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RESEARCH PAPER

STABILITY EVALUATION OF PROTEIN DRUGS IN OPTIMISED THIOLATED-CHITOSAN BASED BUCCAL XEROGELS

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ABSTRACT
The stability profiles of BSA and INS incorporated in lyophilised chitosan-based buccal xerogels have been evaluated using Bradford's Assay as well as SE-HPLC and RP-HPLC quantification for BSA and INS respectively. Chromatographic conditions were; Agilent 1100 series, Tosoh TSK-GEL PW column (7.8 x 300mm, 10 µm), mobile phase PBS, flow rate 1 mL/min, wavelength 215 nm (SE-HPLC); Agilent 1200 with a Jupiter 5-µm C18 column (250mm x 4.6 mm), mobile phase-water: acetonitrile (67:33v/v) 0.1%TFA; flow rate-1 mL/min; and wavelength-220nm (RP-HPLC). The use of SE-HPLC and RP-HPLC methods for BSA and INS respectively showed chromatograms indicating that storage of xerogels using accelerated stability conditions at 25 °C/ 60 % RH for six months resulted in substantial loss of native protein. Storage of xerogels at 5 °C in a refrigerator maintained protein stability up to six months. Bradford's assay for protein quantification was not stability indicating method as it was not specific for the determination of degraded protein products from all xerogels.

Keywords: *Thiolated chitosan, SE-HPLC, RP-HPLC, protein, stability evaluation*

INTRODUCTION
The development and characterisation of chitosan based xerogels for the delivery of proteins *via* the buccal mucosa has become a major area of study for the pharmaceutical scientist in recent years (Bernkop-Schnürch *et al*., 2013; Ayensu *et al*., 2012) It is however, essential that the model protein drug incorporated into the xerogels remain conformationally stable during and after lyophilisation and over a long storage period. Proteins by nature have a narrow stability window when exposed to conditions that lead to aggregation, hydrolysis and denaturation caused by heat, moisture and other organic solvent (Estey *et al*., 2006). It is therefore critical that appropriate conditions of storage be established for the novel chitosan-based protein delivery systems developed. Bradford's assay and HPLC procedures were employed to estimate the protein content of the xerogels. The Bradford assay for protein quantification is a rapid and accurate method with less interference by non-protein components (Kruger, 2008). The method relies on the binding of Coomasie blue G250 dye (Bradford reagent) to protein. Although different ionic forms of the

dye exist, the more anionic blue form of the dye binds to protein with a characteristic absorption maximum at 590 nm.

Although drug content is a crucial stability indicating parameter, the method of determination must also be able to identify and quantify possible degradation products in order to assure safety and efficacy. Accordingly, the International Committee for Harmonisation (ICH, 2003) recommends that stability studies must include testing of those attributes of the drug substance that are liable to change during storage and are likely to affect quality, safety and/ or efficacy. These may include physical, chemical, biological and microbiological attributes, using validated stability indicating analytical procedures. It is essential that the development of polymer based delivery systems for controlled release of therapeutic proteins takes into consideration the stability of the protein drug. In addition, to improve stability of proteins within chitosan based systems, formulations must be protected from potentially extreme hydrolysing conditions to ensure maintenance of the native structure.

This work details accelerated stability studies of bovine serum albumin (BSA) and insulin (INS) incorporated into optimised thiolated (TG)-chitosan xerogels using ICH conditions (ICH, 2003). Results over six month's period are presented.

METHODS AND MATERIALS

Materials

Bovine Serum Albumin (BSA), sodium hydroxide, Phosphate Buffered Saline (PBS) tablets (pH 7.4), human recombinant insulin (INS), Bradford's reagent, Copper (II) chloride, acetonitrile, trifluoroacetic acid, chitosan (CS), thioglycolic acid (TGA), glycerol, D-mannitol, Float-A-Lyzer G2 dialysis device (8-10 kDa Molecular weight cut-off), and N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDAC) were obtained from Sigma-Aldrich, Gillingham, UK. Acetic acid, hydrochloric acid and potassium dihydrogen phosphate were purchased from Fisher Scientific (Leicester UK). All order reagents were of analytical grade.

Preparation of BSA/INS xerogels

BSA loaded xerogels were prepared as previously reported (Ayensu *et. al*., 2012) and briefly described. Chitosan-4-thioglycolic acid (CS-TGA) was synthesized by dissolving 500 mg of CS in 50 mL of 0.1 M HCl. An equivalent of 500 mg of TGA was added followed by 50 mM EDAC in order to activate the carboxylic acid moieties of TGA. The pH was adjusted to 5 with 1 M NaOH at room temperature and stirred for 3 h. Purification to remove unconjugated TGA and sulfhydryl immobilization determination of CS-TGA was by the method reported in literature (Bernkop-Schnurch *et al.*, 2003), using Float-A-Lyzer G2 and Ellman's reaction with standard cysteine HCl curve ($R^2 > 0.99$) respectively. Ten percent (per polymer weight) each of glycerol and D-mannitol as plasticizer and cryoprotectant respectively were added to the recovered dialysed gel and loaded with BSA as a model protein drug in a 2:1 CS-TGA: BSA ratio. The final formulated gel was stirred continuously for 30 min at room temperature to obtain a uniform gel which was kept under ambient conditions to remove all air bubbles. Six grams (6.0 g) of the homogeneous gel was transferred into a mould (diameter 35 mm) and freeze-dried using a novel freeze-drying cycle developed with differential scanning calorimetry (DSC) (Ayensu *et al.,* 2011) on a Virtis AdVantage XL 70 freeze dryer (Biopharma Process Systems, Winchester, UK). An annealing temperature of -25 °C (3 h) was applied during the freezing phase (- 55 °C). Primary and secondary drying were at -30 °C and 20 °C respectively, with a vacuum of 20 torr for 34 h.

Additionally, INS containing xerogels for stability studies were prepared using the method described in (Boateng *et. al*., 2014). Briefly, drug loaded gel formulations were prepared by loading the dialysed TG-chitosan gel with 5 mg/mL of a solution of INS in 0.01 M HCl. The final mixture containing 1 % w/v TG-chitosan was stirred continuously for 15 min to obtain a uniform gel at room temperature and kept for 30 min to remove all air bubbles. 1 g each of the homogeneous gels was poured unto dried hydrophobic EC laminate (Boateng and Ayensu, 2014) backing film in the moulds and lyophilised using the automated lyophilisation cycle described above. Completion of the pre-

programmed primary drying phase was confirmed using sample probes which determined when both the product and shelf temperatures were the same.

Storage of BSA/INS xerogels for stability studies

The lyophilised products (xerogels) were stored in desiccators over silica to ensure low water content ideal for protein stability or stored according to ICH conditions (ICH, 2003) in Table 1 until ready for analysis at selected time points. The samples were either kept in a refrigerator (long-term study) or kept under ICH conditions (for drug products intended for storage in a refrigerator) in a humidity chamber with 60 % relative humidity (RH) ± 5 % RH maintained with $CuCl_2$ (solubility 0.70 g/mL) at 25 °C (ICH, 2003) for accelerated stability studies (Table 1).

Assay of BSA and INS content by Bradford's assay

The BSA and INS contents in xerogels at the various time points (0, 3 and 6 months) were determined using the Bradford's assay (Ayensu, 2013). Briefly, 35 mg of drug loaded xerogel was dissolved in 25 mL of 1% (v/v) acetic acid. The solution was magnetically stirred for 10 min at room temperature at 50 rpm to avoid bubble formation. 50 µL of the solution was withdrawn and treated with 1 mL Bradford's reagent and the absorbance measured at 595 nm and 450 nm for linearization of absorbance (Ernst and Zor, 2010) using a Multiskan EX microplate photometer equipped with Ascent software (Thermo Scientific, Hampshire, UK). The amount of BSA and INS in their respective xerogels were determined by interpolation from their linearized calibration curve ($R^2 > 0.99$). Each experiment was run in triplicate. The xerogels were instantly analysed for protein content immediately after harvesting from freeze-drying to represent the time zero-point determination.

HPLC assay of BSA and INS content

The amount of BSA at time zero was determined with an Agilent 1100 series size-exclusive high performance liquid chromatography (SE-HPLC) analysis system equipped with an isocratic pump and a UV detector. Samples were dissolved in 0.01 M PBS pH 7 (Estey *et al*., 2006) filtered (0.45 µm membrane, Minisart Biotech.) and eluted through a Tosoh TSK-GEL PW column (7.8 x 300 mm, 10 µm) with a flow rate of 1 mL/min at ambient temperature and detected at a wavelength of 215 nm using the same PBS as mobile phase and pure BSA powder as reference standard. Collected SE-HPLC data were analysed with Agilent Chemstation software and content of BSA was estimated from a calibration curve with linearity verified over concentration range of 0.1–1.00 mg/mL ($R^2 > 0.99$). The procedure was repeated for the time points of three (3) and six (6) months for the various storage conditions (Table 1) as specified in the ICH protocol.

The amount of INS loaded in the xerogels was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) (Agilent Technologies, Santa Clara, CA, USA) with a Jupiter 5-µm C18 column (250 mm × 4.6 mm, 300 A°) (Phenomenex, USA) with the following conditions: mobile phase - water: acetonitrile (67: 33, v/v) containing 0.1% (v/v) of TFA; flow rate - 1 mL/min; detection wavelength - 220 nm and injection

Table 1: ICH conditions for drug products intended for storage in a refrigerator and under accelerated conditions

Study	Storage condition	Minimum time covered by data at submission
Long-term	5 °C ± 3 °C	12 months
Accelerated	25 °C ± 2 °C/ 60 % RH ± 5 % RH	6 months

volume - 20 µL (Giovinio *et al*., 2012). All samples were analysed in triplicate and the protein concentration was determined based on interpolation from a calibration curve with linearity verified over concentration range of 50–250 µg/mL ($R^2 > 0.99$). The procedure was carried out for the time points of zero (0), three (3) and six (6) months for the various storage conditions (Table 1).

Statistical analysis

A two tailed Student's t-test at 95% confidence interval (p value < 0.05) as the minimal level of significance was used to evaluate the data statistically.

RESULTS AND DISCUSSION

The BSA and INS contents of the optimised TG-chitosan xerogels were initially estimated by Bradford's assay and HPLC methods. Xerogels were kept in 24 well plates sealed with parafilm and lids to limit moisture absorption, stored in the refrigerator (RF) at 5 °C ± 3 °C and analysed for BSA and INS content at 3 months intervals. The accelerated stability studies of BSA and INS incorporated in the chitosan based xerogels was by the International Committee for Harmonisation (ICH)'s conditions for drug products intended for storage in a refrigerator. The exposure of the xerogels to varying conditions of temperature and moisture (relative humidity) had a profound effect on the stability of the protein model drugs. Generally, xerogels stored in the refrigerator showed no signs of protein degradation/fragmentation throughout the entire period of evaluation with the assayed protein content within the range of 95 – 105 % as specified by in British Pharmacopoeia (BP 2012). This was however, not the case with xerogels stored at ambient temperature at a relative humidity of 60 % in a humidity chamber.

BSA and INS content by Bradford's assay

Fig. 1 shows the stability curves for TG-chitosan xerogels containing BSA and INS

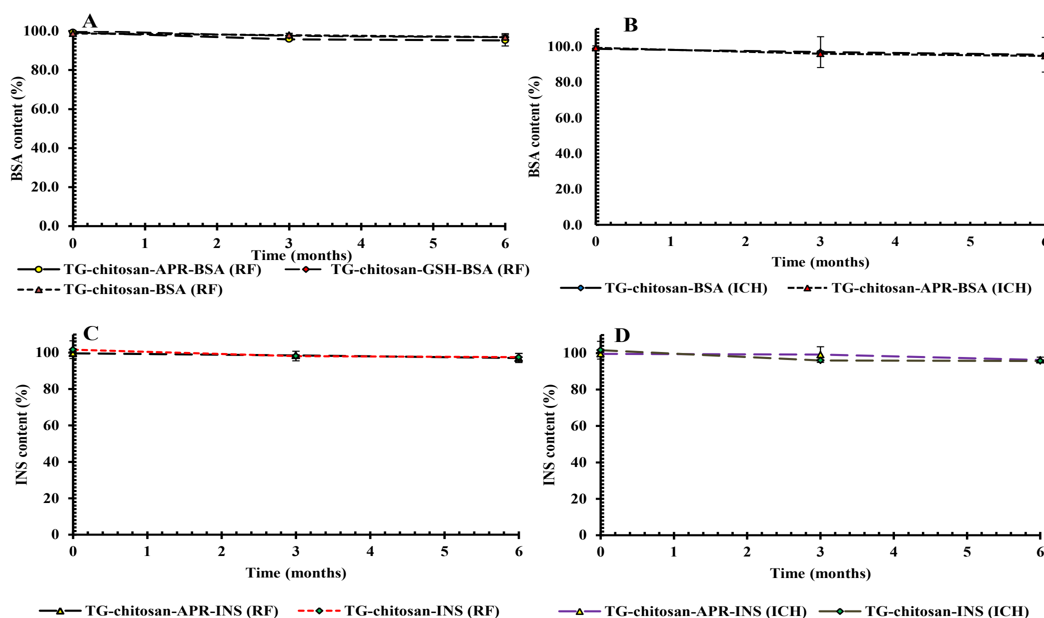

Fig. 1: Stability curves obtained by Bradford's assay. (A) and (B) show the content for BSA while (C) and (D) show the content of INS in TG-chitosan xerogels after six months storage in the refrigerator (RF) and under ICH conditions

determined using Bradford's assay. No significant differences were observed between the mean protein content of all xerogels irrespective of storage conditions ($p < 0.05$). In addition, all time points of analysis showed that the protein content of all xerogels were intact indicating product stability after storage under ICH conditions. However, since the Bradford method of protein estimation depends on binding with proteins, the possibility of binding all degraded/fragmented protein products may exist, thus giving a determination of total protein present. This constitutes a limitation of the method if all fragmented products contain arginyl and lysyl residues of proteins which bind readily with the Coomasie blue dye (Congdon *et al*., 1993 cited in Kruger, 2008). Such a method cannot be used to detect the presence of degraded proteins and thus will not be suitable to differentiate between the protein products in the xerogels after storage under the ICH conditions.

HPLC determination of BSA and INS content

Fig. 2 shows the calibration curves for BSA 'A' and INS 'B' using SE-HPLC and RP-HPLC respectively. The respective SE-HPLC and RP-HPLC estimation of BSA and INS contents provided evidence for the existence of fragmented proteins from xerogels stored under ICH conditions. Storage of xerogels in the refrigerator resulted in the maintenance of their initial content after six months storage. No significant loss in protein was detected when stored in the refrigerator (Fig.3 'A' and 'C'). However, storage at 25 °C and relative humidity of 60 % showed significant reduction in protein content down to 40 % and 20 % for BSA and INS respectively (Fig. 3 'B' and 'D') after six months of storage.

The representative HPLC chromatograms in Fig. 4 also show the peaks of chemically degraded BSA and INS along with the remaining BSA and INS originally present in the xerogels (Fig. 4 'C' and 'F'). The exposure of the xerogels to extreme moisture conditions leads to protein hydrolysis. The fragmented protein species from the BSA containing TG-chitosan xerogels stored under ICH conditions are most likely the result of hydrolysis of peptide bonds (Manning *et al*., 1989, cited in Estey *et al*., 2006). They appeared as unresolved species eluting before the minimised BSA peak as a result of progressive BSA loss. The reduced content of BSA detected at the end of the incubation suggests that the hydrolysis process was continuous throughout the incubation period and was incomplete at the last time point of six months. The presence of glycerol as a plasticizer as well as cryoprtectant is essential for struc-

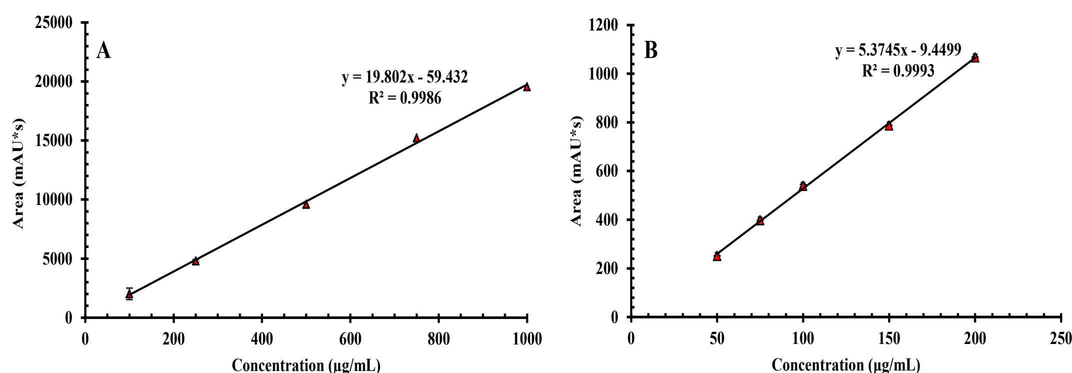

Fig. 2: BSA 'A' and INS 'B' calibration curves using SE-HPLC and RP-HPLC respectively

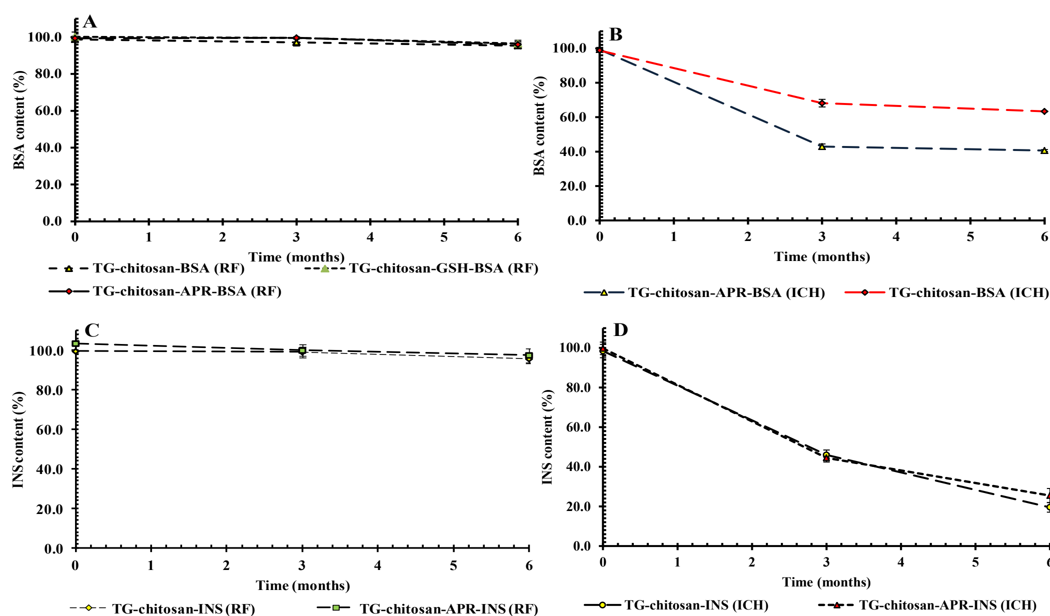

Fig. 3: Stability curves obtained by SE-HPLC and RP-HPLC for BSA and INS respectively. (A) and (B) show the content for BSA while (C) and (D) show the content of INS in TG-chitosan xerogels after six months storage in the refrigerator (RF) and under ICH conditions

tural stability however its hygroscopic nature allows for moisture ingress that has capacity to induce hydrolysis of proteins during storage at room temperature (Takamura *et al.*, 2012).

The stability of INS in the xerogels stored in a refrigerator could stem from formulation TG-chitosan, which as a hydrophilic polymer and would deposit on the substrate surface, preventing moisture from reaching the proteins and thus limiting continuous INS degradation (Maroni *et al.*, 2009). In addition, the formulation process incorporating D-mannitol and glycerol as cryoprotectants prevents protein precipitation at the freezing temperature thus enhancing INS stability during refrigeration (Gokce *et al.*, 2014) However the poor stability of most therapeutic proteins under room conditions is well documented (Strickley & Bradley, 1997 cited in Maroni *et al.*, 2009). Non- enzymatic degradation reactions occur with both solid and aqueous state INS leading to the formation of degradation products. Several INS degradation products including A21-desamido insulin, Other Insulin Related Compounds (OIRCs) and High Molecular Weight Proteins (HMWPs) have been described in literature (Brange, 1992, cited in Maroni *et al.*, 2009). The degradation of INS from the TG-chitosan xerogel at 25 °C/60 % RH could stem from hydration and hydrolysis of the polymer matrix allowing moisture to reach the exposed large surface area of INS. Furthermore, the primary packaging of parafilm sealing on the 24 well plates does not seem to have limited moisture absorption as originally perceived. Therefore, the instability of the loaded protein drugs could be due to poor air and moisture tightness of the sealing material that allowed moisture entry for hydrolysis of the protein drugs to occur during storage.

The foregoing discussion alludes to the fact that lyophilisation of protein drugs leads to

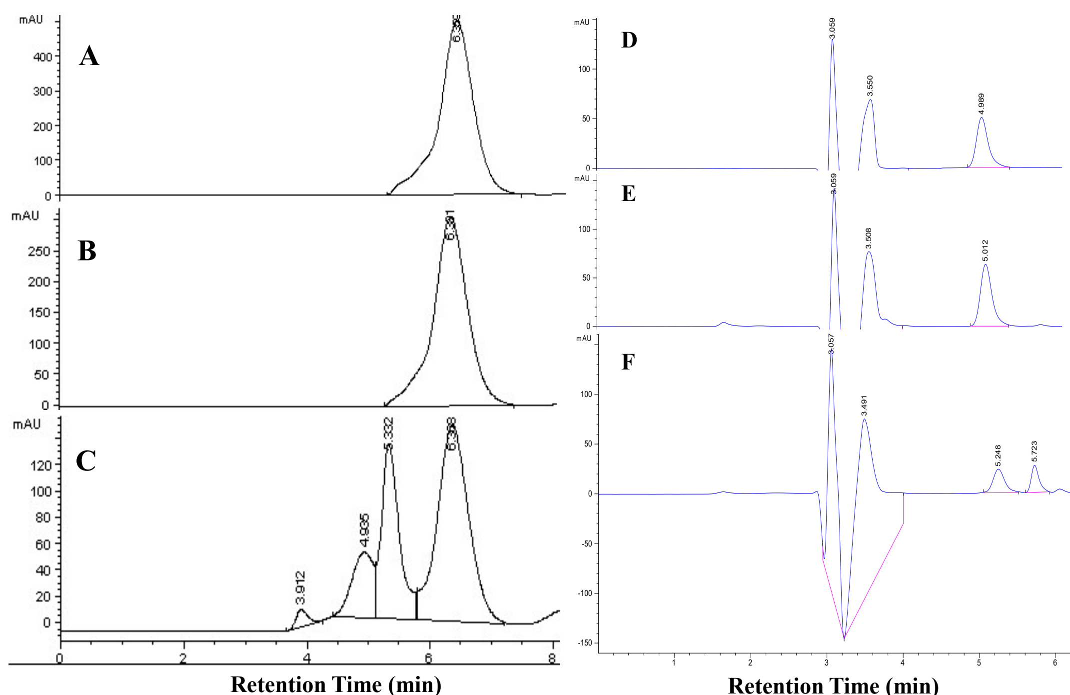

Fig. 4: SE-HPLC chromatograms 'A' shows BSA powder peak (retention time around 6.3 minutes), 'B' BSA released from TG-chitosan xerogels stored at 5 °C and 'C' BSA and degraded BSA products released from TG-chitosan xerogel stored under ICH accelerated stability conditions for six months. The RP-HPLC chromatogram 'D' shows the peak of pure INS powder (retention time around 5 min), 'E' the peak of INS from TG-chitosan stored in a refrigerator and 'F' INS and degraded INS products from xerogels stored under ICH conditions

stability however, the storage conditions must be selectively indicative to prevent degradation during storage. An appropriate stability indicating method of analysis must be employed to detect the least concentration of break down protein products.

CONCLUSION

The stabilities of BSA and INS incorporated in chitosan based xerogels have been critically examined. The results showed that storage of xerogels using accelerated stability conditions at 25 °C/ 60 % RH for six months resulted in substantial loss of native protein. Such reduction in protein content observed may have resulted from increased peptide bond hydrolysis due to enhanced moisture ingress during storage. Storage of xerogels at 5 °C in a refrigerator maintained protein nature up to six month. The use of SE-HPLC and RP-HPLC methods respectively for BSA and INS estimation yielded chromatograms that suggested the presence of degraded protein products for xerogels under accelerated stability studies. The employment of Bradford's assay for protein quantification, however, could not be used to determine the presence of protein degraded products from all xerogels. However, further studies to establish the full extent of stability after storage in the refrigerator for up to 12 months is required.

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