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Spray-dried insulin particles retain biological activity in rapid in-vitro assay

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Abstract

The purpose of this study was to rapidly determine, without the use of extensive animal studies, whether biological activity is retained after spray drying insulin with two excipients, lactose and xanthan gum. This was achieved by the detection of protein kinase B (PKB), which is activated by phosphorylation in response to insulin binding to cellular receptors. A myeloid cell line was cultured and stimulated with the reconstituted insulin powders. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was then utilised to allow in-vitro detection of phosphorylated PKB using an anti-phospho-PKB antibody. A single band specific to phosphorylated PKB was found on the Western blots, indicating that the active conformation of insulin was retained when spray dried in combination with lactose and with xanthan gum over the spray-drying inlet temperature range of 110–170°C. Evidence of inactivation/denaturation was observed when insulin was spray dried at an inlet temperature of 200°C. The assay may be of use as a more rapid and economic means to screen insulin formulations for inhalation and other purposes as opposed to conventional monitoring of blood glucose levels in animals.

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Introduction

Protein powders intended for pulmonary delivery can be prepared by a variety of means, including freeze drying, milling, supercritical fluid precipitation and spray drying (Niven 1995). Single and double evaporation methods have also been described in the literature (Ben-Jebria et al 2000). In many cases, spray drying has proven to be an optimal method of production for such powders. The ability of this technique to allow modification of particle size and morphology are particularly advantageous in terms of allowing controlled release pulmonary delivery (Edwards et al 1997). Another advantage of spray drying is the ability to readily incorporate stabilizing or controlled-release excipients into formulations.

Simple spectroscopic methods used to assess protein aggregation or structural integrity do not provide information with respect to biological activity of powders following processing. Pharmacopoeial methods are based on HPLC and may be used to detect degradation of insulin, as a result of deamidation for example, as well as aggregation (EP 1996), but do not directly assess the integrity of the active site.

In this study we have incorporated a known protein stabilizer, lactose, into respirable insulin particles and have directly measured the retained biological activity using an in-vitro assay. The effect of incorporating another excipient, xanthan gum (McConville et al 2000), on this system is also presented.

Materials and Methods

Materials

Insulin (from bovine pancreas) was purchased from Sigma Chemical Company (Poole, UK). Lactose (Meggle Crystalac 40) was obtained from Meggle (Wasserburg, Germany). Xanthan gum was obtained from Penwest Pharmaceuticals Company (Patterson, NY). Single batches were used in each case.

Bovine serum albumin (BSA) was from Boehringer Mannheim (Lewes, UK). Glutamine, fetal bovine serum, Hank's buffered saline solution (10 ×), penicillin, RPMI 1640 growth medium and streptomycin were from Gibco BRL (Life Technologies Inc., Paisley, UK). Bio-Rad Protein Assay Dye Reagent Concentrate and 2-mercaptoethanol were from Bio-Rad Laboratories (Hemel Hempstead, UK). All other chemicals and reagents were from BDH Laboratory Supplies (Poole, UK), Fisons Scientific Equipment (Loughborough, UK), or Sigma Chemical Company (Poole, UK). Water used was Milli-Q grade (Compact Milli-Q Water System, Millipore (UK) Ltd, Watford, UK).

Preparation of spray-dried powders

Insulin and insulin–excipient powders were prepared using a laboratory scale co-current spray dryer (Model B-191, Büchi Labortechnik AG, Switzerland). Aqueous suspensions of 1% (w/w) insulin were atomized at a rate of 5 mL min^{-1} using compressed air (600 L h^{-1}, 0.7 mm nozzle). Insulin–excipient (insulin–lactose or insulin–xanthan gum) feeds were co-spray dried under the same conditions following aqueous solubilization of both components together, at ratios of 1:10 and 1:100 each. The effect of spray-drying temperature was investigated by processing at different inlet temperatures. Outlet temperatures were recorded during each drying process following equilibration.

Insulin activity assay of spray-dried powders

Insulin activity before and after spray drying was assessed using a specific in-vitro assay. Insulin treatment of responsive cells leads to rapid phosphorylation and activation of protein kinase B (PKB) downstream of the insulin cell-signalling pathway (Cross et al 1995). Since PKB is activated by phosphorylation, activation of the kinase was detected using antibodies specific for the phosphorylated form.

Preparation of cell extracts

Cells were cultured as described previously (Craddock & Welham 1997). A myeloid cell line was used (FD-6), grown in the presence of interleukin-3. Cells were washed three times with Hanks' buffered saline solution, and then re-suspended in serum-free RPMI, at 37°C, to a final cell density of approximately 2×10^6 mL^{-1} and incubated at 37°C for 1 h before stimulation. Portions (0.5 mL) of the cell suspension were stimulated for 2 min with insulin (concentrations equivalent to 0.1, 0.5 or 1.0 μg mL^{-1} of insulin). Cell pellets were lysed in 40 μL of solubilization buffer (50 mM Tris-HCl; 150 mM NaCl; 1% (v/v) Nonidet P-40; 10% (v/v) glycerol; 5 mM EDTA; 1 mM sodium vanadate; 1 mM sodium molybdate; 10 mM sodium fluoride; 40 μg mL^{-1} phenylmethylsulfonyl fluoride; 0.7 μg mL^{-1} pepstatin A; 10 μg mL^{-1} aprotinin; 10 μg mL^{-1} leupeptin; 10 μg mL^{-1} soyabean trypsin inhibitor; pH 7.5). The lysates were centrifuged for 2 min at 13 000 rev min^{-1} (Jouan A14 Centrifuge, Jouan SA, France) to pellet the debris and the supernatants were denatured by boiling (Grant QB T2, Grant Instruments (Cambridge) Ltd, UK) for 2 min in 10 μL of 5 × SDS-sample buffer (10% (w/v) SDS; 50% (v/v) glycerol; 200 mM Tris-HCl; bromophenol blue; pH 6.8).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE and immunoblotting were carried out as described previously (Craddock et al 1999). Before denaturation, the protein concentration of the cell extracts was determined using the method of Bradford (1976) and 40 μg was loaded in to each lane. The primary anti-phospho-PKB antibody, rabbit anti-mouse polyclonal against phospho-Ser 473 (9270, New England Biolabs), was used at 1:1000. Secondary antibodies conjugated to horseradish peroxidase were used at a concentration of 0.05 μg mL^{-1} (Dako, Cambridge, UK). Immunoblots were developed for 5 min using the ECL system (Amersham International, Buckinghamshire, UK) and Kodak X-AR-5 film (Kodak, Liverpool, UK).

Results and Discussion

Initial examination (by scanning electron microscopy and particle sizing) of the spray-dried particles including excipients showed that they had a mean particle size below 5 μm. This size range is of a similar order of magnitude to that which would be expected to reach the

deep lung, the proposed site of lung absorption for insulin. Although further formulation (and perhaps further size reduction) would be required to ensure deep lung deposition of such particles, it would appear that they are in the relevant size range.

Spray-dried insulin alone did not reach the respirable size range and indeed the spray-dried particles were indistinguishable, in size and morphology, from unprocessed particles. This may indicate that they did not solubilize in the feed.

From Figure 1 it can be seen that the antibody, specific for the phosphorylated form of PKB, recognizes a single band on Western blots. Inactivated insulin will not bind to its receptor, hence the signal transduction pathway will not be initiated and the phosphorylation of PKB would not be observed.

The bands obtained due to phosphorylated PKB (Figure 1A) are very similar for the raw material (control) and for insulin spray dried at an inlet temperature of 110°C. However, the bands obtained for insulin spray dried at an inlet temperature of 200°C are noticeably weaker, suggesting a decrease in activity resulting in decreased insulin-receptor binding. Nonetheless, these results are encouraging as activity appears to be unaffected when insulin is spray dried at 110°C, and even at 200°C there is evidence of some activity being retained when the cells were stimulated with the higher concentration of insulin. Furthermore, this demonstrates the quantifiable nature of this method, with the intensity of the band corresponding to the degree of activity retention.

Figures 1B and 1C illustrate that insulin–lactose and insulin–xanthan gum powders spray dried at inlet temperatures of 110, 140 and 170°C all retained insulin activity. There was no apparent effect on this activity as a result of spray drying at the different temperatures and a strong signal for phosphorylated PKB was obtained from all the conditions, indicating that the active conformation of the insulin was retained upon reconstitution.

While lactose and a number of other sugars (particularly trehalose and derivatives) are well characterized as stabilizers for insulin in respirable particles, xanthan gum has previously not been reported widely as such. Xanthan gum is widely used as a controlled-release excipient in oral drug-delivery systems and has recently been demonstrated, in an in-vitro model (McConville et al 2000) to have release-controlling ability for small molecules contained in potentially respirable particles. Its use as a rate-controlling excipient for respirable insulin particles has yet to be demonstrated.

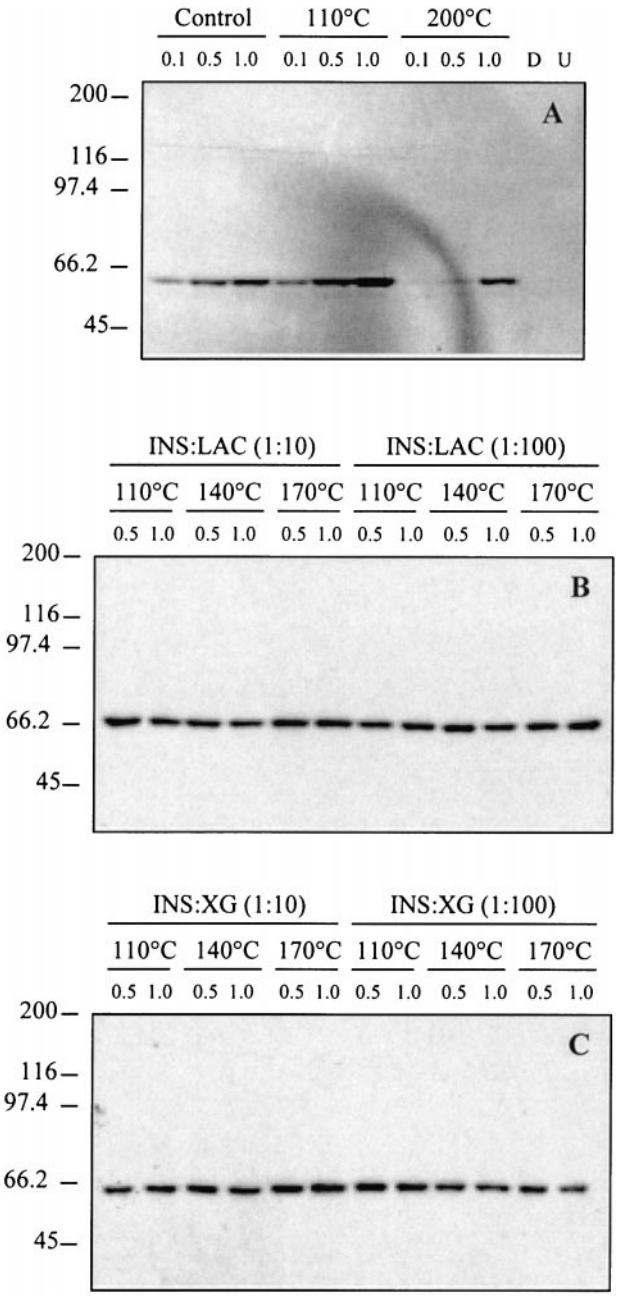

Figure 1 Induction of PBK phosphorylation in FD-6 myeloid cells by insulin (A) as lyophilized raw material (control), and after spray drying 1% (w/w).

Conclusion

The results presented here indicate that insulin co-spray dried with two model excipients retained biological activity, as determined by a rapid in-vitro assay. The incorporation of an excipient with the potential to afford

controlled release of drug in the lungs did not lead to a significant loss of activity. Furthermore the presence of this excipient, which may enhance the viscosity in the environment of the in-vitro assay, did not appear to influence the efficiency of the technique.

This method of determining specific insulin activity in-vitro is not a substitute for full animal or human studies. However, its use as a rapid screen for materials and processes or for future stability studies is demonstrated.

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