-Thio-Butylamidine, Chitosan-Cysteine and Chitosan-2-Iminothiolane conjugates.**



**Fig. 3: Release profile of cefuroxime axetil matrix tablets.**

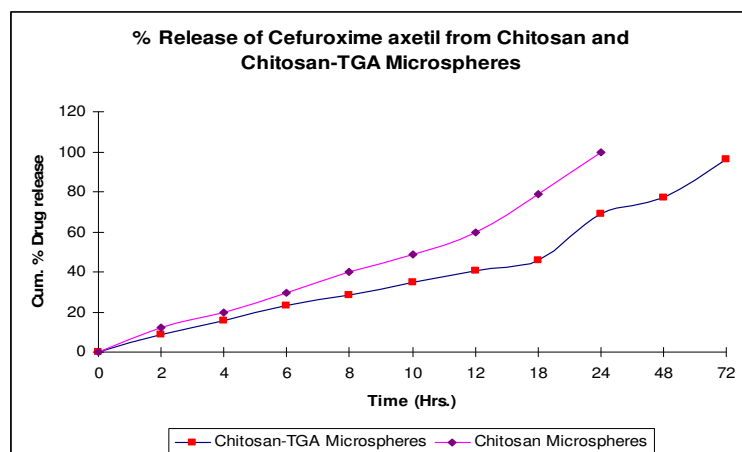


Fig. 4: Release profile of cefuroxime axetil from mucoadhesive microspheres.

Table 1: Main biofeedback parameters after oral administration of tablets containing all equal amounts of Salmon Calcitonin to rats (n=5).

Tablet composition	Maximal reduction of Ca-level (% of initial value)	Time point of maximal reduction of Ca-level (hour)	Pharmacological efficacy (%)
Small Intestine targeted tablets (2 mg) <i>Chitosan-TBA conjugate</i> , Citosan-BBI conjugate, Chitosan-elastatinal conjugate, glutathione.	89.9	12	1.3
<i>Chitosan-TBA conjugate</i> , Citosan-BBI conjugate, Chitosan-elastatinal conjugate.	91.0	12	0.9
<i>Unmodified chitosan.</i>	a	-	0
Stomach targeted tablets (5 mg) <i>Chitosan-TBA conjugate</i> , glutathione Chitosan-pepstatin A conjugate.	88.8	6	1.3
<i>Unmodified chitosan.</i>	a	-	0

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