Measurement of Nasal Residence Time by Microdialysis

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Nasal microdialysis using sodium fluorescein as a dialyzable marker is introduced as a means of screening formulations to predict the residence time of nasally administered insulin. Exogenous fluorescein and insulin concentrations were correlated with the pharmacodynamic effect of insulin by monitoring serum glucose levels in alloxan-induced diabetic rabbits. Nasal insulin (5 U/kg) depressed blood glucose levels for at least three hours after administration. Although insulin could not be determined in the dialysates, fluorescein was easily measured and gave residence times consistent with glucose depression after insulin administration. A viscosity-enhancing polymer increased residence times of fluorescein and this, too, was consistent with blood glucose depressions. The present approach for nasal sampling may possibly be used for other substances of low molecular weight as well.

Although the acceptance of the nasal route for drug administration is reflected in increased numbers of drug approvals using this route of administration, and although the residence time of drug at the site of deposition influences bioavailability, no *in vitro* method simulates the mucocilliary clearance mechanism of the nasal mucosa. The growth rate of ~30% per year in approvals for systemically active drugs is three times higher than approvals for locally active products, and more than half of these products were approved during the 1990s. The nasal route is an effective means of administration of biotechnology-produced pharmaceuticals because it avoids the first-pass metabolism by the liver and thereby increases bioavailability relative to oral administration. Heightened interest in this route of administration is due, in part, to the size of the adult nasal cavity which has about a 20 mL capacity, with a large surface area of 180 cm^2 of highly vascularized tissue, providing an attractive site for rapid and efficient systemic absorption (1). The absorption of a drug in humans via the nose depends in large part on a ciliary clearance mechanism that affords an average residence half-life in the nose of approximately 15 minutes in humans and is comparable to the rabbit or sheep model. The former is an ideal taxonomic form for these studies because its nose is anatomically similar to humans and is well suited to delivery devices such as metered dose spray pumps. These species exhibit microvilli along the pseudostratified luminar epithelial cells of the nasal mucosa, which remove drug from this window of absorption via a highly efficient ciliary clearance that sweeps xenobiotics trapped in the turbinates back toward the esophagus where they are eliminated. Low permeability secondary to transient contact time at the site of deposition by the action of mucocilliary clearance may pose a significant limitation of nasal delivery. Therefore, a methodology to evaluate residence time of drug in the nasal passage would have great value in the dosage form development process.

A fundamental strategy to increase bioavailability is to modify the viscosity of the mucus by addition of viscosity-enhancing polymers or by mucoadhesive particulates. An optimal dosage form would be a solution of low viscosity that sprays well but undergoes a viscosity change or interacts with the mucosa at the site of deposition to limit drug clearance rate. Nasal delivery of biotechnological drugs as powders has two advantages, namely: most proteins and peptides are more stable in a solid state, and powder formulations remain longer on the nasal mucosa than liquid formulations (2). The clearance of putative bioadhesive polymer gels from the nasal cavity has been measured by following the removal of fluorescent-labeled microspheres incorporated into the formulation (3). Dondetti et al. (4) developed a

method to rank order formulations by measuring the appearance of marker molecules in the back of the throat. This method utilized sodium fluorescein as marker because it was, in comparison to other fluorescent probes, nontoxic and easily analyzed. Though effective, such procedures are somewhat invasive, requiring swabbing the esophagus with cotton applicators which in all likelihood affects the integrity of subsequent samples.

Particulates of proteins can be produced by the rapid expansion of solvent (RESS) process, in which the decompression of supercritical fluids containing dissolved substances can be accomplished under benign conditions. Insulin and other products of biotechnology have been studied in this process as model substrates with biological activity for optimization of a supercritical fluid carbon dioxide-based method of formulating bioadhesive particles (5-8). The goal is a new approach to make the transition from biotech candidate to viable dosage form. Microdialysis has been shown useful to the study of local metabolic action of these large proteins in tissues (9) and is opening new avenues for *in vivo* metabolic research. Because no *in vitro* method exists to simulate the cleansing action of the microvilli lining the nasal mucosa, biopharmaceutical studies of nasal formulation are the only means to identify formulation factors that influence pharmacological or physiological response. This report introduces a simple nasal microdialysis method as a means of screening nasal formulations prior to extensive biopharmaceutical testing to assure optimum therapeutic activity.

Experimental Procedures

Chemstrip bG® test strips and sodium fluorescein were purchased from Roche Diagnostics (Indianapolis, IN) and Sigma Chemical, respectively. ELISA insulin kits were purchased from Alpco (Windham, NH). Kelcogel® (Gelan gum) was a gift of Kelco US (San Diego, CA), and metered dose sprayers (50 µL actuation volume) were supplied by Valois (France). Human insulin powder (26.8 IU/mg) was a gift from Novo Nordisk (Denmark). All chemicals used were of analytical grade. Four male New Zealand rabbits (7.3-8.6 kg) were obtained from Charles River Labs (Amherst, MA). The study protocol was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act Regulations and approved by the Institutional Animal Care and Uses Committee of St. John's University (Jamaica, NY). The subject received an initial intraperitoneal injection of ketamine hydrochloride (35 mg/kg: Ketaset® Fort Dodge Animal Health, IA) and xylazine (5mg/kg; Rompun® Bayer AG, KS). Once sedated, a CMA/20 4mm membrane polycarbonate microdialysis probe with 20,000 Dalton cutoff was immobilized in the anterior region of the nasal cavity through the right nares of the anaesthetized rabbit and immobilized with swimmer's earwax. Aqueous solutions of sodium fluorescein (1 mg/mL) were prepared without and with 0.4% or 1.5% ion-sensitive viscosity-enhancing agent, Gelan gum. Because of intrasubject weight variability, insulin was dissolved in these solutions in a manner such that the dosing volume of 100 µL delivered an insulin dose of 5 units per kilogram. The four animals were dosed in randomized, crossover design with a two-day rest period between treatments in the left nostril with two 50 µL sprays from the Valois metered dosing spray device. The animals were kept sedated by administering 25% of the initial ketamine/xylazine dose every hour for the duration of the experiment. The probe was perfused with a modified Ringers solution (155.0 mM Na^+, 1.1 mM Ca^{++}, 2.9 mM K^+, 132.76 mM Cl^-, and 0.83 mM Mg^{++}) at 5 µL min^{-1} and collected at 30-minute intervals in a refrigerated fraction collector (CMA 107). Blood samples (0.5-0.8 mL) were collected every 30 minutes from the marginal ear vein cannulated with a 28-gauge butterfly needle firmly fastened to the ear using adhesive tape. Subjects experienced no distress as the probe was simply pulled out of the nose, permitting the rabbit to serve as its own control after a one-week washout period.

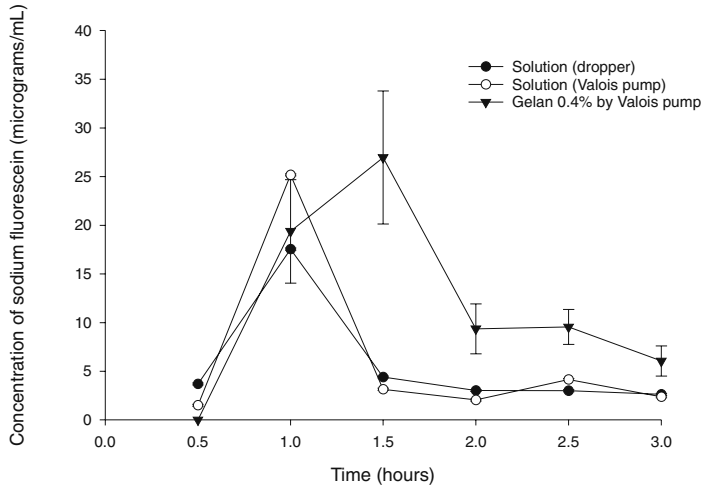
Fluorescein determination was performed in a HTS fluorescent plate reader (Perkin Elmer). Blood glucose determination was performed immediately after sampling using Chemstrip bG test strips and an Accu-Chek II blood glucose monitor. In this method the glucose concentration in whole blood is determined as a function of the color intensity produced by the glucose oxidase/peroxidase reaction occurring on Chemstrip bG test strips. The intensity of the colors formed is proportional to the glucose concentration in the specimen. The linear dynamic range was 20-500 mg/dl. Serum and nasal insulin levels were determined with Mercodia Insulin ELISA Kits (Alpco). The linear dynamic range validated with rabbit serum prior to the experiments was 0.1-5 ng/mL and 5-180 ng/mL for 10 µL and 50 µL samples, respectively.

Results and Discussion

A primary method of increasing bioavailability of peptides delivered by the nasal route is to increase the viscosity of the mucus produced by the nasal epithelium so that the intensity and beat frequency of the cilia are decreased. Gelan gum is a water-soluble polysaccharide extremely effective at low use levels in forming gels for mucosal drug delivery. In particular, the Kelcogel grade of Gelan gum is a novel gelling agent with the unique property of gelling in the presence of sodium and potassium ions. This lab has established the critical parameters that utilize this property while simultaneously conforming to other experimental constraints such as optimum metered dosing device, dose volume, etc. Our dosage form development

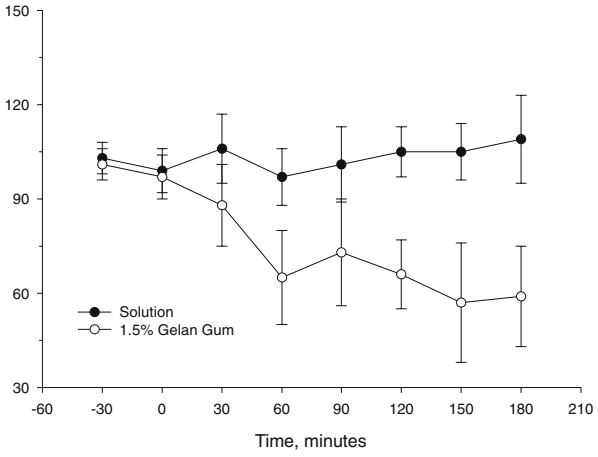
F1

Concentration of sodium fluorescein in dialysate from right rabbit nares following nasal administration of solutions by dropper or Valois pump, n = 4 (standard deviations for circles removed for clarity).



F2

Mean blood glucose levels in four diabetic rabbits (+/- s.d.) following nasal adminstration of insulin in Gelan vehicle versus solution.



program was predicated on the observation that because viscosity of the formulation is thin prior to actuation of the dosing device, the dose assumes a robust plume pattern that more efficiently covers the nasal cavity. Post-actuation, the finely distributed aerosol gels *in situ* upon contact with extracellular fluid cations cause the drug to persist longer at the deposition site. When the effect of solution viscosity on variability in dosing volume was studied gravimetrically and analyzed by ANOVA using SAS software (Cary, NC), it was concluded that viscosity enhancement due to addition of gum to the formulation had no effect on the dose delivered by the metered dose spray pumps ($p < 0.05$). It must be noted that both the *in vitro* probe efficiency for fluorescein and insulin and the effect of human versus rabbit blood matrix on Chemstrip bG glucose determinations were omitted from this initial study.

The results, shown in ***F1***, show the average amount of fluorescein (± s.d.) collected at the microdialysis probe tip in the four animals. The results are consistent with this line of reason, with fluorescein persisting significantly longer in the dialysate when administered as the Gelan 0.4% solution. In comparison, solutions delivered by dropper and Valois metered dose pump gave dialysates that rapidly decreased in fluorescein content one hour post dosing. ***F1*** shows the concentration of fluorescein in dialysates of the four animals in real time, that is to say uncorrected for the length of the 30 minute sampling period. Thus, the "apparent" lag time of 0.5 hour is actually the combined effects of rapid ciliary clearance toward the anterior nasal turbinate (i.e., the site of probe placement) and the average experience of the microdialysis probe over the 30 minute sampling interval. ***F2*** shows the effect of formulation factors on blood glucose levels in the four animals following nasal administration of insulin. In this case Gelan 1.5% solution showed a marked prolongation of glucose level depression. Taken in conjunction with the fluorescein results, it is not unreasonable to conclude that the significant difference between formulations on blood glucose arise from enhanced bioavailability of insulin secondary to prolonged residence time in the nasal cavity of Gelan-based formulations.

The selection of a proper test animal is critical, since interspecies differences exist (10-12). Pharmaceutical research and development aimed toward optimizing retention time in the nasal cavity typically utilize sheep and require that nasal dosage formulations be sent to commercial centers for radioactive labeling with neutron activated samarium-containing ligands, followed by timed gamma scintigraphy. This expensive procedure requires both a staff trained in nuclear medicine and proximity to a particle accelerator. A further disadvantage is that the dose must be formulated on site with equipment unfamiliar to the parent drug company by staff not trained in the art of extemporaneous compounding.

Insulin could not be determined in the nasal dialysates despite previous reports demonstrating dialysis membranes employed to measure insulin concentration in subcutaneous interstitial fluid (13) and muscle (14). The molecular weight cutoff of the probe membrane controls the selective sampling of proteins (15), and the probes used in our study were the largest molecular weight cutoff (20,000 Dalton) commercially available at the time. Insulin (MW = 6,000) should dialyze across this membrane but insulin exists primarily as a hexamer and in practice probe efficiency, as measured by

relative microdialysis recovery, for interstitial insulin *in vivo* is 3.0 ± 0.3% (16). In those reports, increased effective surface areas permitted 20 minute microdialysis samples to be obtained from the abdominal subcutaneous tissue using two custom made 45-mm polypropylene dialyzing tubes (o.d. ~0.5 mm, pore size 0.2 m). Unfortunately, such dimensions are inappropriate to the nasal anatomy. Insulin might be measured if larger nominal cut-off membranes (which facilitate entry of macromolecules into the perfusion fluid) had been used, but this advantage is negated by the excessive loss of perfusion fluid which could, in turn, alter microviscosity of the mucus and cilia beat frequency. Microdialysis catheters with a large pore-size membrane (100 kDa) made possible macromolecule sampling in muscles by increasing the colloid osmotic pressure of the perfusate with 40 g/L of dextran-70 to prevent perfusate loss across the dialysis membrane (17). Another difficulty encountered was that the insulin concentration was below the experimentally determined limit of detection of this ELISA, which used a chromogenic rather than radiolabeled or fluorogenic substrate. However, the objective was less one of insulin determination in the nose, but rather one of developing a robust screening tool. Use of the surrogate probe (i.e., sodium fluorescein) is aligned with this objective and even preferred, with fluorescein being free of the extensive binding, enzymatic degradation, etc., that biotech drugs frequently experience. ELISAs based on one of three fluorogenic substrates for common enzymes e.g., fluorescein diphosphate, carboxyumbelliferyl galactoside and Amplex Red are more sensitive reagents than chromogenic substrates. Amplex Red, a new substrate for peroxidase which is chemically stable and nonfluorescent until it reacts with the enzyme (18), will improve the limit of detection of insulin in the nasal dialysates. Finally, large molecular weight compounds that bind and trap insulin in the perfusate also could be used to permit the direct measurement of insulin residence time in the nose. The effect of slower flow rates and dialysate-additives on probe efficiency for insulin collection is the topic of ongoing work in this laboratory.

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Biographical Sketch

Emilio Squillante is an Assistant Professor. He uses supercritical fluid technology to formulate drugs with poor water solubility into lipid-based self-emulsifying systems and solid dispersions to improve bioavailability. In addition to CNS, cell culture and pharmacokinetic applications, his group uses microdialysis to study the role of dermal inflammatory mediators in transdermal permeation enhancement, transplant rejection and chronic inflammatory skin diseases.

References

1. *Y.W. Chien, Nasal Systemic Drug Delivery, Marcel Dekker, Inc., New York, (1989).*
2. *L. Illum, The nasal delivery of peptides and proteins. Trend Biotech. 9 (1991) 284-289.*
3. *Z. Mengpinga, M.D. Donovan, Intranasal mucocilliary clearance of putative bioadhesive polymer gels. Int. J. Pharm. 135(1-2) (1996) 115-125.*
4. *P. Dondeti, H. Zia, T.E. Needham, L.A. Luzzi, Development of a new non-surgical perfusion technique for evaluation of nasal drug delivery. Pharmazie 49(7) (1994) 505-9.*
5. *A.K. Alavi, E. Squillante, and S. Bagchi, The preservative action of polymers on the activity of lactase enzyme treated with supercritical carbon dioxide. AAPS Annual Meeting, November 14-18, 1999, Poster 2210.*
6. *E. Squillante and S. Neduvelil, Preparation of sustained-release chlorpheniramine maleate powders using supercritical fluid processing technology. AAPS Annual Meeting, November 14-18, 1999, Poster 3617.*
7. *H. Zia, P. Dondeti, and T.E. Needham, Comparison of nasal insulin powders prepared by supercritical fluid and freeze-drying techniques. Part. Sci. Tech. 15 (1997) 217-244.*
8. *P. Dondeti, H. Zia, and T.E. Needham, In vivo evaluation of spray formulations of human insulin for nasal delivery. Int. J. Pharm. 122 91-105 (1995).*
9. *H. Rosdahl, K. Hamrin, U. Ungerstedt, and M. Henriksson, Metabolite levels in human skeletal muscle and adipose tissue studied with microdialysis at low perfusion flow. Am. J. Physiol. 274 (1998) E936-E945.*
10. *S. Gizurarson, Animal models for intranasal drug delivery studies. Acta Pharm. Nord. 2(2) (1990) 105-122.*
11. *W.A. Ritschel, In vivo animal models for bioavailability assessment. S.T.P. Pharma, 3(2) (1993) 125-141.*
12. *F.W. Merkus, J.C. Verhoef, S.G. Romeijn, and N.G. Schipper, Interspecies differences in the nasal absorption of insulin. Pharm. Res. 8(10) (1991) 1343.*
13. *P.A. Jansson, J.P. Fowelin, H.P. von Schenck, U.P.Smith, and P.N. Lonnroth, Measurement by microdialysis of the insulin concentration in subcutaneous interstitial fluid. Importance of the endothelial barrier for insulin. Diabetes 42(10) (1993) 1469-73.*
14. *M. Sjostrand, A. Homang, and P. Lonnroth, Measurement of interstitial insulin in human muscle. Am. J. Physiol. 276(1Pt1) (1999) E151-154.*
15. *J.M. Schneiderheinze, and B.L. Hogan, Selective in vivo and in vitro sampling of proteins using miniature ultrafiltration sampling probes. Anal. Chem. 68 (1996) 3758-3762.*
16. *A. Holmang, K. Mimura, P. Bjorntorp, and P. Lonnroth, Interstitial muscle insulin and glucose levels in normal and insulin-resistant Zucker rats. Diabetes 46(11) (1997) 1799-1804.*
17. *H. Rosdahl, K. Hamrin, U. Ungerstedt, and J. Henriksson, A microdialysis method for the in situ investigation of the action of large peptide molecules in human skeletal muscle: detection of local metabolic effects of insulin. Int. J. Bio. Macromolecules 28(1) (2000) 69-73.*
18. *N.G. Hentz, Synthesis and characterization of insulin-fluorescein derivatives for bioanalytical applications. Anal. Chem. 69 (1997) 4994.*