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Guinea Pig Animal Model for Predicting Immunogenicity of Insulin Analogues

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ABSTRACT
Our objective is to use the guinea pig model to evaluate the immunogenicity of recombinant insulin and three specific insulin analogs: Glargine, Aspart, and Lispro. These analogs have been structurally modified from the original insulin sequence to enhance their pharmacokinetic and pharmacodynamic properties, leading to better blood glucose management in diabetic patients. This study aims to determine whether these commercially available analogs can trigger an immune response, potentially leading to the production of antibodies. Such immune responses could reduce the effectiveness of the insulin analogs and cause adverse effects. Additionally, we aim to gain valuable insights into the immunogenicity of these insulin analogs, thereby enhancing our understanding of their safety and efficacy. This knowledge will support the development of improved insulin therapies for diabetes management.

Keywords: Neutralizing Nab, Lispro, Glargine, Aspart, AIA, and HRP Conjugated Secondary Antibody

INTRODUCTION:

Insulin analogs have transformed the treatment of diabetes by offering enhanced properties in terms of how they are processed and how they act in the body, surpassing regular human insulin. Nonetheless, as with any medication, it is crucial to be aware of the safety considerations that come with using insulin analogs. Key safety issues associated with these analogs include immunogenicity, low blood sugar risk, obesity, and allergic reactions on the injection site. Among these concerns, immunogenicity is particularly significant, as it has the potential to impact other organs and result in long-term complications [1]. It's crucial to recognize that not every insulin analog or structural modification automatically triggers an immune response. The likelihood of immunogenicity varies among different analogs and is influenced by factors such as the degree of structural modification and individual genetic variation although insulin and related products on the market are recognized to be produced to high standards and to have received regulatory authority, temperature fluctuations can still occur during storage, transportation, or patient administration when given over an extended period [2]. Insulin is highly sensitive to temperature, and these fluctuations can potentially impact its effectiveness by causing structural alterations. Consequently, there is a legitimate concern regarding the safety of the drug if its potency is compromised due to such changes occurring in the hands of patients and other patient-specific factors. Intentional or unintentional structural modifications in insulin analogs can potentially induce an immune response through epitope exposure or altered binding to insulin receptors [3].

Epitope exposure: The changes made to the structure of insulin analogs can uncover novel or altered regions on the insulin molecule known as epitopes. Epitopes refer to distinct regions recognizable by the immune system. These modified epitopes have the potential to activate immune cells like T cells and B cells, leading to the production of antibodies targeting the insulin analog [4]. Additionally, the structural modifications in insulin analog can sometimes create resemblances or mimicry with other foreign substances or antigens. If the altered epitopes share resemblances with epitopes present in pathogens or other external substances, there is a possibility that the immune system could misinterpret the insulin analog as a potential threat, leading to the activation of an immune response against it.[5].

Altered binding to insulin receptors: Structural modifications in insulin analog can cause changes in their ability to bind to insulin receptors found on target cells. These alterations in binding can lead to modifications in receptor activation, signaling pathways downstream, and the activation of immune cells. Consequently, these modifications have the potential to initiate an immune response as the immune system identifies and reacts to the modified interaction between the insulin analog and the receptor [6,7].

Insulin and its analogs are produced as drug substances and subsequently formulated with buffers and preservatives to create drug products that have a prolonged shelf life. Apart from alterations in the structure of insulin or its analogs, which may lead to potential immunogenicity, it is crucial to acknowledge that impurities related to the manufacturing process, such as enzymes, host cell-related proteins, and DNA, could also trigger an immune response [7]. In specific instances, these contaminants might serve as adjuvants, inadvertently eliciting an undesirable immunogenic response in humans. This could potentially result in the generation of antibodies and adverse effects related to the immune system [8].

To assess the potential immune reaction induced by insulin analogs, researchers carry out comprehensive investigations through preclinical and clinical studies. These studies are designed to assess the immune reactions and safety profiles of these drugs as part of preclinical toxicity studies or assessed in phase 3 clinical trials to identify and address any concerns related to immunogenicity before approving them for use in clinical settings [8].

Animal models have been employed to investigate insulin analogs. The selection of a specific model depends on various factors, including physiological relevance, availability, ethical considerations, and specific research objectives. Here are some commonly used animal models for studying the immunogenicity of insulin analogs [4].

Non-human primates, such as cynomolgus monkeys, possess genetic and physiological resemblances to humans, rendering them valuable for investigating immunogenicity. They offer advantages in terms of their immune system similarity and predictive value for human responses. However, their use is often limited due to ethical concerns and practical considerations.

Rodents (e.g., mice, rats): Rodents are widely used due to their availability, cost-effectiveness, and ease of handling. Although their immune systems differ somewhat from humans, they still provide valuable insights into immunogenicity. Transgenic mouse models expressing human insulin receptors or possessing humanized immune systems can enhance the relevance of these models.

Pigs: Pigs have anatomical and physiological similarities to humans, including a comparable insulin structure. They can provide valuable data on the immunogenicity and pharmacokinetics of insulin analogs, although their use is relatively less common compared to other models.

Guinea pigs: Guinea pigs have historically been used in insulin immunogenicity studies due to their susceptibility to developing immune responses against insulin. They share certain similarities with humans in terms of immune responses and have proven valuable in assessing the immunogenicity potential of insulin analogs [9].

The selection of an appropriate animal model should take into account specific research objectives, including desired outcomes, available resources, and ethical considerations. It is also crucial to validate the findings in relevant human studies to ensure their translational relevance and applicability to human patients. Among the animal models used for insulin immunogenicity studies, guinea pigs have demonstrated benefits, particularly due to their suitability for insulin immunogenicity assessments.

Guinea pigs offer several advantages over non-human primate models in terms of accessibility, cost-effectiveness, and ease of handling. They can be conveniently maintained in laboratory settings, allowing for experimental studies with larger sample sizes. The primary structure of human insulin and guinea pig insulin differs by 16 amino acids, increasing the relevance of using guinea pigs as a model for investigating insulin immunogenicity. Guinea pigs have demonstrated a heightened susceptibility to developing immune responses against insulin, resulting in a greater likelihood of antibody production compared to other animal models like rodents. This heightened sensitivity makes guinea pigs an invaluable model for evaluating the potential immunogenicity of insulin analogs [10].

Furthermore, guinea pigs possess immunological characteristics that are advantageous for studying insulin immunogenicity. They have a well-developed immune system that exhibits similarities to human immune responses. Additionally, guinea pigs have been extensively utilized in various immunological studies, establishing them as a familiar and reliable model for assessing immunogenicity.

Intentional Structural characteristics:

The insulin analogs' nomenclature offers insights into alterations within their structure, yet it may not offer a thorough comprehension of precise structural adjustments or the underlying mechanisms. When a particular amino acid in the insulin sequence is replaced with another, the analog's name typically incorporates the substituted amino acid's name.

As an illustration, the amino acid sequence in lispro is modified by reversing the positions of proline and lysine at positions twenty-eight and twenty-nine of the beta region. This alteration is intended to decrease the occurrence of insulin dimers and hexamers, facilitating faster absorption and a quicker onset of action.[11]. Similarly, the process of formulating aspart entails replacing the proline located at position twenty-eight of the beta chain with aspartic acid. This modification leads to an increased absorption rate and a quicker onset of action when compared to standard human insulin[12].

Regarding glargine, two structural modifications occur. In the modification process, glycine replaces the asparagine at the A21 position in the alpha chain. Furthermore, two molecules of arginine are added to the amino acid threonine at position B30. The modifications lead to the development of insulin glargine, which is distinguished by its prolonged duration of action due to the gradual release of insulin from subcutaneous deposits. Our research aimed to investigate the immunogenicity of insulin analogs, utilizing guinea pigs as an animal model. The study had three primary objectives. Firstly, we aimed to assess whether the modified insulin analogs induce an immune response, leading to the generation of antibodies against these analogs[13].

Secondly, our objective was to ascertain whether the antibodies produced in guinea pigs specifically attach to the altered epitope of the analogs or if they also display reactivity with other insulin-related epitopes. Lastly, we sought to evaluate the implications of these findings, to offer valuable insights to researchers and clinicians. These insights can aid in making well-informed decisions regarding the development, formulation, dosing, and monitoring strategies for insulin analogs. This consideration involves taking into account their potential to elicit an immune response.

Insulin and its analogues like lispro, aspart, and glargine, when stored in vials, exist as hexametric proteins. After administration, they transform into monomers, affecting their absorption rate post-injection and their duration of action. The pharmacokinetic characteristics of human insulins are compared with those of insulin analogues.

Table 1: Pharmacokinetics of insulin and its Analogues

S.No	Human regular insulin	Insulin Lispro	Fast acting insulin Aspart	Insulin glargine
In vial	Hexamer	Hexamers	Hexamers	Hexamers
After administration	Hexamer dimers and monomers	Hexamer dimers and monomers	Hexamer dimers and monomers	Concentrated hexamer and aggregates
In the capillary	Monomer	Monomer	Monomer	Monomer
Rate of absorption	Fast	Very fast	Very fast	Very slow
Duration of action	Short	Short	Short	Short

The graph analyzed the plasma characteristics of Insulin/analogue over time, detailing their respective durations of action. Aspart and lispro were found to be active for approximately 4-6 hours, regular insulin exhibited activity for 6-10 hours, and glargine had a longer duration of action lasting from 20-24 hours.

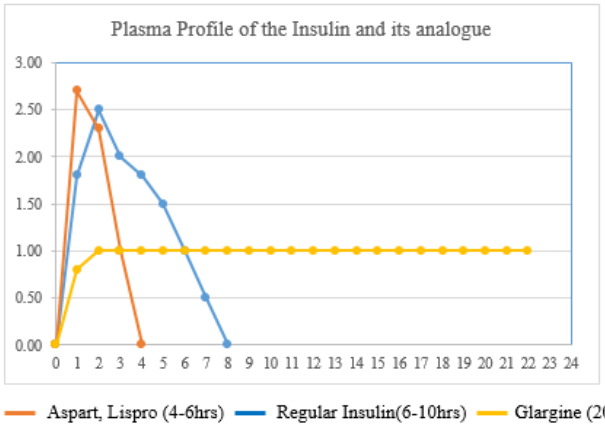


Figure 1: Plasma profile of the insulin and its analogues

MATERIALS AND METHODS

To generate antibodies against insulin analogs in animals, ethical approval was received from the institutional biosafety committee. Following procedures outlined in reference [14] eight adult female animals, weighing between 250 to 300 grams and lacking pre-existing antibodies to analogs, were selected for the experiment. These animals were housed in pairs in cages and provided with feed and reverse osmosis water. The animal was kept in a controlled environment with an ambient temperature and a relative humidity of around thirty to seventy percent. During both the initial dose and additional follow-up doses, 5 percent glucon D powder was added to the animals' drinking water. This addition aimed to mitigate any potential hypoglycaemic effects caused by the recombinant analogs employed in the research.

Immunogen preparation

We procured commercially available Lispro, Aspart, and Glargine and conducted dialysis against phosphate-buffered saline. Subsequently, they underwent treatment with reducing agents to deactivate them by disrupting the disulfide bonds.

To make the immunogens, we generated two distinct concentrations, approximately 100 µg, and 200 µg, utilizing insulin analogs (Glargine, Lispro, Aspart, and human insulin) treated with DTT from MP Biomedical. These immunogens were mixed with 0.9% saline to achieve a final volume of 500 µl.
For the initial primary immunization, we gradually added 0.5ml of FCA (Immuno Potentiators- SLBZ9885) to the immunogens, ensuring thorough mixing to avoid any clustering. In the following three supplementary doses, the FCA was replaced with FICA (SLCP1057) on the day of immunization.

Immunization protocols
Guinea pigs were immunized in two groups, each comprising four animals. Initial doses of 0.1mg and 0.2mg of the immunogens (Glargine, Lispro, Aspart, and human insulin) were administered to each animal in both groups.

After fourteen days, blood samples were collected, and the serum was separated and stored as test bleed 1. Supplementary doses were given on day twenty-two, with group 1 receiving 0.075mg and group 2 receiving 0.15mg g of the immunogen. Test bleed two was collected on day thirty-six.

Additional supplementary doses were administered on days forty-four (0.075mg for Group 1 and 10.15mg for Group 2) and sixty-three (0.1mg for Group 1 and 0.2mg for Group 2). Production bleed 1 was collected on day fifty-six, and a final bleed was conducted on day 70, collecting 8 ml of blood from each animal. The serum was separated, aliquoted, and stored at a low temperature.

Indirect ELISA was performed on the serum to determine antibody titers, and the final terminal bleeds were stored for subsequent antibody purification [14].

Table 2: Immunisation protocol for the development of Antibody

Day	Immunization	Antigen concentration /Animal	Antigen Volume	Nacl	FCA	FIA	Route of administration
0	Pre bleed collection	-	-	-	-	-	-
1	Primary Immunization and Test bleed on day 14	0.1mg	0.145ml	0.355ml	0.5ml	-	Thoroughly mix and administer 0.5ml of the antigen subcutaneously.
22	Supplementary dose – I	0.075mg	0.11ml	0.39ml	-	0.5ml	Thoroughly mix and administer 0.5ml of the antigen subcutaneously.
36	Test Bleed - II	We will draw two ml of blood from each animal, with 0.5 ml of serum reserved for titer examination.					
42	Supplementary dose – II	0.075mg	0.11ml	0.39ml	-	0.5ml	Thoroughly mix and administer 0.5ml of the antigen subcutaneously.
56	Production bleed I	We will draw two ml of blood from each animal, with 0.5ml of serum reserved for titer examination.					
62	Supplementary dose – III	0.1mg	0.11ml	390 µl	-	0.5ml	Thoroughly mix and administer 0.5ml of the antigen subcutaneously.
75	Final bleed	Around 8-10 millilitres of serum were gathered from each animal to determine and record the titer. The collected serum was then stored until the purification process.					

The immunization timetable aims to generate antibodies against Insulin Analogues in Guinea pig models, ensuring the immunogenicity of these analogues is accurately assessed.

Antibody titer analysis using Indirect ELISA techniques
We modified the standard indirect ELISA procedure for our experiment. The antigen stock at a concentration of 5 µg/mL was used to coat 96-well high-binding ELISA plates using 100 µL of the antigen solution per well, followed by a 90-minute incubation at RT.

The high binding wells were washed two times with 1X PBST and blocked by 3% PEG solution for 45 minutes. Following blocking, the wells underwent two washes with 1X PBST buffer.

The hyperimmune sera were serially diluted in the buffer and added to the wells. After one hour of incubation at ambient temperature, the wells underwent five washes using 1X PBST.

Next, a secondary antibody (Invitrogen TL276796) was introduced to each well at a 1:10000 dilution, with an incubation period of 30 minutes.

After performing an additional five washes with a wash buffer, the color development process commenced by introducing 100 µL of substrate (Kementech 4395E) into each well.

The process continued for 20-25 minutes, concluding with the addition of 100 µL of sulfuric acid per well to halt the reaction. Subsequently, the absorbance at 450 nm was promptly determined [14,15].

The indirect ELISA procedure starts by coating a microplate well with insulin antigen to facilitate antibody interactions. To ensure specificity, blocking agents are applied to prevent non-specific binding.
Following this, hyperimmune serum samples containing anti-insulin antibodies are introduced, allowing them to bind to the coated antigen.
An enzyme-linked anti-IgG antibody is then introduced to specifically bind to these anti-insulin antibodies. Upon adding a substrate solution, a color change occurs if anti-insulin antibodies are present, which is measured for absorbance.
This method provides accurate quantification of anti-insulin antibodies, giving valuable insights into the immune response against insulin analogues.

Table 3: Indirect ELISA Protocol for anti-insulin antibody titre analysis

S.No	ELISA Steps	Concentration and Reagent	Vol per/well (µl)	Incubation temperature	Incubation time minutes
1	Coating	5µg/ml of insulin analogues in 1x PBS	100	RT	90 minutes
2	Washing	Wash buffer 1xPBST	200,2times	NA	NA
3	Blocking	In house blocking buffer 3% PEG	200	RT	45 ± 5
4	Washing	Wash buffer 1XPBST	200, 2 times	NA	NA
5	Primary	Serum/Purified Ab dilution	100	RT	60 ± 5
6	Washing	Wash buffer 1XPBST	200,5times	NA	NA
7	Secondary	Anti-Guinea pig Ab HRP Conjugate	100	RT	45 ± 5
8	Washing	Wash buffer 1XPBST	200,5times	NA	NA
9	Substrate	TMB	100	RT	20minutes
10	Stop solution	H2So4	100	RT	NA
11	Read out	450nm	-	-	-

Analog-specific antibody purification- Step 1
The analog-specific antibody purification procedure was adopted from the reference without any modification [14]. The purified antibodies underwent assessment for their ability to bind to insulin and its analogs using an indirect ELISA method. The objective was to ascertain the level of cross-reactivity among insulin analogs.

Target Affinity Purification Method- Step 2
To isolate analog-specific antibodies, Protein A purified anti-lispro, anti-aspart, and anti-glargine Guinea pig IgG was further purified by passing through the respective antigen affinity column, and affinity- eluted antibody was passed again through insulin-immobilized Affigel-10 to deplete any insulin binders. Flow through that got depleted of insulin antibody binders will be specific to the structural epitope of the analogs.
To immobilize the insulin or its analogs onto a solid support, the drugs were chemically cross-linked to Affigel-10 beads by following the manufacturer's instructions. The required amount of 1ml of Affigel beads was taken from the stock and the gel within 20 minutes using a cold 10-millimolar concentration of sodium acetate at pH 4.5. Around 12 mg of Insulin or analogues were aliquoted per ml of Affigel 10, which was made up to 10 mL with coupling buffer and allowed to rock for 4 hrs at 4 degrees. Next, the moist gel is into a Bio-Rad affinity column, and the top and bottom caps of the column allow the solution to drain into a clean tube. To block any remaining uncoupled sites on the gel, 0.1 ml of one molar ethanolamine hydrochloride at pH 8 was added per ml of gel and incubated for 1 hour. After incubation, the gel was transfer to water or coupling buffer until there were no remaining reactants, which was confirmed by measuring OD280. Finally, the gel with elution buffer to prepare the column for equilibration and sample application. Cleansed the packed column using 50 millmolar of specific buffer with the neutral condition and added Protein A purified analog specific antibodies to the respective insulin or analogue affinity columns and allowed to incubate overnight. The next day, after a couple of washing the drug-bound target antigen analogue specific antibodies were released using 0.2 molar glycine with the acidic condition. After dialysis of eluted antibodies, the target affinity purified antibodies were passed through an insulin affinity column, to trap the antibodies common to insulin and respective analogs, the flow through collected from the column would be specific for analogs structural epitopes [16].

Results:

The graphs illustrate the titer values of hyperimmune serum samples derived from various insulin analogs and their corresponding animal bleeds.

A graph was generated using the OD values for all four analogs. The OD at 450nm of 4 Guniea pigs' bleed was graphed linearly against their bleed type, with the X and Y axis labeled as Titer and Absorbance, respectively.

All four animals exhibited a robust response to the immunization with insulin analogs (200g/mL). Consequently, the serum from these animals was selected for antibody purification, aiming to assess insulin immunogenicity.

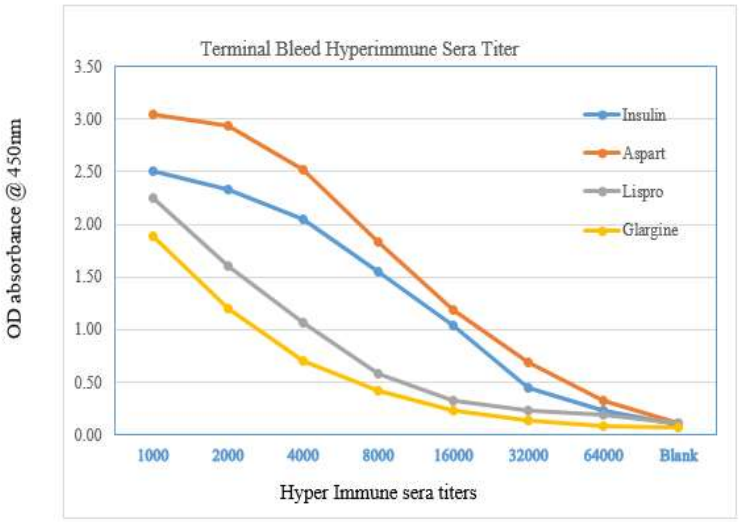


Figure 2: Terminal bleed hyperimmune sera titre for insulin and its analogues

Guinea pigs were immunized with insulin and insulin analogues over 70 days, resulting in varying degrees of immune response. Among the four drugs used as immunogens along with adjuvants, aspart, and insulin produced a relatively higher level of antibody titers compared to lispro and glargine. The differences in eliciting an immune response and antibody titers cannot be attributed to any structural dissimilarities between the drugs but may be due to inherent variability among individual animals. On average, the antibody titers for aspart and insulin were around 8000, whereas for lispro and glargine, the titers were 2-3 times lower.

The sequence of human insulin and analogs with receptor binding amino acids highlighted in different colour and mutation of the analogs boxed in the C terminus with a different color. This detailed depiction aids in understanding the structural modifications in insulin analogs and their impact on receptor interactions, providing insights into their therapeutic efficacy and potential differences compared to native human insulin.

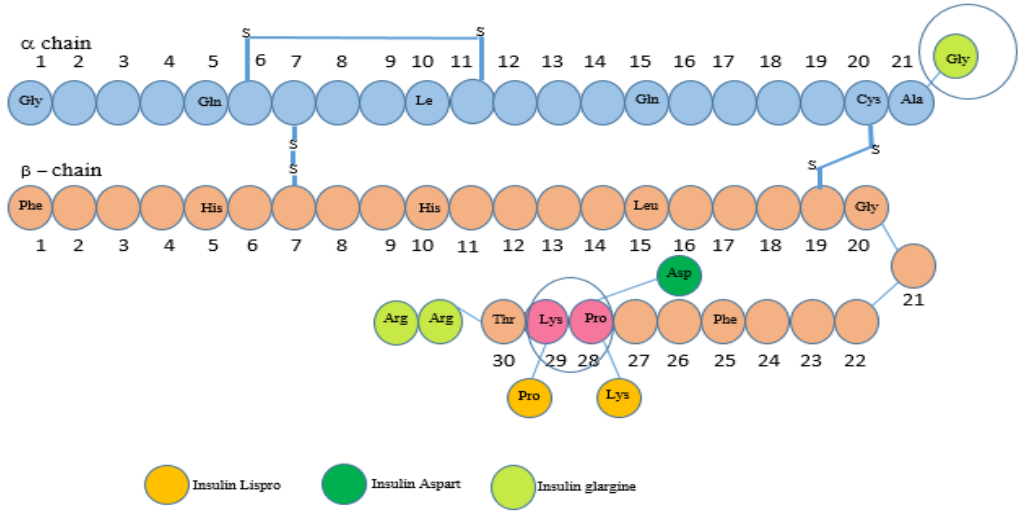


Figure 3: Structure of Insulin and its analogues with amino acid mutation

The analog-specific B-chains antibodies from the guinea pig hyperimmune sera were purified by passing through an insulin column (negative or subtractive purification) to eliminate those antibodies that bind to common epitopes found in both insulin and analogs. Unbound antibodies collected as flow-through are a mixture containing the B- chain specific antibodies along with non-specific guinea pig IgG was further purified by an analog-specific affinity column. The analog-specific antibodies were eluted from the affinity column, and further concentrated by passing through a Protein A columns, and the antibody purification yield was determined using the UV 280 spectrophotometric method.
To determine the percentage yield of anti- insulin (A and B chain-specific) and analog (B-chain specific) antibodies, the typical yield of total IgG/mL of serum was determined by passing the hyperimmune sera through a Protein A column.The specificity of the antibody eluate and flow-through from the insulin column were assessed for binding to insulin and other analogs in an ELISA assay using the indirect ELISA format (as depicted in Figure 4). Further purification of analog-specific antibodies was accomplished by affinity purification in their respective analog-specific affinity columns. The binding specificity to insulin and other analogs were determined by indirect ELISA (Figure 4). The antigen and antibody binding profile is represented in the graph below. The ELISA data reveal that guinea pigs have developed antibodies specifically to respective analogs and most probably to the B-chain epitopes.

This detailed analysis through Indirect ELISA allows researchers to precisely determine the specificity of purified antibodies against insulin or its analogues. It provides insights into the immune response generated against these molecules, aiding in evaluating their efficacy and potential immunogenicity in therapeutic applications.

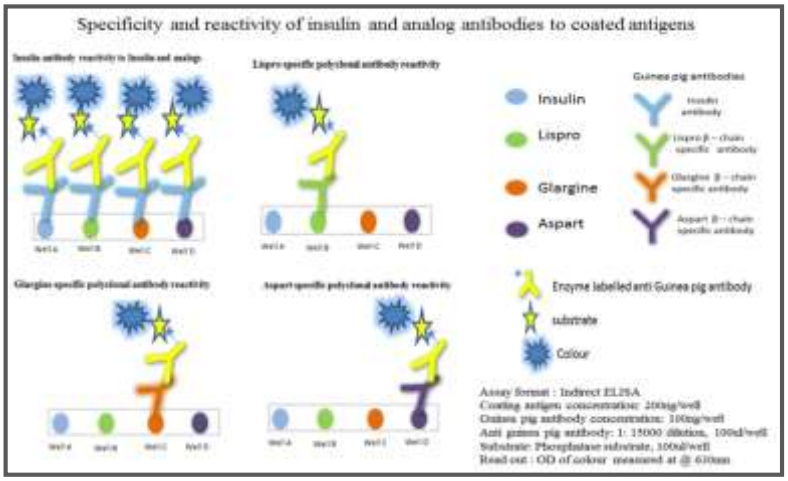


Figure 4: Specificity and reactivity of insulin and its analogue antibodies to the respective antigens

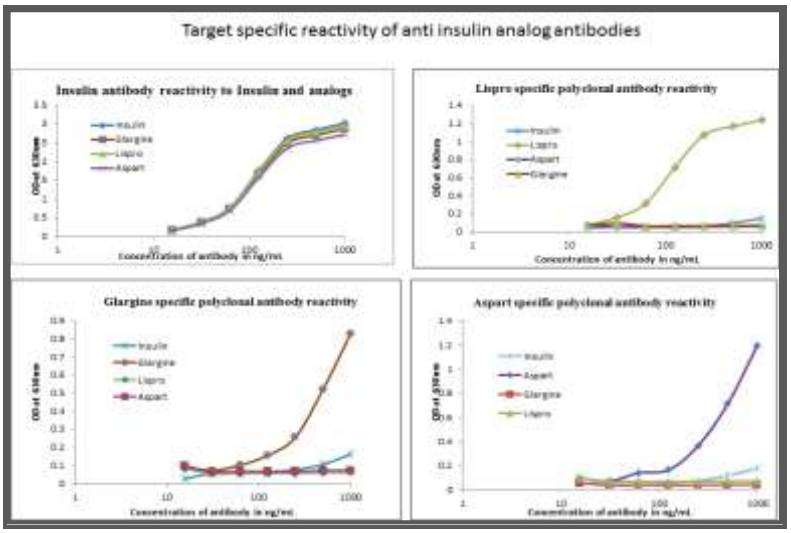
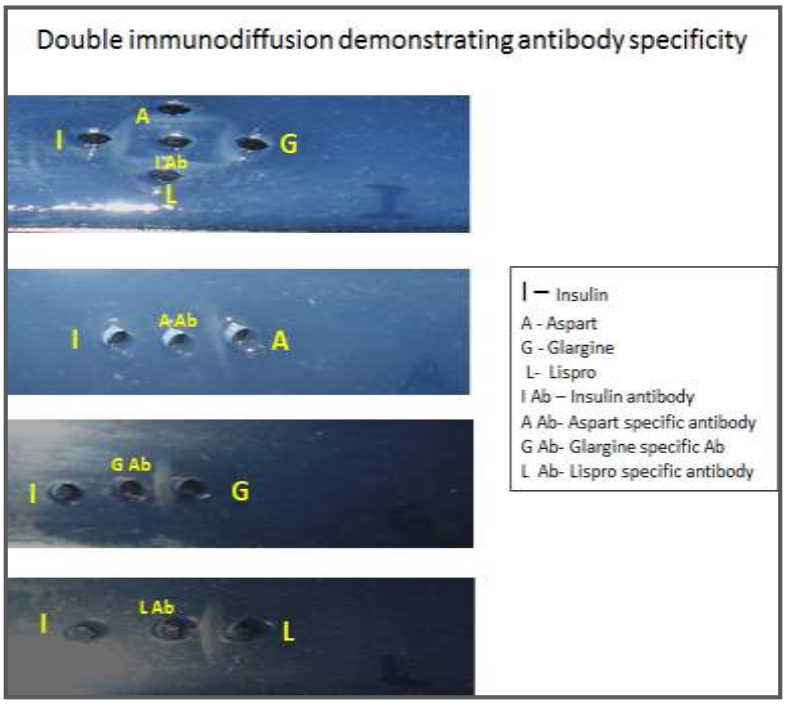


Figure 5: Double Immunodiffusion demonstrating antibody specificity

We used the Ouchterlony technique to demonstrate that the purified antibodies are specific to their respective analogs. Specific (analog- B chain-specific) polyclonal antibodies or complex polyclonal antibodies (Insulin A and B chain specific) were placed in a central well in an agarose slab/slide and allowed to interact with antigens (analogs and Insulin) diffusing towards it from two or four peripheral wells. In the figure below, slide 1 demonstrates that the precipitin lines formed in a square shape touching each other indicate that the antibodies can recognize the common epitope found in

insulin and all the other three analogs (Lispro, Aspart, and Glargine). In Figure 5, slides 2, 3, and 4 precipitin lines found only close to the analogs (right well) and not to the insulin (left well) is an indication of immunological distinctness, thus proving that the antibodies recognize only the linear and/or structural epitope of analogs as result of mutations introduced in the B-chain C-terminal end of the insulin sequence.

A double immunodiffusion assay is conducted to assess the interaction between insulin and its analogues with specific antibodies from guinea pigs. This assay involves placing samples containing insulin or its analogues in one well and the guinea pig antibodies in an adjacent well. Over time, a visible precipitin line forms where the antibodies and antigens interact, indicating the presence of a specific immune response.



We interchangeably assessed the specificity and cross-reactivity of the B chain-specific antibodies of Lispro, Aspart, and Glargine by comparing them using the Ouchtterlony double diffusion techniques. The qualitative results of precipitin line formation as a result of an antigen-antibody reaction in agarose slab/slide are indicated as positive(+ : reactive/binding) or negative(- : non reactive/non-binding)

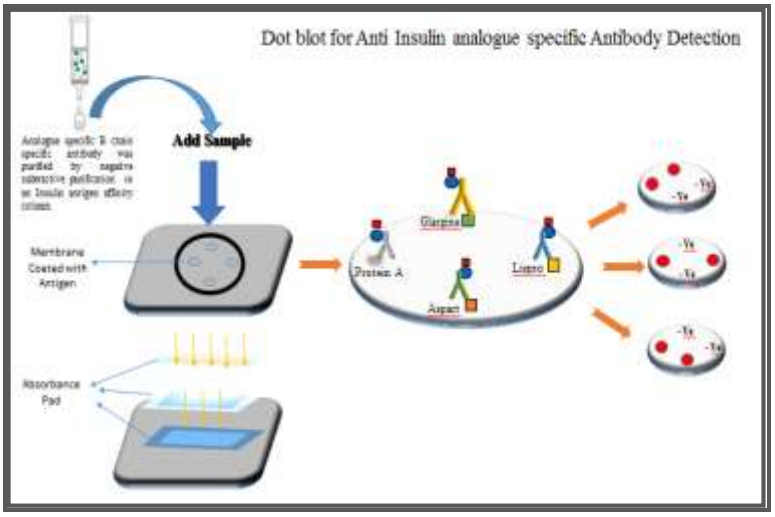
Provide flowcharts/system architecture diagrams/ snapshots for supporting
To further validate the proof-of-mechanism, insulin detection and anti-insulin or analog-specific antibody detection were demonstrated using guinea pig anti-insulin and analog B-chain-specific antibodies in a prototype disposable cassette-type kit which works on the principle of dot-blot technique.

Insulin Detection in the sample:
The description outlines a solid-phase bridge assay that is straightforward, sensitive, rapid (completed in 3 minutes), and consistently replicable. It is designed for detecting insulin in human serum within the low nanogram range (4ng). The nitrocellulose membrane was dot-blotted with guinea pig anti-insulin B- B-chain specific antibodies and blocked in the unbound region with a blocking buffer containing 2% BSA. When serum samples containing insulin were added to the membrane the coated antibody was captured or bound to the insulin B-chain and after upon a quick rinse with wash buffer (PBST), the detection of guinea pig anti-insulin A-chain specific antibodies conjugated to HRP was immobilized by the insulin which bridged the capture and detection antibodies. Following a rinse with the wash buffer and upon addition of the TMB substrate, the enzyme HRP catalyzes and the substrate forms a perceptible product on the membrane. The observed color intensity in the dot-blot is directly proportionate to the concentration of the particular antigens.

Dot Blot Assay:
For proof-of-mechanism, anti-insulin antibodies and analog-specific B-chain antibodies were differentially purified using antigen-specific affinity columns, and the eluate containing the specific antibodies was used for the demonstration. The antigens were immobilized onto the nitrocellulose membrane in the cassette as a dot and when respective antibody-containing samples were added, the antibodies in the sample formed a complex with the antigen specifically (Figure 6). The presence of antibodies in the complex was detected using a gold-conjugated protein A. The protein A-bound

antibody-antigen complex will form a round spot with an intensity that is visible to the naked eye (Figure 6). Even though this is a qualitative technique, the presence of anti-insulin analog-specific antibodies can be attributed to the loss of efficacy of the drug to a certain degree. At a time, in a single cassette, we can specifically determine whether the analogs also bind to the insulin antibody; thereby, it is possible to conclude that negative results indicate an absence of antibodies to an analog, and it is the best substitute insulin analog drug that can be used for diabetes management [16].

Figure 6: A dot blot assay is used to detect anti-insulin analogue antibodies, focusing on Lispro, Aspart, and Glargine. The process involves spotting target antigens onto a membrane, blocking nonspecific binding, and adding Guniea pig hyperimmune samples containing the antibodies. Bound antibodies specifically attach to their corresponding antigens. After washing, Protein A labeled secondary antibody is added to visualize the bound antibodies. This assay helps assess the immune response to insulin analogues and their immunogenicity.



For commercial launch, we intend to develop dot-blot devices. The device will reveal a very specific way to detect anti-analog-specific antibodies. We plan to introduce in-built insulin as a trapper to trap all antibodies against the common epitope shared between insulin and insulin analog antibodies. Upon loading the serum into the sample loading well, the serum component moves laterally with the help of a buffer, and all antibodies against shared epitopes of insulin and its analogs will be trapped by the insulin coated onto the membrane. If there are any analog B-chain specific antibodies present, they laterally move along with other non-specific antibodies, bind and carry the gold conjugated Protein A, and precipitate as an antigen and antibody complex at the region in the membrane coated with the specific analogs.

Discussion and Conclusion

The major objective of the investigation is to assess whether the structural alterations in insulin analogs would induce an immune response when a guinea pig was used in an animal model and whether the developed antibodies bind exclusively to structural epitopes and are analog-specific. Generally, guinea pigs are commonly used in insulin antibody production due to their ability to generate a strong immune response against insulin analogs. The yield of antibodies is usually assessed by measuring the antibody titer in the guinea pigs' serum through methods like enzyme-linked immunosorbent assays (ELISA). Among the 4 drugs tested for immunogenicity, all four drugs induced an immune response, and the yield of anti-insulin and analog-specific antibodies in guinea pigs was found to be of the same level of titer. This could be due to the use of identical immunization protocols with the same dose and frequency of immunization and the same adjuvants in the specific guinea pig strain [17].

The average yield of insulin or analog-specific antibodies was found to be only 2% despite using complete and incomplete adjuvants. The amino acid sequence difference between human and guinea pig insulin, which is 18 amino acids, is indeed relatively small, and they share a high degree of similarity in their primary structures. Insulin, a hormone produced in humans and guinea pigs, may develop immune tolerance, dampening the immune response when exposed to human insulin. Although insulin can be immunogenic in some contexts, it may not be as potent in guinea pigs as in other species. The production of antibodies against specific structural epitopes of insulin analogues was found to be only 0.3% of the total guinea pig IgG, which is only 20% of antibodies developed against the whole insulin molecule. The structural epitopes recognized by antibodies are often highly specific to the antigen's three-dimensional conformation [4]. Even minor differences in the insulin molecule's structure between human and guinea pig insulin can result in reduced recognition and binding of guinea pig antibodies to human insulin. The specific structural features and accessibility of the epitope to the immune system can influence its immunogenicity. Guinea pigs, like other animals, may have some degree of immune tolerance to their insulin. This tolerance can extend to certain structural epitopes, further reducing the immune response against those epitopes. Overall, our choice of Guinea pig animal model and the successful isolation of specific structural

epitope-specific antibodies highlight that Guinea pig could help in developing in vivo assays to determine novel as well as biosimilar insulin immunogenicity [18].

Confirming the specificity of structural epitope-specific antibodies is an essential step in antibody characterization and validation. Two common methods used for this purpose are quantitative indirect enzyme-linked immunosorbent assay (ELISA) and cross-reactivity studies using qualitative techniques such as dot blot immunodiffusion methods. The indirect ELISA was used to assess the specificity of isolated antibodies and determine whether they recognize the target antigen's structural epitopes. The ELISA results were analyzed by comparing absorbance values in samples that included the structural epitope-specific antibody binding to insulin and its analogue. The goal was to assess whether the structural epitope-specific antibody exhibits notable binding to other related antigens or epitopes. The findings clearly indicate that analog-specific antibodies demonstrate dose-dependent binding to their target epitope, without any cross-reactivity with other analogs [19]. Qualitative techniques such as the Ouchterlony method and dot blot method also demonstrated that the purified antibodies are specific and selectively bound only to the target antigen epitope, and there is no cross-reactivity to the related antigens [20]. Both dot blot and immunodiffusion are relatively simple and widely used techniques in immunology and serology laboratories. However, it may lack sensitivity and specificity compared to enzyme-linked immunosorbent assays (ELISA). The results obtained correlated and established that the minor structural differences in insulin and analogs are sufficient for guinea pigs to elicit a strong immune response.

However, while animal studies and in vitro analyses can offer valuable insights, caution is necessary when extrapolating the findings to humans. Guinea pigs may respond differently than humans to minor structural changes in insulin and its analogues. Nevertheless, the study highlights the potential of using guinea pigs as an animal model to assess the immunogenicity of insulin and its analogues before conducting clinical trials in humans.

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Declarations

Ethics Approval: IAEC approval number ABS-IAEC-032–2021-22

Participation Agreement: This article is a collaborative effort by all of us.

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