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Use of the ninhydrin assay to measure the release of chitosan from oral solid dosage forms

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Abstract

This study evaluated and optimised the ninhydrin assay as a tool for measuring the in vitro release and dissolution of chitosan from solid dosage forms. The precision and accuracy of the assay for the type of chitosan used in the study were examined by measuring the inter- and intra-sample variation and found to be within acceptable limits. The assay was applied practically to construct a pH/solubility profile for chitosan and subsequently to measure the release and dissolution of chitosan from dosage forms in the presence and absence of a model drug, sodium salicylate. Assay performance was found to be satisfactory over a wide range of physiologically relevant pH values. It is concluded that the ninhydrin assay is an essential aid in the design and testing of solid dosage forms with different chitosan–drug release profiles.
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Keywords: Chitosan; Ninhydrin assay; pH/solubility profile; Tablet dissolution

1. Introduction

Chitosan is the term given to a family of high molecular weight cationic polysaccharides derived from chitin that naturally occurs in crustacean shells. The repeating units in chitosan are a 2-deoxy-2-(acetylamino) glucose and a 2-deoxy-2-amino glucose linked by glycosidic bonds into a linear polymer (Fig. 1). Different chitosans can vary in their degree of polymerisation and their degree of deacetylation.

Chitosan is a weak base and this characteristic gives its distinctive solubility properties. At acidic pH values the amino groups become protonated, causing the chitosan chain to uncoil and become soluble. As pH values increase above its pK_a of approximately 6.5 (Schipper et al., 1996) chitosan loses this charge, coils up and is likely to precipitate from solution.

Chitosan has considerable potential for many pharmaceutical applications (Illum, 1998). Many studies have investigated the use of chitosan in the design of sustained release dosage forms, such as matrix tablets, and have shown that, in general, chitosan provides excellent sustained release properties in vitro especially at low pH values where a gel matrix is formed (Kawashima et al., 1985; Miyazaki et al., 1981). The higher the concentration of chitosan the harder the tablets produced and the more sustained the release of drug (Upadrashta et al., 1992). At higher pH values (above pH 7), where chitosan is insoluble, rapid disintegration of such tablets occurs (Inoyatov et al., 1998). Other researchers have found chitosan to be useful as

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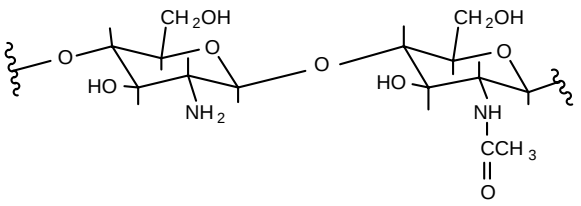

Fig. 1. Structure of chitosan.

a tablet binder (Kepsutlu et al., 1999), or a direct compression excipient (Sawayanagi et al., 1982) in solid dosage forms.

More interestingly, it has been demonstrated that chitosan can act as a transmucosal absorption enhancer for therapeutic compounds that are poorly absorbed through, for example, the nasal or gastrointestinal membranes (Illum et al., 1994). Such compounds are typically hydrophilic with a large molecular weight and include many proteins, peptides and polysaccharides (Illum, 2003). Chitosan needs to be in solution or gelled to exert its absorption-enhancing action. It is believed that its activity results from the interaction of the positively charged amino groups on the C-2 positions of chitosan with negatively charged components in the cell membrane thereby activating the protein kinase C pathway (Natsume et al., 2003), resulting in transient and reversible opening of the intercellular tight junctions followed by enhanced paracellular transport of compounds. The "bioadhesive nature" of chitosan may also play a role by prolonging the contact between the drug compound and the membrane (Soane et al., 1999).

From other studies (Lee et al., 2000), it appears that for successful oral absorption promotion, high local concentrations of drug and enhancer must co-exist at the relevant site in the gastrointestinal tract (GIT). However, unlike the nasal or rectal routes, the motility in the intestine is extensive and large amounts of fluids and mucus are being secreted throughout the GIT. This can lead to rapid dilution of the enhancer and the drug (Baluom et al., 2000). Therefore, for the improvement of drug absorption a formulation that disintegrates and releases the chitosan and drug rapidly and simultaneously is preferable to a sustained release formulation. As a result, an assay method is needed which can measure the rate of release of chitosan and compare it directly to the rate of release of drug from the dosage form.

Most quantitative assay methods for polysaccharides involve hydrolysing the polymers to their constituent monomers and then quantifying those monomers (Lamps et al., 1995; Bosworth and Scott, 1994). However, this approach is difficult for chitosan as the beta-glycosidic linkages between the chitosan monomers are very difficult to hydrolyse due to the presence of positively charged amino groups (Prochazkova and Varum, 1999). Other methods which could be adapted as quantitative assay methods for chitosan include first derivative ultraviolet spectrophotometry or infra-red spectroscopy (Muzzarelli et al., 1997).

Colourimetric methods appear to offer promise as a means of quantifying chitosan. Anionic dyes have been shown to interact with the positively charged amine groups on the chitosan molecule (Roberts, 1997). An assay method using one such dye, Cibacron brilliant red 3B-A was found to be linear and reproducible for six different types of chitosan tested (Muzzarelli, 1998). An alternative colourimetric method involves the use of ninhydrin. The reaction of ninhydrin (triketohydrindene hydrate) with a primary amino group to form a coloured product, diketohydrindylidene-diketohydrindamine (Ruhemann's Purple) has been known and studied for years and is extensively used for amino acid analysis (Moore and Stein, 1948). When this assay was first used to quantify chitosan (Curotto and Aros, 1993) it was demonstrated that the assay could be used reliably in the quantification of the chitosans studied. Overall, it was found that the method was rapid, sensitive and reproducible but very dependent on the type of chitosan used. Chitosans with different degrees of deacetylation and molecular weight gave rise to different results being obtained. It was shown that the colour yield per mole of amino groups decreased with an increase in the percentage of free amino groups. In addition, the colour yield per mole of amino groups decreased with an increase in the degree of polymerisation up to a degree of polymerisation of 14, after which it was essentially constant. The authors concluded that the most likely reason for these phenomena was the occurrence of side reactions between some amino groups and ninhydrin or ninhydrin intermediates (Prochazkova and Varum, 1999). Another difficulty lay in the fact that if the formulation or medium contained materials with amine groups, a false positive result could occur leading to overestimation of the chitosan content.

Since the results obtained using the ninhydrin assay would differ for different types of chitosan, the work presented here involved construction of a calibration curve and evaluation of the performance of the ninhydrin assay with the particular chitosan base used in our formulations. Once this was accomplished, the assay was then used for quantification of chitosan in construction of a pH/solubility profile at a concentration relevant for dissolution testing. Then, the release of chitosan from solid dosage forms at physiologically relevant pH values was measured. This was carried out both for chitosan placebo tablets and also for tablets containing a model drug, sodium salicylate.

2. Materials and methods

2.1. Materials

Chitosan base (ChitoClear®) was obtained from Primex (Haugesund, Norway) with a reported degree of deacetylation of 93% and an approximate average molecular weight of 150,000 g/mol as measured by a sedimentation equilibrium method (Harding et al., 1991). Sodium salicylate was purchased from Fluka (Buchs SG, Switzerland). Ninhydrin, hydrindantin and lithium acetate were purchased from Sigma (Dorset, UK). Mannitol (Pearlitol SD 200) was a gift from Roquette Freres (Lestrem, France). All chemicals reagents and solvents used were of the highest grade available and used as provided.

2.2. Ninhydrin assay

Lithium acetate buffer (10 ml) was prepared by dissolving 4.08 g of lithium acetate dihydrate in approximately 6 ml of deionised water. The pH of the resulting solution was adjusted to 5.2 using glacial acetic acid and the volume adjusted to 10 ml with deionised water. The ninhydrin reagent was freshly prepared on the day of the assay by adding 4 M lithium acetate buffer (10 ml) to 0.8 g ninhydrin and 0.12 g hydrindantin in 30 ml DMSO. For the assay, 0.5 ml of reagent was added to 0.5 ml of the sample in a glass scintillation vial. The vials were immediately capped, briefly shaken by hand and heated in boiling water for 30 min to allow the reaction to proceed. After cooling, 15 ml of a 50:50 ethanol:water mixture was added to each vial. The vials were then vortexed for 15 s in order to oxidise the excess of hydrindantin. The absorbance of each solution was measured on a UV spectrophotometer (Agilent 8453) at 570 nm and the concentration of chitosan in the sample calculated from a standard calibration curve.

2.3. Assay validation

Fresh base standards were prepared each day for 5 days containing 1.5 mg/ml chitosan base in 0.1% acetic acid aqueous solution. This stock solution was diluted with 0.1% acetic acid aqueous solution to give 0.25, 0.50, 0.75, 1.00 and 1.50 mg/ml solutions of chitosan. All were subjected to analysis using the ninhydrin assay. A calibration curve was constructed on each day. In addition the 0.50, 0.75 and 1.00 mg/ml solutions from day one were tested on all subsequent days. Variation within and between samples was analysed in order to measure both the precision and accuracy of the assay.

2.4. pH/solubility profile

A 1.2 mg/ml chitosan solution was prepared by dissolving 120 mg of chitosan base in 0.176N HCl (15 ml of 1N HCl in 70 ml water). Then, 15 ml of 1N NaOH was added to the solution in order to adjust its salt concentration to that of physiological saline (0.15N). The pH of this solution was varied using either 0.1N HCl or 0.1N NaOH. After reaching the desired pH value, 0.5 ml samples were taken in triplicate and filtered through a 0.45 μm filter (Pall Acrodisc®). The samples were assayed using the ninhydrin assay as described above and a pH/solubility profile generated.

2.5. Tabletting

Constituents were bag blended for 10 min. Placebo blends contained 20% chitosan and 80% mannitol. Blends for sodium salicylate tablets contained 50% chitosan, 40% mannitol and 10% sodium salicylate. Tablets were compressed on a single station Manesty F3 tablet press using 6 or 9 mm round normal concave punches. Tablets were compressed to a target weight of 100 mg and a minimum hardness of 4 kp for the 6 mm tablets and 8 kp for the 9 mm tablets. Checks were carried out during tabletting to ensure that both

weight and hardness levels complied with these targets. Tablets of the same batch showed consistent dissolution behaviour with small standard deviations in the subsequent dissolution studies indicating that once a minimum hardness value had been obtained, dissolution was comparable.

2.6. Dissolution testing of chitosan placebo tablets

Tablets were tested in a Vankel Dissolution Bath VK100 in 250 ml of the relevant buffer-simulated gastric fluid USP pH 1.2, acetate buffer pH 3.8 or HEPES buffer pH 7.4. Six tablets were tested in triplicate at each pH value. The paddle apparatus (USP Type 2) was used at 100 rpm. The dosage forms were observed throughout testing and their disintegration behaviour noted. Samples were removed at periodic intervals and filtered through a 0.45 μm membrane filter (Pall Acrodisc®). Absorption of chitosan to the membrane filter was found to be negligible. The samples were assayed using the ninhydrin assay as described above.

2.7. Dissolution testing of chitosan/sodium salicylate tablets

Two chitosan tablets were placed in a gelatin capsule (size 0). Three capsules were tested using 250 ml of dissolution medium in each beaker. Testing was performed in triplicate. The paddle apparatus (USP Type 2) was used at 100 rpm. Dissolution tests were carried out at two different pH values—pH 7.2 HEPES buffer to mimic conditions in the small intestine and pH 5.6 acetate buffer to mimic conditions in the coecum and ascending colon. Two samples were taken at each timepoint and filtered through a 0.45 μm membrane filter (Pall Acrodisc®). One sample was subjected to the ninhydrin assay for chitosan content as described above, the other measured by UV at 297 nm to determine sodium salicylate content. At this wavelength, the gelatin capsules contributed negligibly to the UV absorption (0.04 absorbance units (AU)). Concentrations of the relevant components were determined from standard calibration curves.

Since all materials that contain amine groups can potentially react with the ninhydrin reagent to form Ruhemann's Purple all excipients used in the formulations were tested for false positive effects. It was found that only the gelatin capsules interfered in such a manner. In order to account for their contribution, three gelatin capsules of the same type used in dissolution testing of chitosan dosage forms were dissolution tested in triplicate. Samples were taken at the same timepoints and reacted with the ninhydrin reagent as already described. The "false positive" contribution due to the gelatin capsules at each timepoint was calculated from a standard chitosan calibration curve. When fully dissolved, gelatin capsules reacted with the ninhydrin reagent to give an absorption of 0.26 AU which is equivalent, in chitosan terms, to the absorption from about 20% of a typical chitosan dosage form. The values obtained were subtracted from the chitosan results in any dissolution experiments containing gelatin capsules.

3. Results and discussion

3.1. Inter-assay calibration data

Calibration curves constructed using different stock solutions on successive days gave accurate measures of the concentrations of the stock chitosan solutions (Table 1). Inter-assay precision was very tight ranging from 3.9 to 6.2% for solution concentrations between 0.5 and 1.5 mg/ml. Although the range for the 0.25 mg/ml solutions was somewhat wider in percentage terms, the absolute estimated values varied from 0.17 to 0.28 mg/ml which is acceptable for practical purposes. The correlation (r) of the calibration curves was greater than 0.99 in all cases.

3.2. Inter-day accuracy and precision

Measurement of the same sample on different days also gave acceptable results (Table 2). Accuracy levels were 97.5, 100.6 and 102.1% at the 0.50, 0.75 and 1.00 mg/ml levels, respectively. Corresponding precision values were 5.7, 3.5 and 7.4%.

3.3. pH/solubility profile

The pH/solubility profile (Fig. 2) of the chitosan base used in these studies illustrated that it was soluble, at the chitosan concentration used (1.2 mg/ml), up to pH values of 6.55, roughly corresponding to its pK_a. Above this pH, solubility declined sharply

Table 1
Inter-assay calibration data

Nominal concentration (mg/ml)	Back-calculated concentrations (mg/ml)					Inter-assay precision (%)
	Day 1	Day 2	Day 3	Day 4	Day 5	
0.25	0.19	0.22	0.28	0.26	0.17	20.8
0.50	0.50	0.50	0.50	0.47	0.43	6.2
0.75	0.74	0.75	0.85	0.76	0.79	5.8
1.00	1.01	1.08	1.05	0.92	1.01	6.1
1.50	1.51	1.45	1.41	1.56	1.51	3.9
Correlation (r)	0.999	0.996	0.992	0.996	0.996	

Table 2
Inter-day accuracy and precision

	Nominal concentrations (mg/ml)					
	0.50		0.75		1.00	
	Measured concentration (mg/ml)	Accuracy (%)	Measured concentration (mg/ml)	Accuracy (%)	Measured concentration (mg/ml)	Accuracy (%)
Day 1	0.498	−0.4	0.735	−2.0	1.009	0.9
Day 2	0.501	0.1	0.766	2.1	0.894	−10.6
Day 3	0.447	−10.6	0.706	−5.9	1.064	6.4
Day 4	0.519	3.7	0.767	2.3	1.080	8.0
Day 5	0.473	−5.3	0.755	0.6	1.060	6.0
Precision	5.7%		3.5%		7.4%	

reaching negligible values at pH 7.18. The results obtained correspond very closely to those obtained using a pH/absorbance method which used optical density measurements at 500 nm (Saito et al., 1997) to measure the increase in turbidity of isotonic chitosan solutions as the pH was increased. As chitosan precipitates out of solution, absorbance increases due to the increased turbidity of the solution. Using this method

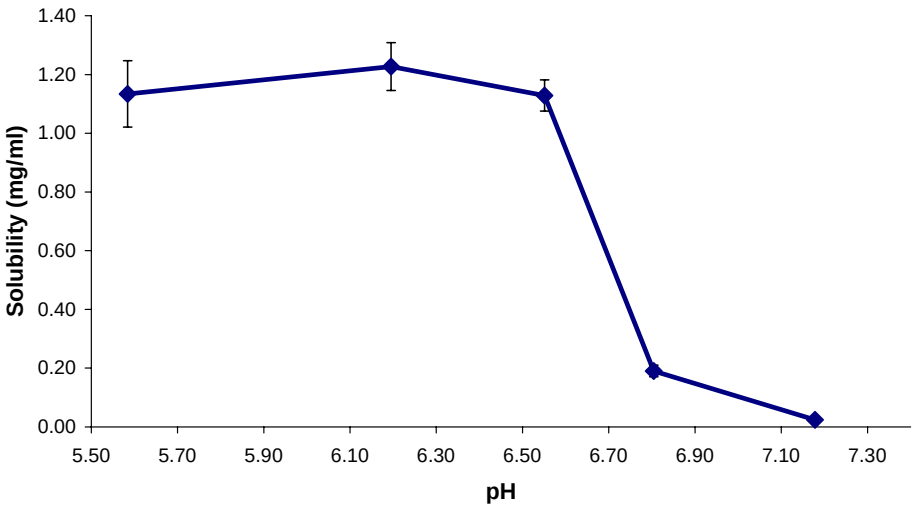

Fig. 2. pH/solubility profile for chitosan base (data shown are the mean ± standard deviations, $n = 3$).

on the chitosan base, a low absorbance, indicative of a clear solution was observed at low pH values up until approximately the pH 6.4–6.5 region beyond which a sharp increase in absorbance was seen corresponding to a sudden precipitation of chitosan out of solution leading to cloudiness in the system (results not shown) supporting the results obtained using the ninhydrin assay.

3.4. Dissolution testing of chitosan placebo tablets

Chitosan tablets showed markedly different disintegration properties at the different pH values tested (Table 3). At neutral pH (7.4), chitosan tablets disintegrated almost immediately. At the lower pH values, gelling of the chitosan was seen leading to a much slower disintegration. In the case of simulated gastric fluid pH 1.2, disintegration was complete within 210 min.

The results of the dissolution testing complemented these observations (Fig. 3). At pH 7.4, negligible levels of chitosan were found in solution at all timepoints. This result is consistent with the solubility properties of chitosan already described. At pH 3.8 a relatively steady release and dissolution of the chitosan from the tablets was seen with nearly 50% in solution within 30 min and over 90% within 90 min. At pH 1.2 dissolution was significantly slower due to the increased gelling of the chitosan at the lower pH value. After 45 min, only approximately one-third of the chitosan had been released and had dissolved and after 4 h the level of chitosan in solution did not exceed 82%. Complete dissolution did not occur at this pH due to the incomplete disintegration of the dosage forms. Gelled fragments of the tablets were still visible in the

Table 3
Disintegration behaviour of chitosan placebo tablets

Buffer pH	Disintegration behaviour
1.2	Gel layer formed. Tablets gradually eroded. Completely disintegrated after 210 min. Small fragments of undissolved material still present at 240 min
3.8	Gel layer formed. Tablets gradually eroded. Completely disintegrated after 120 min
7.4	Instant disintegration to form a fine dispersion

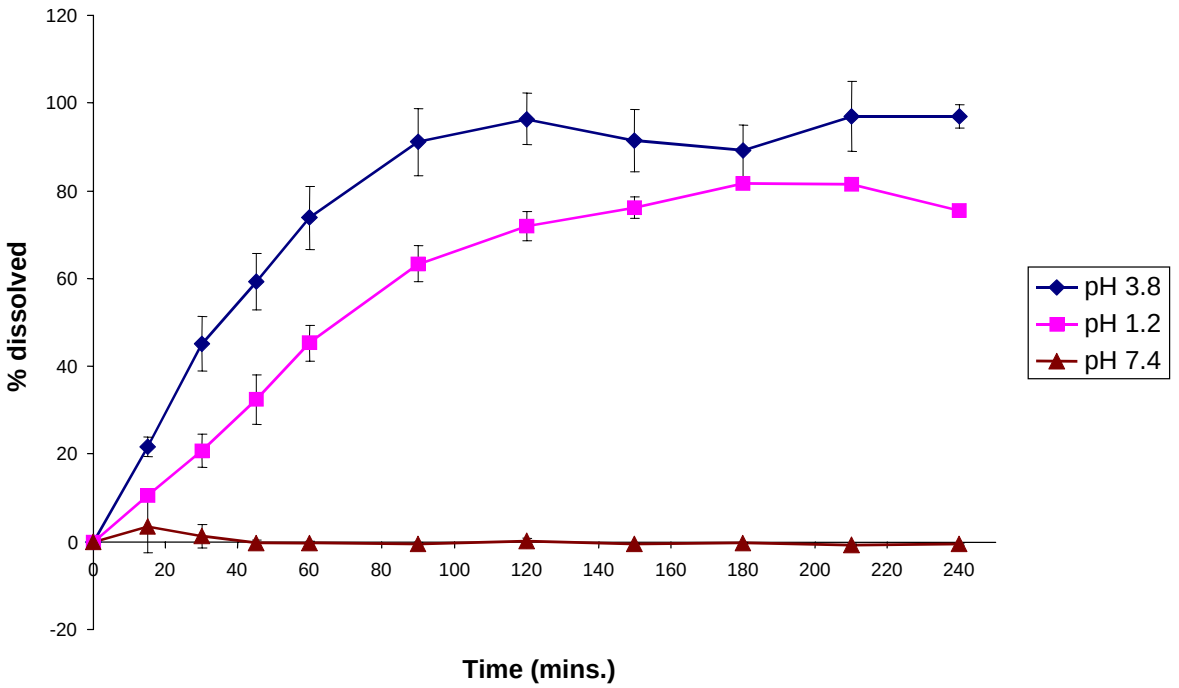

Fig. 3. Dissolution of chitosan from placebo tablets at different pH values (data shown are the mean ± standard deviations, $n = 3$).

dissolution vessel at the end of testing. The ninhydrin assay proved to be very reproducible in practice with low standard deviations seen between the triplicates tested at each pH value.

3.5. Dissolution testing of chitosan/sodium salicylate tablets

When dissolution testing was carried out at pH 7.2, the ninhydrin assay again measured negligible chitosan levels in solution. Levels of chitosan were below 1% at all timepoints up to 3 h (Fig. 4). By contrast, the release of sodium salicylate was very rapid with over 90% in solution within 45 min (Fig. 4). This fast dissolution of drug is most likely due to the rapid disintegration of the chitosan tablet at this pH. However, the disintegration was not as rapid as that seen previously for chitosan placebo tablets.

When dissolution testing was carried out in the lower pH medium (pH 5.6), an increased level of release and dissolution of chitosan was seen. After a brief lag time, the levels of chitosan rose to reach nearly 35% after 3 h (Fig. 5). Sodium salicylate release was very rapid with 87% in solution after 15 min and essentially 100% after 30 min (Fig. 5). Triplicate samples showed standard deviations of less than 10% in all cases.

The difficulty of quantifying chitosan in solution is illustrated by the fact that although there is a large body of research published investigating chitosan and its applications, there are very few papers dealing with its quantification. A recent study in the pharmaceutical literature (Khoo et al., 2003) evaluating the in vitro dissolution of chitosan from films avoided the use of a formal assay altogether. Samples were retrieved, dried and weighed at the end of the dissolution test. The loss in weight of the film was defined as the amount of chitosan dissolved. Such a method allows for only one measurement to be made and runs into difficulties when analysing dosage forms containing more than one component.

In the wider scientific arena, an assay for the isolation of chitin from fungi has been described (Lamps et al., 1995). The assay was found to be highly specific and sensitive but was rather complex and laborious. Initially, the chitin was deacetylated to chitosan. This was hydrolysed to glucosamine monomers which were then deaminated. The resulting 2,5-anhydromannose was reduced using a radioactive reagent to give radiolabelled 2,5-anhydromannitol which could be quantified by liquid scintillation. An additional difficulty in using such a method for chitosan quantification is due to the particular properties of chitosan itself. When describing an assay method for hexosamines

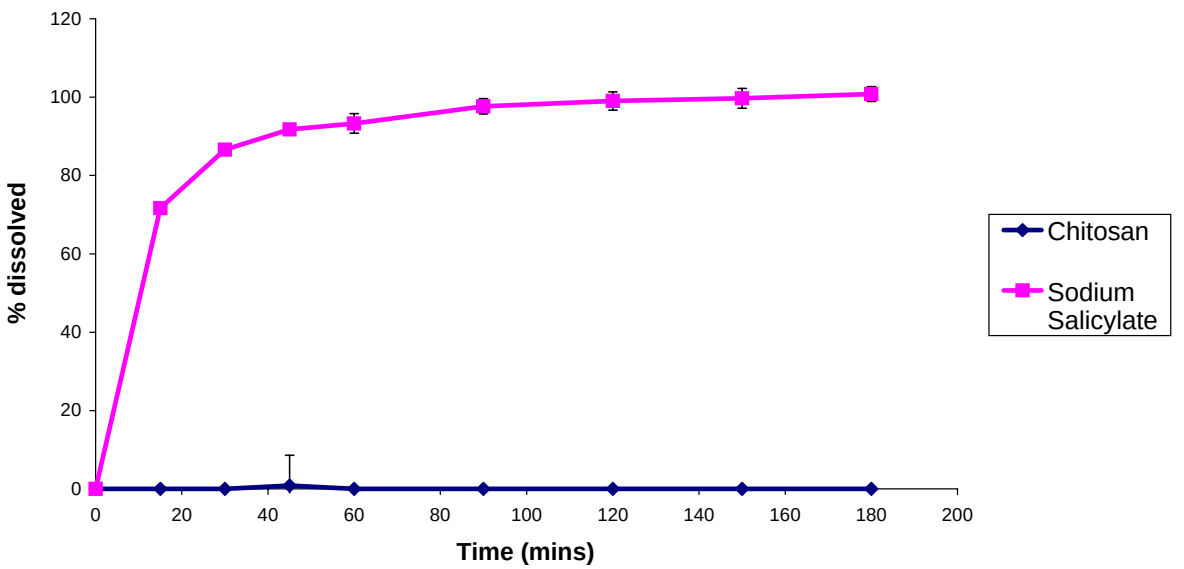

Fig. 4. Chitosan and sodium salicylate dissolution from minitablets at pH 7.2 (data shown are the mean ± standard deviations, $n = 3$).

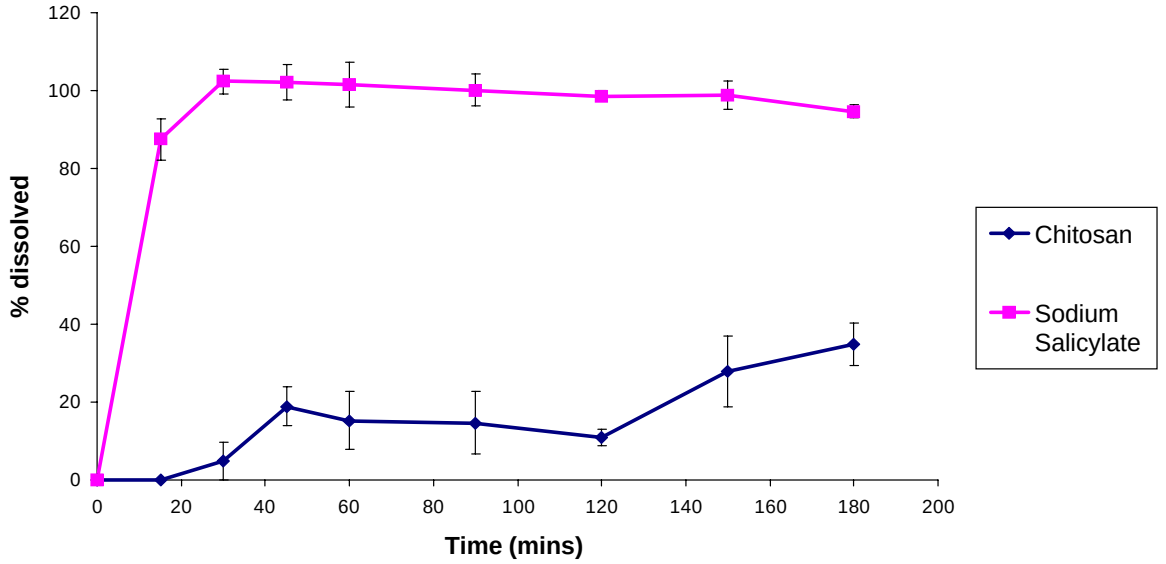

Fig. 5. Dissolution of chitosan and sodium salicylate from minitablets at pH 5.6 (data shown are the mean ± standard deviations, $n = 3$).

other researchers encountered difficulties when trying to hydrolyse and deaminate chitosan (Bosworth and Scott, 1994) since the highly polycationic nature of chitosan led to the existence of an electrostatic barrier counteracting the proceeding of the reaction. The hydrolysation and deamination processes took 23 h to reach completion as determined by measurements of free sugars using the Elson–Morgan and 3,5-diaminobenzoic acid assays. This was almost six times longer than the time necessary for purified anionic glycosaminoglycans.

The use of a colourimetric assay avoids such complications with the ninhydrin method proving particularly advantageous. Other colourimetric reagents such as picric acid can be rather laborious to use in practice with a reaction time of up to 6 h (Neugebauer, 1989). By contrast, the ninhydrin reaction is complete in 30 min allowing the efficient processing of a large number of samples. It has been shown to be suitable for the highly deacetylated chitosans commonly used in pharmaceuticals and is also relatively inexpensive. All these elements combine to make the ninhydrin method the procedure of choice for the quantification of chitosan. However, it should be emphasized that the ninhydrin assay is very sensitive to changes in degree of deacetylation and molecular weight of chitosan and that hence a validation of the assay has to be performed for each type of chitosan (or batch of chitosan) used.

4. Conclusions

The ninhydrin assay has proven to be a rapid, convenient and reproducible method for quantification of the release of chitosan into solution from solid dosage forms. The validation experiments carried out here have shown that the assay is both precise and accurate within the concentration range for chitosan that would normally be encountered in tablet or capsule dissolution experiments. Furthermore, the ninhydrin assay has been shown to be applicable for determining the pH/solubility profile for this type of chitosan and for measuring its dissolution at a range of physiologically relevant pH values both in the absence and presence of a drug. It must be borne in mind, however, that the results presented in this chapter are only applicable for this particular type of chitosan. A fresh calibration curve and validation should be carried out if a different type of chitosan is being assayed. Future studies will utilise the ninhydrin assay to design dosage forms which will release both chitosan and

drug rapidly and simultaneously in order to achieve optimum absorption-enhancing activity in the GIT.

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