

For reprint orders, please contact reprints@future-science.com

Insights in regulated bioanalysis of human insulin and insulin analogs by immunoanalytical methods

Despite the long and illustrious history of insulin and insulin analogs as important biotherapeutics, the regulated bioanalysis (in this article, regulated bioanalysis refers to the formalized process for generating bioanalytical data to support pharmacokinetic and toxicokinetic assessments intended for development of insulin and insulin analogs as biotherapeutics, as opposed to the analytical process used for measuring insulin as a biomarker) of these peptides remains a challenging endeavor for a number of reasons. Paramount is the fact that the therapeutic concentrations are often low in serum/plasma and not too dissimilar from the endogenous level, particularly in patients with insulin resistance, such as Type 2 diabetes mellitus. Accordingly, this perspective was written to provide helpful background information for the design and conduct of immunoassays to support regulated bioanalysis of insulin and insulin analogs. Specifically, it highlights the technical challenges for determination of insulin and insulin analogs by immunoanalytical methods that are intended to support evaluations of pharmacokinetics and toxicokinetics. In a broader sense, this perspective describes the general bioanalytical issues that are common to regulated bioanalysis of peptides and articulates some of the bioanalytical differences between conventional monoclonal antibodies and peptide therapeutics.

Introduction & historical perspective

Clinical use of insulin has grown exponentially following its discovery in 1921 [1,2] and initial administration in man as nonpurified animal insulin [3]. Today, insulin and **insulin analogs** represent the standard of care for treatment of both Type 1 and 2 diabetes mellitus (DM) and comprise the largest selling category of peptide therapeutics with projected 2010 worldwide sales approaching US\$15 billion. Furthermore, the demand for new and improved insulin therapeutics continues to grow due in large part to the global epidemic in DM with an estimate of 438 million affected individuals by 2030 [201].

Historically, the first report of the detection of circulating insulin by immunoassay methodology occurred in 1956 [4]. In fact, the subsequent 1960 publication by this same group was reported to be one of the most-cited articles ever published in the *Journal of Clinical Investigation* [5,6]. Today the topics of early insulin research and development of the immunoassay are linked inextricably as this peptide was the first analyte to be quantified by immunoassay and, as such, cultivated wide usage of radioimmunoassay (RIA) as a major diagnostic tool, which culminated in the Nobel Prize in 1977 [6–9]. In the early years, attention in insulin drug development focused

on increasing the purity of extracted animal insulins and improving its time–action profile through introduction of formulation changes. The 1980s marked the beginning of commercial production of human insulin by means of **recombinant DNA** methodology [10–12]. At about this same time technology for the production of monoclonal antibodies spurred the era of modern immunodiagnosics [13]. This culminated in broad application of noncompetitive ELISAs as a major analytical tool for routine determination of analytes in biological matrices [14–16]. Reports of monoclonal antibody-based sandwich ELISAs for insulin first appeared in the mid-to-late 1980s [17–24]. Today, commercial kits are available widely for determination of insulin by both RIA and ELISA. However, most commercial kits are intended for use in measuring **endogenous concentrations** of insulin. As such, they are typically not designed ideally for conducting insulin measurements to support pharmacokinetic (PK) assessments, particularly for good laboratory practice (GLP)-compliant determinations. In addition, the discordance in reported concentrations for endogenous insulin detected by different commercial kits has led to creation of a working group for analytical standardization of insulin immunoassays [25–27]. Despite its long history in

Ronald R Bowsher^{1,2}
& William L Nowatzke³

¹B2S Consulting, 6656 Flowstone Way, IN 46237, USA

²EMD Millipore, BioPharma Services, 15 Research Park Dr., St Charles, MO 63304, USA

³Worldwide Clinical Trials, TX, USA

[†]Author for correspondence:

Tel. +1 317 787 2213

Fax. +1 888 769 2017

E-mail. ronb@b2s-stats.com

**FUTURE
SCIENCE** part of
fsg

Key Terms

Insulin analog: A recombinant DNA-derived protein that possesses a high degree of homology with native human insulin and which has been modified systematically to possess specific pharmacokinetic/ pharmacodynamic attributes.

Recombinant DNA: A sequence of nucleic acid consisting of introns that is transcribed by a host organism to ultimately generate a recombinant protein.

Endogenous concentration: Concentration of analyte that was produced by the host organism and is identical or similar to the biopharmaceutical of interest.

Regulated bioanalysis: Bioanalysis conducted to generate preclinical/clinical data to support a drug-development program and usually intended for submission to a regulatory agency.

Heterogeneity: Consisting of several parts that lack uniformity.

clinical medicine, reference values for circulating concentrations of insulin are not readily available. Notwithstanding a report by Chevenne *et al.* [28], some typical insulin concentrations in different species are reported in **TABLE 1**. Inspection of the serum insulin values in **TABLE 1** indicates the baseline level is about 10–15 $\mu\text{U/ml}$ with slightly higher values in the rat.

Regulated bioanalysis of insulin and insulin analogs is challenging for a number of reasons. First, while insulin is an endogenous peptide that is largely conserved across species [29–31], it does display sequence **heterogeneity**, particularly in rodents [30,32–34]. Second, insulin analogs often display a lack of parallelism in insulin immunoassays, especially when the reagent antibodies are produced against native human insulin. Third, therapeutic concentrations for insulin analogs are not too dissimilar from the endogenous serum concentrations of native insulin. This often necessitates measurement of insulin analogs concentrations in a biological matrix that contains a similar level of native insulin. Lastly, insulin immunoassays are characterized by the same technical challenges that generally impact ligand-binding assays (LBAs) for regulated bioanalysis, such as matrix interference due to a lack of sample cleanup prior to analysis [35–37].

Whereas the analytical attributes of insulin immunoassays have been reviewed a number of times, these publications are focused on clinical diagnostic applications for insulin as a biomarker and not intended for supporting PK evaluations

in animals and man [28,38–40]. Hence, this perspective was written to specifically address the challenges inherent in regulated bioanalysis of insulin and insulin analogs and provide insights into the design and application of immunoassays for supporting PK/toxicokinetic (TK) assessments. Consequently, this perspective indirectly addresses key bioanalytical method validation (BMV) differences between assays used for drugs (i.e., GLP bioanalysis) [41–43,202] and clinical biomarkers (i.e., Clinical Laboratory Improvement Amendments [CLIA]/Clinical Laboratory Standards Institute [CLSI]) [44–48]. For this reason, it may help to broadly communicate some bioanalytical points to consider for peptide therapeutics beyond those just for insulin and insulin analogs.

Current guidelines for regulated bioanalysis of protein therapeutics

Good laboratory practices for conducting regulated nonclinical laboratory studies were established in the USA in 1976 [202]. The purpose of these rules is to ensure the quality and integrity of the safety data generated during the conduct of the study. The GLP regulations were intentionally written to be vague, defining practices in a broad overview, and permitting some degree of flexibility for implementing processes. Using the US FDA regulations as a template, the Organization for Economic Co-operation and Development (OECD) Principles of GLP [49] were developed by an Expert Group on GLP established in 1978.

Table 1. Reported endogenous levels of insulin in various species.

Species	Matrix	Model	Description	Insulin ($\mu\text{U/ml}$)	Method	Ref.
Human	Serum	Normal 12 h fasting	Newborn	3–20	Not reported	[134]
Human	Serum	Normal 12 h fasting	Adult	2–25	Not reported	[135]
Human	Serum	Normal 12 h fasting	>60 years	6–35	Not reported	[212]
Human	Serum/plasma	Not reported	Not reported	2–20	Not reported	[213]
Human	Serum	Not reported	Not reported	<25	Abnova	[210]
Human	Not reported	Normal healthy fasting	Adult (n = 137)	2–25	ELISA	[211]
Human	Extracted serum (free insulin)	Normal healthy fasting	Adult (n = 15)	4.1–28.5	RIA by Phadebas	[136]
Human	Extracted serum (total insulin)	Normal healthy fasting	Adult (n = 15)	9.4–26.6	RIA by Phadebas	[136]
Human	Extracted serum (free insulin)	Normal healthy nonfasting	Adult (n = 17)	5.4–47.8	RIA by Phadebas	[136]
Human	Extracted serum (total insulin)	Normal healthy nonfasting	Adult (n = 17)	8.2–42.2	RIA by Phadebas	[136]
Human	Extracted serum (free insulin)	Diabetic with AIA	Adult (n = 21)	0–230	RIA by Phadebas	[136]
Human	Extracted serum (total insulin)	Diabetic with AIA	Adult (n = 21)	0–10888	RIA by Phadebas	[136]
Rat	Heparin plasma	Sprague-Dawley nonfasting	Males, 2.5–5 months (n = 8)	25.3	RIA	[137]
Dog	Serum	Canine	Mongrel (n = 6)	4.2–28.2	Not reported	[138]

To convert from $\mu\text{U/ml}$ to pmol/l (pM), multiply the values by 6. AIA: Anti-insulin antibody; RIA: Radioimmunoassay.

The first American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop was held in 1990 [42]. The scope of this meeting was to address important topics related to bioanalytical method validation, with an emphasis on chromatographic–MS platforms. In the mid 1990s, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) published a method validation Industry Guideline [203] and a follow-up Guidance [204] with nonbinding recommendations for US, European and Japanese investigators.

An important roundtable session was held at the 1998 AAPS Annual Meeting that specifically highlighted some of the unique challenges for application of LBAs in supporting regulated bioanalysis of macromolecules. This roundtable prompted several attending scientists to collaborate in the development of a manuscript that specifically focused on the unique BMV attributes of LBAs [35]. In March 2000, a key AAPS/FDA Workshop was held in Crystal City, VA, USA [50]. At the conclusion of this conference the Ligand-Binding Assay Bioanalytical Focus Group (LBABFG) was established within AAPS shortly after conclusion of the Crystal City Workshop to create a forum for ongoing discussions and education. Numerous important consensus publications have since been co-authored by members of LBABFG for recommending best BMV practices to support regulated bioanalysis in preclinical and clinical phases of drug development for biotherapeutics. These White Papers include LBA methods to support PK assessments [42], detection and characterization of antidrug antibodies (ADAs) [51–53] and quantitative determination of novel biomarkers [45].

In 2001, the FDA issued the Guidance for Industry, BMV [54]. This is currently the only US regulatory guidance document that must be

adhered to when validating bioanalytical methods to support human clinical pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring PK evaluation. This guidance also applies to bioanalytical methods used for nonhuman PK/TK studies and preclinical studies. A revised version of this document is expected in the near future. In addition to the 2001 US FDA Guidance, companies conducting studies in the USA are expected to follow recommendations published in conference reports published after 2001. Namely, the recommendations contained within the conference report of the third AAPS/FDA Bioanalytical Workshop, which was held in 2006 to “...identify, review, and evaluate the existing practices, White Papers and articles, and clarify the FDA Guidance” [43]. Best practice documents for conducting bioanalytical method validation are in place or under consideration in several additional countries (e.g., Singapore and Brazil). The EMA issued a draft Guideline on Validation of Bioanalytical Methods in 2009 [55]. The end of the consultation period for public feedback was May 2010.

■ Future regulations

There is currently an effort underway to harmonize a global set of standards for BMV. Led by the Global Bioanalysis Consortium [54] the mission statement is “...to harmonize and merge existing or emerging bioanalytical guidance documents to create one, unified consensus document that can be presented to the regulatory bodies/health authorities in various countries”.

Structural features of human insulin & insulin analogs

Native human insulin is a 51-amino acid peptide (MW 5807.6 Da) that consists of an A-chain (21 amino acids) and B-chain (30 amino acids),

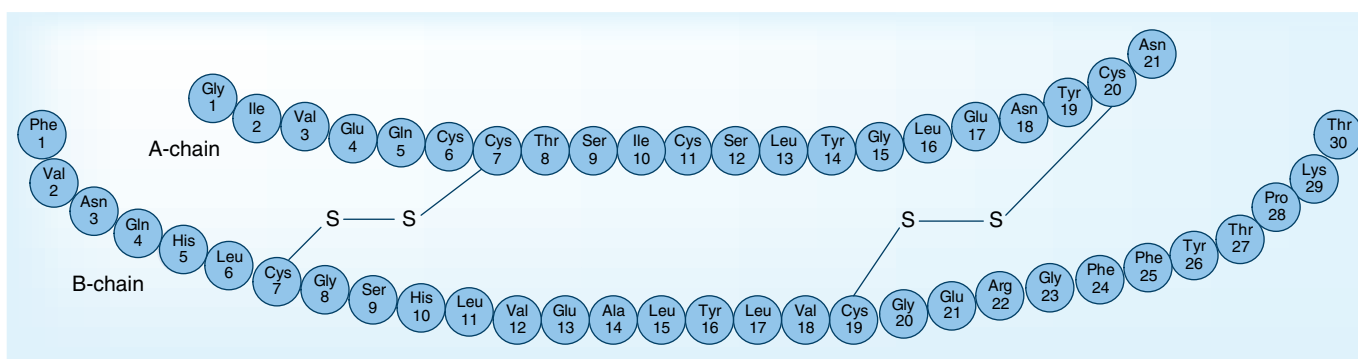


Figure 1. Primary structure of native human insulin.

Key Term

Anti-insulin antibody: Host antibody that is specific to epitopes on native insulin or insulin analogs.

which are connected by two sets of disulfide bonds between amino acids A₇–B₇ and A₂₀–B₁₉, respectively (FIGURE 1). While there is some debate [40,55,56], the commonly accepted assigned potency for native insulin based on highly purified standards is 1 IU = 6.00 nmol (0.0347 mg) or (1 μIU/ml = 6 pM) [57–59]. All marketed insulin analogs, except insulin detemir, have reported potencies similar to native insulin (TABLE 2).

Over the past one-to-two decades, numerous reviews have been published focused on the design of new insulin analogs that possess improved PK and pharmacodynamic (PD) properties for treating type 1 and 2 DM [59–70]. These novel insulin therapeutics are classified as either rapid-acting (i.e., used for meal time glycemic control) or long-acting (i.e., used for maintenance of basal metabolism) (TABLE 2). To date, four general strategies have been employed successfully to design customized insulin therapeutics. First, in the early years improvements in animal-sourced insulin peptide included crystallization, changes in formulation and improved purification [71,72]. Second and most recently, pulmonary delivery of insulin has been utilized as an alternative to subcutaneously administered peptides to provide a rapid onset and time action for use at meal times [73,74]. Third, a number of insulin products have been marketed in which their primary amino acid sequence was altered systematically to yield analogs with optimized PK/PD properties (TABLE 2) [59,75–84]. Finally, in one instance, insulin detemir, the insulin peptide was modified structurally by selectively appending a C₁₄ fatty acid moiety at B₂₉ to extend its time–action profile via binding to serum albumin [85–87].

Unlike a change in formulation or pulmonary delivery, alteration in the primary sequence of insulin or the addition of a moiety (i.e., fatty acid) to the peptide backbone would be expected to alter immunoreactivity *vis-à-vis* native insulin. This notion has been confirmed in numerous reports that describe decreased detection of insulin analogs in various insulin immunoassays used to support clinical laboratory testing [88–95]. The inconsistency in the detectability of insulin analogs is case-by-case across different commercial kits and a direct consequence of differences in the specificity of antibodies used in these commercial assays. In addition, despite the minor structural changes in some analogs, there are published reports of analog-specific immunoassays that lack crossreactivity with native insulin [96,97]. These publications further support the view that subtle changes in peptide sequence have the potential to

alter an analog's reactivity in conventional insulin immunoassays. Therefore, unless the circulating levels of native insulin are expected to be low (e.g., fasted subjects or patients with Type I DM), application of a commercial insulin kit for supporting PK of an insulin analog requires that the analog and native insulin display comparable crossreactivity [98,99]. Otherwise, the reported result can reflect an interpolated value consisting of multiple insulin peptides (i.e., therapeutic and native) that possess different immunoreactivity and lack parallelism. An alternate approach for supporting PK/TK of insulin analogs has been the establishment of analog-specific immunoassays (i.e., ones that lack crossreactivity with native human insulin and other insulin-related peptides, such as proinsulin and proinsulin cleaved intermediates) [96,97].

■ Antigenic characteristics

The general antigenic features of human insulin have been appreciated for 50 years or more, due to immunogenic differences seen in patients following administration of insulin products that were derived from different species, such as porcine and bovine [100]. Two discrete regions on insulin are recognized by T cells and comprise major binding epitopes [101,102]. These include the A-chain loop (A₈–A₁₀) and the C-terminus of the B-chain (B₂₇–B₃₀) [103–107]. The A-chain loop comprises one of the most evolutionarily diverse regions in insulin across different species [103] and is a well-known immune-dominant region in which substitution of a single amino acid was sufficient to elicit antibody formation in a transgenic mouse model [108]. Undoubtedly, this knowledge combined with the well-known greater incidence of **anti-insulin antibody** formation in patients on beef insulin therapy as compared with pork and human insulin, is one factor that has prompted innovator companies to minimize amino acid changes to this region of the A-chain.

The C-terminus of the B-chain is the second immune-dominant region in which monoclonal antibodies have been capable of detecting subtle changes in peptide structure, particularly involving the B₃₀ terminal amino acid [105,107,109]. Bowsher and co-workers produced a polyclonal antiserum in guinea pigs that was specific for the human insulin analog, insulin lispro, in which the amino acids at B₂₈ and B₂₉ are reversed [96]. In an analog specific RIA, native insulin demonstrated a crossreactivity of <0.05% *vis-à-vis* insulin lispro. Based on detailed crossreactivity

experiments involving structurally related peptides, the authors concluded the antibody binding epitope was comprised of the amino acid sequence -X_{aa}-Pro-Thr-COOH (where X_{aa} is a basic amino acid) at B₂₈-B₃₀. In addition, optimal crossreactivity was only achieved when the peptide chain terminated at B₃₀; this reinforced the importance of a free carboxyl-terminus for defining the antigenic reactivity of the C-terminus of the B-chain. Similarly, a specific monoclonal antibody, X14-6F34, was raised that recognizes the C-terminus of the B-chain of insulin aspart, an analog in which the proline at B28 is replaced by an Asp residue [104]. Thus, experiments involving epitope mapping of native insulin and the documented lack of human insulin crossreactivity in analog specific immunoassays clearly indicate that amino acids residues B₂₈-B₃₀ comprise an important binding epitope region in insulin.

The C-terminus of the B-chain warrants special attention as an antigenic epitope for the design of immunoassays to support regulated bioanalysis of insulin analogs. This peptide region represents the predominant area for structural modification(s) in all marketed insulin products (TABLE 2). For this reason, use of antisera/antibodies that recognize this peptide region should be done judiciously to ensure method validity (i.e., comparable potency and parallelism). As indicated previously, the bioanalytical goal for method development should be to either identify an antiserum/antibody that detects the analog and native insulin comparably or obtain one that recognizes the analog's B-chain C-terminus region specifically with minimal crossreactivity from native insulin.

In addition to binding epitopes formed by linear peptidyl sequences, there is good evidence that insulin also possesses one or more conformational epitopes that are formed by peptide folding [103,104]. Models indicate the B-chain C-terminal region lies on the surface of insulin in close proximity to the N-terminus of the A-chain [110,111]. Accordingly, several groups of investigators have reported that some insulin antibodies recognize this conformational epitope [103,104,111,112]. Consequently, peptide modifications, particularly at the C-terminus of the B-chain, that alter an analog's conformation relative to native insulin have the potential to affect an analog's antibody crossreactivity with antibodies produced against native insulin. Likewise conformational differences could provide the basis for generation of antibodies specific for insulin analogs.

Table 2. Properties of marketed insulin therapeutics^{††}.

Insulin active pharmaceutical ingredients	Human insulin	Insulin lispro	Insulin aspart	Insulin glulisine	Insulin glargine (HOE-901)	Insulin detemir
Name and manufacturer	Humulin® (Lilly), Novolin® (Novo Nordisk)	Humalog® (Lilly)	NovoLog® (Novo Nordisk)	Apidra® (sanofi-aventis)	Lantus® (sanofi-aventis)	Levemir® (Novo Nordisk)
Type of insulin	Native peptide	Fast-acting analog	Fast-acting analog	Fast-acting analog	Long-acting/basal analog	Long-acting/basal analog
Structural change(s) relative to native insulin	None	Ly ⁵ _{B28} , Pro ⁹²⁹	ASP ^{B28}	Ly ⁵ _{B37} , Glu ¹⁰²⁹	Gly ^{A21} , Arg ^{B31} , Arg ^{B32}	Des-Thr ^{B30} , C ₁₄ fatty acid at B ₂₉
PK attributes (T _{max} and elimination T _{1/2})	50–120 min 90 min (sc.)	30–90 min 30–90 min (sc.)	30–90 min 30–90 min (sc.)	30–90 min 30–90 min (sc.)	0–22.5 h N/A	6–8 h 5–7h (sc.)
PD attributes (onset, peak and action duration)	0.75 h ^s , 2–3 h, and ~4–6 h	0.25–0.5 h, 1–2 h, and ~3–4 h	0.25–0.5 h, 1–2 h, and ~3–4 h	0.25–0.5 h, 1–2 h, and ~3–4 h	1–2 h, no pronounced peak, up to 24 h	
Empirical formula	C ₂₅₇ H ₃₈₃ N ₆₅ O ₇₇ S ₆	C ₂₅₇ H ₃₈₃ N ₆₅ O ₇₇ S ₆	C ₂₅₆ H ₃₈₁ N ₆₅ O ₇₉ S ₆	C ₂₅₈ H ₃₈₄ N ₆₄ O ₇₈ S ₆	C ₂₆₇ H ₄₀₂ N ₇₂ O ₇₈ S ₆	C ₂₆₇ H ₄₀₂ N ₆₄ O ₇₆ S ₆
Molecular weight (Da)	5807.6	5807.6	5825.8	5823	6,063	5916.9
Potency (U/mg)	28.8 ^{††}	28.8 ^{††}	-	28.65 [#]	27.49 ^{††}	7.04 ^{††}

[†][209].
^{††}[210].
[§][83].
[#][211].
^{††}[139].
^{††}[132].
^{††}[140].

PD: Pharmacodynamic; PK: Pharmacokinetic; sc.: Subcutaneous.

■ Peptide heterogeneity across species

While the primary sequence homology of insulin is highly conserved across most mammalian species [29–31], differences in primary sequence have been known for at least five decades [32–34,113]. For many mammals, insulin only differs from the human peptide at a few residues with typical identity in the range of 92–98% [114]. **TABLE 3** summarizes the primary amino acid sequence homology across a number of species, including those that are used often to support PK/TK investigations of insulin and insulin analogs [116,117]. In contrast to other species, the insulin sequences in some rodent species, such as the guinea pig and mouse, are known to differ more and have lower identity relative to human insulin [32–34]. Because of the known primary sequence difference in rodent insulin, it should not be too surprising that repeated administration of human insulin and insulin analogs in rodents is capable of inducing generation of anti-insulin antibodies. Moreover, if anti-insulin antibodies reach a critical threshold level, they can cause assay interference [90,115]. For these reasons, we recommended that anti-insulin ADA be monitored in studies involving repeated administration of human insulin or insulin analogs in preclinical species, especially in rodent studies involving daily administration for one month or longer. If anti-insulin ADA are detected, strategies can be employed to minimize their analytical interference. The peptidyl regions of structural heterogeneity among different species include the aforementioned A-chain loop (A₈–A₁₀) and the C-terminus of the B-chain. As noted, both of these regions constitute important binding epitopes for insulin's antigenic recognition [103–107]. Thus, despite insulin's conserved nature, antisera are often capable of detecting the small differences in the peptide's primary sequence across different species. Consequently to ensure acceptable method performance for supporting regulated bioanalysis in nonclinical species, particularly rodents, careful consideration should be given to assay design and the relative extent of crossreactivity by the therapeutic insulin and the species native insulin.

Immunoanalytical considerations for insulin & insulin analogs

■ Immunoassay design

Strategy & format

Both competitive and noncompetitive immunoassay formats are suitable to support regulated bioanalysis of human insulin and insulin analogs. Design of an optimized immunoanalytical

strategy requires consideration of three categories of factors. One includes the peptide's structure, one concerns study-specific factors and the last one involves the species, subject or patient factors. In addition, the analytical strategy for an insulin analog often needs to evolve over time, as the various factors impacting assay performance (i.e., specificity) change when the peptide progresses from nonclinical PK/TK evaluation through clinical testing. Some important factors to consider when formulating an immunoanalytical strategy for regulated bioanalysis of insulin and insulin analogs are listed in **TABLE 4**.

Antibody specificity

Antibody specificity is the critical parameter that governs assay specificity and ultimately dictates the accuracy and precision of an insulin immunoassay. However, immunoassays of insulin and insulin analogs are complicated by the presence of endogenous insulin and the structural differences between the therapeutic insulin and the native form. Thus, selection of a reagent antibody or antibody pairs is more complicated than selection simply based on affinity (i.e., sensitivity). Because of the factors listed in **TABLE 4**, some insulin antibodies may be well suited for use as reagents in certain bioanalytical circumstances but not others.

As depicted in **FIGURE 2**, insulin analogs can be viewed at a high level as possessing two general types of binding epitopes, those that are unique to the analog (i.e., analog-specific) and others that are common to native human insulin. Furthermore, it is the relative specificity of the reagent antibodies for these distinct types of insulin antigenic epitopes that ultimately dictates the specificity and performance of an insulin analog immunoassay. While the use of commercial anti-insulin antibodies is often appealing for use in the development of an analog immunoassay, because of convenience and avoidance of the time required for antibody generation, analytical issues can arise due to the differences in peptide structure. Despite subtle structural differences, what is often found with conventional anti-insulin antiserum is that the analog displays decreased potency and a lack of parallelism *vis-à-vis* native human insulin. Because of the presence of endogenous insulin, this issue can limit the utility of commercially available insulin antibodies for establishment of an immunoassay to support regulated bioanalysis of an analog. The same assay specificity issue also occurs when native insulin is administered to some preclinical species, such as rodents. A difference in

Table 3. Insulin primary sequence homology across different species*.

Species	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21										
Human/ monkey	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	Asn	Tyr	Cys	Asn										
Pig/dog	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	Asn	Tyr	Cys	Asn										
Cow	Gly	Ile	Val	Glu	Gln	Cys	Cys	Ala	Ser	Val	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	Asn	Tyr	Cys	Asn										
*Rat/ mouse (I & II)	Gly	Ile	Val	Asp	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	Asn	Tyr	Cys	Asn										
Rabbit	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	Asn	Tyr	Cys	Asn										
Guinea pig	Gly	Ile	Val	Asp	Gln	Cys	Cys	Thr	Gly	Thr	Cys	Arg	His	Gly	Leu	Gln	Ser	Tyr	Cys	Asn											
Horse	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Gly	Ile	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	Asn	Tyr	Cys	Asn										
Cat	Gly	Ile	Val	Glu	Gln	Cys	Cys	Ala	Ser	Val	Cys	Ser	Leu	Tyr	Gly	Leu	Glu	His	Tyr	Cys	Asn										
Species	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25	B26	B27	B28	B29	B30	B30
Human/ monkey	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr	
Pig/dog	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala	
Cow	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala	
*Rat I/ mouse I	Phe	Val	Lys	Gln	His	Leu	Cys	Gly	Pro	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ser	
*Rat II/ mouse II	Phe	Val	Lys	Gln	His	Leu	Cys	Gly	Pro	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Met	Ser	
Rabbit	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ser	
Guinea pig	Phe	Val	Ser	Arg	His	Leu	Cys	Gly	Ser	Asn	Leu	Val	Glu	Thr	Leu	Tyr	Ser	Val	Cys	Gln	Asp	Asp	Gly	Phe	Phe	Tyr	Ile	Pro	Lys	Asp	
Horse	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala	
Cat	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala	

*Amino acids that differ from native human insulin are shown in bold.

[†]In rats and mice two distinct circulating forms of insulin (I and II) are expressed as products of different nonallelic preproinsulin genes [33–35].

Rat and mouse insulins I and II are identical and differ from human insulin at the positions A4, B3, B9 (I only), B29 (II only) and B30 [35].

Data taken from [31–35].

Table 4. Three categories of factors to consider in the design of an immunoanalytical strategy to support regulated bioanalysis of human insulin and insulin analogs.

Peptide-related factors	Study-related factors	Subject/patient-related factors
Any unique structural features or ADME attributes that provide the basis for a peptide's novel PK/PD properties	Species used/structural homology between endogenous insulin and therapeutic insulin	Normal healthy subjects Patients with Type 1 DM Patients with Type 2 DM
Type of insulin (i.e., native human, fast acting analog or long acting basal)	Limitations in the volume of serum/plasma for study test samples	Glycemic state (fed or fasted)
Structural homology between therapeutic insulin and the endogenous peptide	Route(s) of administration (i.e., sc., iv or pulmonary)	Previous exposure to insulin therapeutics
Type and location of the therapeutic peptide's structural modification(s)	Frequency, duration of dosing and time period over which samples are collected	Prevalence of pre-existing insulin ADA
Specificity of reagent antiserum or antibodies (degree of crossreactivity for native human insulin and analog)	Potential administration of multiple types of insulins (e.g., meal time and basal) with a study	Potential for analytical interference from co-administered medications

ADA: Antidrug antibody; DM: Diabetes mellitus; iv: Intravenous; PD: Pharmacodynamic; PK: Pharmacokinetic; sc.: Subcutaneous.

immunoreactivity between the human insulin drug and endogenous animal insulin results in undesired recovery (%RE) in experiments to assess method accuracy.

To overcome the crossreactivity issue, a reagent antibody strategy should be conceptualized at project inception to deal with differences in immunoreactivity between the analog and endogenous insulin. As shown in **FIGURE 3**, three developmental scenarios are possible for establishing an insulin analog immunoassay. Of these options, two represent acceptable scenarios. These include where the antibody is specific for the insulin analog, and where the analog and native insulin crossreact and are equipotent. The one scenario that is not desirable is where the analog and native insulin crossreact, but differ in potency and parallelism. Establishment of an analog-specific immunoassay that incorporates

use of analog-specific antiserum/antibody yields an assay in which the analog is essentially treated as a **xenobiotic** drug. In this case subcutaneous single-dose administration of the analog results in a PK profile where the terminal elimination phase approaches zero (i.e., below the quantitation limit, no baseline endogenous insulin). The alternate acceptable bioanalytical strategy is one in which the analog and endogenous insulin display full crossreactivity (i.e., equipotent and parallel) with the reagent antibodies. In this case, subcutaneous single-dose administration of the analog results in a PK profile that possesses a terminal elimination phase, which approaches the circulating concentration of the endogenous insulin (e.g., often below the quantitation limit). Since circulating insulin levels are transient and change in response to fasting and food intake, this approach typically leads to greater inter-subject variability in the PK terminal elimination phase. The undesired scenario is that the therapeutic and endogenous insulin differ in their immunoreactivity. Unless the endogenous insulin concentration is kept low (e.g., fasted animals or patients with Type 1 DM), it can be challenging to demonstrate acceptable assay performance that meets current BMV expectations for LBAs. For example, selectivity assessments near the LLOQ can be particularly challenging due to the differences in crossreactivity.

Key Term

Xenobiotic: A foreign substance that is not normally present in an organism.

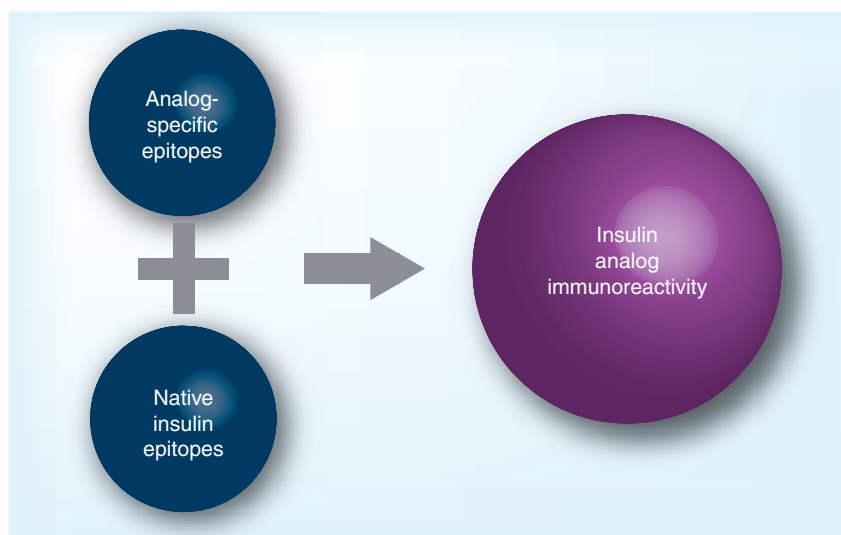


Figure 2. Different binding epitopes present in an insulin analog.

Matrix calibration

One feature of regulated bioanalysis that makes insulin immunoassays particularly challenging is the requirement for 'matrix-based' calibration. In practice, because insulin is an endogenous peptide, it is difficult to obtain insulin-free matrix (i.e., serum or plasma that is devoid of endogenous

insulin) for preparing standard curves and validation samples. This is made more challenging by the fact that pharmacologic levels of insulin are not too dissimilar from its endogenous level. While the requirement for matrix-based calibration does not present an issue for analog specific immunoassays (i.e., no crossreactivity from endogenous insulin), it is often problematic for insulin/analog immunoassays that employ conventional anti-insulin antibodies.

A number of strategic approaches can be potentially considered to address the issue of dealing with an endogenous component [35]. First, simple introduction of a minimal required dilution is often used for some biotherapeutics to overcome interference from endogenous component(s). Dilution of test samples will lessen interference from endogenous insulin, but at the same time it can compromise assay sensitivity. This strategy, therefore, is not an option as a highly sensitive assay is required to measure therapeutic concentrations of insulin that are typically in the picomolar to nanomolar range. A second strategy might be to employ buffer-based solution matrix, instead of using a biological matrix, for preparing standard calibrators. This approach, while used widely in research and clinical diagnostic kits for measurement of insulin as a biomarker, is discouraged for regulated bioanalysis, because it can lead to assay bias and is not consistent with published GLP bioanalytical recommendations [41,202]. A third strategy is to deplete the matrix of insulin to obtain 'insulin-free' serum or plasma. The obvious goal for depletion is to treat the matrix to efficiently remove the endogenous insulin without appreciably altering the matrix so that it remains representative of plasma/serum. Another possible strategy for obtaining a lot of matrix that has a low endogenous insulin concentration (e.g., ~5 µl/ml or 30 pM) is to collect the matrix from individuals or animals after an overnight fast. While this strategy does not completely deplete insulin, it is a reliable means for consistently obtaining a pool of matrix with a low insulin concentration. Even though this strategy can be of value for preparing quality control (QC) samples, it is not suitable for preparing matrix calibrators as it does not completely eliminate endogenous insulin. While it is not practical, pharmacological suppression of circulating insulin is yet another strategy for obtaining animal plasma/serum that is low in endogenous insulin. As stated in the 2001 FDA BMV guidance [202] and some publications

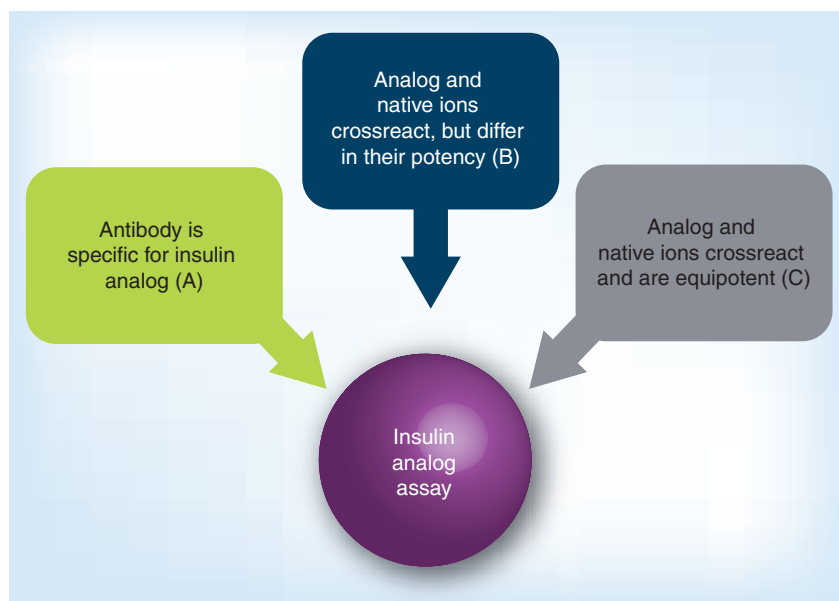


Figure 3. Immunoanalytical strategic options for design of an insulin analog assay.

for LBAs [35], it is permissible to use “alternate matrices of equivalent performance”. For this reason, we recommend the use of immunodepletion or charcoal treatment (with or without heat treatment at 56°C for ~1 h) as an effective means to deplete matrices of insulin and other endogenous peptides for preparing insulin calibrators [35,117]. In our experience, this depleted matrix is a suitable surrogate for both serum and EDTA plasma and provides an acceptable approximation of authentic ‘unaltered’ matrix (i.e., acceptable spike recovery and parallelism). In addition the use of insulin-depleted matrix facilitates determination of basal insulin concentrations, a key objective for assays employing conventional anti-insulin antisera, and to define the immunoassay’s LLOQ.

Preparation of validation & QC samples

To demonstrate acceptable immunoassay performance of a surrogate matrix or alternate calibration matrix strategy, it is important to prepare the validation samples in both the depleted matrix (i.e., altered) and authentic matrix (i.e., unaltered). Because the endogenous insulin concentration is often greater than an immunoassay’s sensitivity limit, validation samples prepared in the depleted matrix are useful for documenting assay performance (i.e., accuracy and precision following spike recovery) at the expected LLOQ (i.e., sensitivity limit). In contrast, validation samples in authentic matrix are needed during prestudy validation

to demonstrate suitable accuracy (mean bias, %RE) and method precision (intra- and inter-assay, %CV) within the immunoassay's anticipated validated range. Furthermore due to the presence of endogenous peptide, we recommend that the set of validation samples (LOQ, MOQ, HOQ and ULOQ levels) also include one that is 'unspiked' to reflect the endogenous insulin level. In this way suitable method performance (i.e., accuracy and precision) can be demonstrated by computing the % recovery after subtracting the estimated endogenous concentration from the measured total concentrations in the validation sample (i.e., endogenous + spike). Upon completion of prestudy validation, the low, mid and high validation samples can be assigned a total insulin concentration (i.e., endogenous + spike) and then be used during in-study to judge the acceptability of assay runs during analyses of test samples.

PK/TK study-related factors

An important design consideration for insulin immunoassays intended to support regulated bioanalysis concerns the planned PK/TK studies. For convenience, it is helpful to discuss these study related factors separately based on whether they occur in a preclinical and clinical setting.

As already noted for preclinical studies, insulin's primary amino acid sequence is heterogeneous across species and reagent antibodies are known to detect these subtle differences [110–114]. This issue can create a problem for assessments of selectivity/recovery (accuracy) and parallelism, particularly at low concentrations near the LLOQ, during prestudy BMV of an insulin therapeutic. Thus for application in a preclinical setting, assay design should take into account any differences between the species insulin and the human therapeutic peptide. This is especially important for studies involving rodents, but less so for canine and porcine due the similar structure of their native insulin (TABLE 3). A related issue in the preclinical setting concerns the duration of exposure for a human insulin or insulin analog. Because these peptides are dosed at higher levels in toxicology studies and are foreign, they are often immunogenic (i.e., capable of eliciting antibody formation) upon repeated administration. Since insulin ADAs are known to interfere in both competitive and noncompetitive immunoassay formats, it is advisable to monitor for the presence of insulin ADA to minimize risk of erroneous PK/TK data due to assay interference.

Regulated bioanalysis of insulin or insulin analogs in a clinical setting also requires that certain factors be given careful consideration for optimal assay implementation. As already mentioned and depicted in FIGURE 3, an analytical strategy for clinical evaluation of an insulin analog must consider the extent of crossreactivity for both the analog and native insulin. The study patient population can further complicate this issue, as patients with Type 2 DM are characterized by insulin resistance and have elevated levels of circulating insulin, proinsulin and cleaved intermediates [118,119]. This means the baseline of insulin-like peptides is increased *vis-à-vis* fasted normal healthy adults and creates a greater challenge for bioanalysis of an analog, unless of course, the reagent antibodies are analog-specific. In contrast, sera from patients with Type 1 DM are theoretically devoid of insulin. However, these patients are often positive for the presence of insulin ADA due to prior exposure to therapeutic administration of insulin and insulin analogs. If sera are insulin ADA positive, analytical strategies, such as pretreatment by polyethylene glycol, must be implemented to minimize their interference [115,116].

Disposition & metabolism

Characterization of *in vitro* catabolism of native human insulin has been described in numerous publications [120–121]. While the *in vivo* metabolism of insulin is understood less well, the major mechanism for insulin clearance from the circulation is thought to involve receptor mediated endocytosis followed by intracellular degradation. From a bioanalytical perspective, insulin is generally regarded as being stable in plasma and serum when appropriately stored frozen at -20°C to -70°C [122,123].

Whereas the *in vivo* catabolic pathways for most insulin analogs is thought to be similar to native insulin [124], one analog is unique in that it is known to undergo extensive metabolism following subcutaneous injection. Insulin glargine (Lantus[®]) (TABLE 2) [125] is used widely as a long-acting basal insulin [125–126,208]. In addition, due to its structural differences, this therapeutic has been reported to display much reduced immunoreactivity in some clinical insulin assays [90–92,95,127,128]. Furthermore, glargine is known to undergo catabolism in both the subcutaneous space and circulation with sequential cleavage of the Arg residues at the carboxy-terminus of the B-chain to yield two predominant active metabolites, M1 (A_{21} -Gly-insulin) and

M2 (A₂₁-Gly-des-B₃₀-Thr-insulin) [129]. The metabolites M1 and M2 have been reported to possess equivalent glucose-lowering potency as the parent peptide *in vitro* and *in vivo* [130]. The proteolytic degradation of glargine is initiated at the injection site and continues in the systemic circulation and is catalyzed at least in part by plasma/serum carboxypeptidase(s) [127,130]. Thus, insulin glargine is one analog that warrants additional discussion concerning the potential impact of metabolism on its quantitative determination by immunoassay. Structurally the metabolites M1 and M2 are more ‘insulin-like’ than parent glargine and reported to be pharmacologically active *in vivo*. Because the C-terminal region of the B-chain comprises an important insulin antigenic epitope, it is likely that these metabolites will differ in their immunoassay potency relative to the parent peptide. Moreover, as suggested in recent publications [127,129], it is interesting to speculate that these metabolites may show greater potency in some assays than parent glargine. Thus, these data raise provocative questions about appropriate design and calibration of immunoassays to quantify the different ‘pharmacologically active’

forms (i.e., parent peptide and active metabolites) of insulin glargine in the circulation following subcutaneous injection. For these reasons, one should consider strategically whether the immunoassay needs to detect parent peptide specifically (i.e., no crossreactivity from metabolites) or also quantify the various known active metabolites of glargine. Either immunoanalytical approach will dictate defined bioanalytical requirements and impact assay design and the selection of reagent antibodies.

Recommendations for analytical validation of immunoanalytical methods for insulin bioanalysis

The purpose of BMV is to demonstrate that the bioanalytical method consistently generates reliable data for the intended purpose of the study [41,43]. There are three sequential steps towards the establishment of a validated method. method development, prestudy validation and in-study validation [42]. The evaluation of specific method characteristics for each phase of validation are summarized in TABLE 5. A detailed description of these processes has been published previously [42,205,131]. In addition,

Table 5. Summary of prestudy validation requirements for GLP bioanalysis of protein.

Performance characteristic	Methods development	Prestudy validation	In-study validation
Assay reagent selection/stability/assay format/batch size	Identify; establish	Confirm	Monitor; lot changes require confirmation of performance
Specificity and selectivity	Establish	Confirm	Confirm in diseased states as available
Matrix selection/ sample preparation/minimum required dilution	Establish	Confirm; for modified matrices, quality controls must be prepared in relevant neat matrix	Monitor; with changes to lot of matrix, comparability must be demonstrated
Standard calibrators and standard curves	Select model	Confirm	Monitor
Precision and accuracy	Evaluate imprecision and mean bias	Establish imprecision (%CV) and bias (%RE)	Total error (4–6–30)
Range of quantification	Evaluate	Establish	Apply
Sample stability	Initiate	Establish	Ongoing assessment and extension
Dilutional linearity	Evaluate	Establish	Monitor and establish dilutions not covered in prestudy validation
Parallelism	N/A	Investigate where possible	Establish with incurred sample
Incurred sample reproducibility	N/A	Investigate where possible	Confirm
Robustness/ruggedness	Evaluate	Establish	Monitor
Partial validation/method transfer	N/A	N/A	Apply as appropriate
Run acceptance criteria	N/A	Runs accepted based on standard curve acceptance criteria	Standard curve and QC acceptance criteria Apply 4–6–XX rule or other appropriate statistical criteria that aligns with the prestudy acceptance

CV: Coefficient of variation; N/A: Not applicable; RE: Relative error.

interassay precision is evaluated during in-study validation using incurred study samples. In the USA, recommendations on incurred sample reproducibility have been recently published [43,132,133].

Conclusion

The primary purpose of this perspective was to offer insights into some of the common technical challenges and hurdles in the establishment of immunoassays to support regulated bioanalysis for assessing the PK/TK of new novel insulin therapeutics. Despite insulin's long and illustrious history as a peptide therapeutic and wide availability of reagent antibodies and commercial diagnostic kits for human insulin, routine establishment of regulatory-compliant immunoassays for new analogs remains a challenging endeavor. Furthermore the challenges for bioanalysis of insulin and insulin analogs will persist, as new and novel analogs continue enter drug development in the search for improved insulin therapeutics, and the BMV requirements for LBAs to support regulated bioanalysis continue to evolve over time.

Future perspective

While this perspective provides some useful insights into the technical considerations for insulin and insulin analogs, it likely describes in a broader sense some of the general bioanalytical issues that are common to peptide therapeutics. In this regard, it underscores the growing heterogeneity and complexity in biotherapeutics and reinforces some of the key differences in regulated bioanalysis between monoclonal antibodies and peptide therapeutics. Accordingly, the utility of the information contained in this perspective may increase over time, as clinical utility of this class of biotherapeutic continues to grow.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Insulin was the first recombinant DNA-derived biologic therapeutic manufactured. The success of recombinant insulin commercialization lead the way for the successful development of subsequent novel therapeutics produced by microorganisms.
- The regulated bioanalysis of insulin and insulin analogs is confounded by a number of technical challenges, including biochemistry, endogenous peptide, structural differences between an analog and native insulin, differences in potency for analogs, interference from antidrug antibodies and the pathology of diabetes mellitus that often results in insulin resistance an elevated levels of circulating.
- Insulin and insulin analogs contain epitopes that can be utilized to develop specific immunoassays.
- The presence of endogenous insulin represents a substantial challenge for conducting regulated bioanalysis, and is a common issue when supporting therapeutic peptides that are present endogenously.
- Insulin analogs present unique bioanalytical challenges when employing immunoassay methods and can behave differently in the immunoassay than with the native protein or peptide; particularly when bioanalytical antibodies were generated to the native molecule.
- As new insulin analogs are developed and technologies advance (e.g., routes of administration, formulation, etc) the bioanalytical methods will undoubtedly need to evolve to meet these new challenges.
- The innovation in the design and development of recombinant insulin and insulin analogs will be exploited and utilized in the future design of new and clinically improved biopharmaceuticals.

Bibliography

Papers of special note have been highlighted as:

- of interest

- 1 Bliss M. *The Discovery of Insulin*. McClelland & Stewart Limited, Toronto, Canada (1982).
- 2 Rosenfeld L. Insulin. Discovery and controversy. *Clin. Chem.* 48, 2270–2288 (2002).
- 3 Banting FG, Best CH, Collip JB, Campbell WR, Fletcher A. Pancreatic extracts in the treatment of diabetes mellitus. A preliminary report. *Can. Med. Assoc. J.* 12(3), 141–146 (1922). Also see Encore publication in *Can. Med. Assoc. J.* 145(10), 1281–1286.
- 4 Berson SA, Yalow RS, Bauman A, Rothchild MS, Newerly K. Insulin-I131 metabolism in human subjects. demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J. Clin. Invest.* 35, 170–190 (1956).
- 5 Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.* 39, 1157–1175 (1960).
- 6 Kahn CR, Roth J, Berson, Yalow and the JCI. the agony and the ecstacy. *J. Clin. Invest.* 114(8), 1051–1054 (2004).
- 7 Yalow RS. Radioimmunoassay. A probe for the fine structure of biologic systems. *Science* 200(4347), 1236–1245 (1978).

- 8 Berson SA, Yalow RS. General principles of radioimmunoassay. 1968. *Clin. Chim. Acta* 369(2), 125–143 (2006).
- 9 Blumenthal S. The insulin immunoassay after 50 years. A reassessment. *Perspect. Biol. Med.* 52(3), 343–354 (2009).
- 10 Johnson IS. Human insulin from recombinant DNA technology. *Science* 219(4585), 632–637 (1983).
- **Production of recombinant human insulin from prokaryotic host cells.**
- 11 Chance RE, Frank BH. Research, development, production and safety of biosynthetic human insulin. *Diabetes Care* 16(Suppl. 3), 133–142 (1993).
- 12 Ladisch MR, Kohlmann KL. Recombinant human insulin. *Biotechnol. Prog.* 8(6), 469–478 (1992).
- 13 Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256(5517), 495–497 (1975).
- **The generation of monoclonal antibodies.**
- 14 Martinis J, David GS, Bartholomew RM, Wang R. Novel applications of monoclonal antibodies. *Basic Life Sci.* 25, 129–154 (1983).
- 15 Payne WJ, Marshall DL, Shockley RK, Martin WJ. Clinical laboratory applications of monoclonal antibodies. *Clin. Microbiol. Rev.* 1(3), 313–329 (1988).
- 16 Borrebaeck CA. Antibodies in diagnostics – from immunoassays to protein chips. *Immunol. Today* 21(8), 379–382 (2000).
- 17 Tolonen E, Hemmila I, Marniemi J, Jorgensen PN, Zeuthen J, Lovgren T. Two-site time-resolved immunofluorometric assay of human insulin. *Clin. Chem.* 32, 637–640 (1986).
- 18 Comitti R, Racchetti G, Guocchi P, Marandi E, Galeuti YM. A monoclonal based two-site enzyme immunoassay for human insulin. *J. Immunol. Methods* 99, 25–37 (1987).
- 19 Burgi W, Briner M, Franken N, Kessler AC. One-step sandwich enzyme immunoassay for insulin using monoclonal antibodies. *Clin. Biochem.* 21(5), 311–314 (1988).
- 20 Sobey WJ, Beer SF, Carrington CA *et al.* Sensitive and specific two-site immunoradiometric assays for human insulin, proinsulin 65–66 split and 32–33 split proinsulins. *Biochem. J.* 260, 535–541 (1989).
- 21 Alpha B, Cox L, Crowther N, Clark PMS, Hales CN. Sensitive amplified immunoenzymometric assays (IEMA) for human insulin and intact proinsulin. *Eur. J. Clin. Chem. Clin. Biochem.* 30, 27–32 (1992).
- 22 Andersen L, Dinesen B, Jorgensen PN, Poulsen F, Roder ME. Enzyme-immunoassay for intact human insulin in serum or plasma. *Clin. Chem.* 39, 578–582 (1993).
- 23 Clark PMS, Hales CN. How to measure plasma insulin. *Diabetes Metab. Rev.* 10(2), 79–90 (1994).
- 24 Deberg M, Houssa P, Frank BH, Sodoyez-Goffaux F, Sodoyez JC. Highly specific radiimmunoassay for human insulin based on immune exclusion of all insulin precursors. *Clin. Chem.* 44, 1504–1513 (1998).
- 25 Robbins DC, Andersen L, Bowsher R *et al.* Task Force Report. Report of the American Diabetes Association's task force on standardization of the insulin assay. *Diabetes* 45, 242–256 (1996).
- 26 Marcovina S, Bowsher RR, Miller WG *et al.* Standardization of insulin immunoassays. Report of the American Diabetes Association Workgroup. *Clin. Chem.* 53, 711–716 (2007).
- 27 Miller WG, Thienpont LM, Van Uytanghe K *et al.* *Clin Chem* 55(5), 1011–1018 (2009).
- 28 Chevenne D, Trivin F, Porquet D. Insulin assay and reference values. *Diabetes & Metabolism (Paris)* 25, 459–476 (1999).
- 29 Young FG. Experimental research on diabetes mellitus. *Br. Med. J.* 2(5265), 1449–1454 (1961).
- 30 Peterson JD, Steiner DF. The amino acid sequence of the insulin from a primitive vertebrate, the Atlantic hagfish (*Myxine glutinosa*). *J. Biol. Chem.* 250(13), 5183–5191 (1975).
- 31 Shuldiner AR, Bennet C, Robinson EA, Roth J. Isolation and characterization of two different insulins from an amphibian, *Xenopus laevis*. *Endocrinol.* 125, 469–477 (1989).
- 32 Clark JL, Steiner DF. Insulin biosynthesis in the rat. Demonstration of two proinsulins. *Proc. Natl Acad. Sci. USA* 62(1), 278–285 (1969).
- 33 Zimmerman AE, Yip CC. Guinea pig insulin I, purification and physical properties. *J. Biol. Chem.* 249(13), 4021–4025 (1974).
- 34 Wentworth BM, Schaefer IM, Villa-Komaroff L, Chirgwin JM. Characterization of the two nonallelic genes encoding mouse proinsulin. *J. Mol. Evol.* 23, 305–312 (1986).
- 35 Findlay JWA, Smith WC, Lee JW *et al.* Validation of immunoassays for bioanalysis. A pharmaceutical industry perspective. *J. Pharm. Biomed. Anal.* 21, 1249–1273 (2000).
- 36 Tate J, Ward G. Interferences in immunoassay. *Clin. Biochem. Rev.* 25, 105–120 (2004).
- 37 Jones AM, Honour JW. Unusual results from immunoassays and the role of the clinical endocrinologist. *Clin. Endocrinol.* 64, 234–244 (2006).
- 38 Clark PM. Assays for insulin, proinsulin(s) and C-peptide. *Ann. Clin. Biochem.* 36, 541–564 (1999).
- 39 Sapin R. Insulin assays. previously known and new analytical features. *Clin. Lab.* 49(3–4), 113–121 (2003).
- 40 Sapin R. Insulin immunoassays. Fast approaching 50 years of existence and still calling for standardization. *Clin. Chem.* 53(5), 810–812 (2007).
- 41 Shah VP, Midha KK, Dighe S *et al.* Analytical methods validation. Bioavailability, bioequivalence and pharmacokinetic studies. Conference report. *Pharm. Res.* 9(4), 588–592 (1992).
- 42 DeSilva B, Smith W, Weiner R *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* 20(11), 1885–1900 (2003).
- **Detailed White Paper which complements the 2001 US FDA Guidance.**
- 43 Viswanathan CT, Bansal S, Booth B *et al.* Quantitative bioanalytical methods validation and implementation. Best practices for chromatographic and ligand binding assays. *Pharm. Res.* 24(10), 1962–1973 (2007).
- **Supplements the FDA 2001 Guidance on bioanalytical method validation.**
- 44 Hannon WH, Atkinson MA, Ball DJ *et al.* Assessing the quality of immunoassay systems. Radioimmunoassay and enzyme, fluorescence, and luminescence immunoassays; approved guideline. *CLSI I/ LA23-A* 24(16), 1–36 (2004).
- 45 Lee JW, Devanarayan V, Barrett Y-C *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm. Res.* 23(2), 312–328 (2006).
- 46 Kricka LJ, Master SR. Validation and quality control of protein microarray-based analytical methods. *Mol. Biotechnol.* 38, 29–31 (2008).

- 47 Warnick GR, Kimberly MM, Waymack PP, Leary ET, Myers GL. Standardization of measurements for cholesterol, triglycerides, and major lipoproteins. *Labmedicine* 39(8), 481–490 (2008).
- 48 Jennings L, VanDeerlin VM, Gulley ML. Recommended principles and practices for validating clinical molecular pathology tests. *Arch. Pathol. Lab. Med.* 133, 743–755 (2009).
- 49 Organization for Economic Co-operation development. OECD series on principles of Good Laboratory Practice and compliance monitoring number 4 (revised). GLP consensus document. Quality Assurance and GLP environment directorate. Organization for economic co-operation development, Paris (1999).
- 50 Miller KJ, Bowsher RR, Celniker A *et al.* Workshop on bioanalytical methods validation for macromolecules. Summary report. *Pharm. Res.* 18(9), 1373–1383 (2001).
- 51 Mire-Sluis AR, Barrett YC, Devanarayan V *et al.* Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J. Immunol. Meth.* 289(1–2), 1–16, (2004).
- One of the inaugural papers to discuss investigations into antidrug antibodies.
- 52 Gupta S, Indelicato S, Jethwa V *et al.* Recommendation for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutic. *J. Immunol. Meth.* 321, 1–18, (2007).
- 53 Shankar G, Devanarayan V, Amaravadi L *et al.* Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J. Pharm. Biomed. Anal.* 48(5), 1267–1281 (2008).
- 54 Abbott R. The broadening scope of validation. Towards best practices in the world of bioanalysis. *Bioanalysis* 2(4), 703–708 (2010).
- 55 Heinemann L. Insulin assay standardization. leading to measure of insulin sensitivity and secretion for practical clinical care. *Diabetes Care* 33(6), e83 (2010).
- 56 Staten MA, Miller WG, Bowsher RR, Steffes MW. Insulin assay standardization. leading to measure of insulin sensitivity and secretion for practical clinical care. *Diabetes Care* 33(6), E84 (2010).
- 57 Volund A, Brange J, Drejer K *et al.* *In vitro* and *in vivo* potency of insulin analogs designed for clinical use. *Diabetic Med.* 8, 839–847 (1991).
- 58 Volund A. Conversion of insulin units to SI units. *Am. J. Clin. Nutr.* 58(5), 714–715 (1993).
- 59 DiMarchi RD, Chance RE, Long HB, Shields JE, Sliker LJ. Preparation of an insulin with improved pharmacokinetics relative to human insulin through consideration of structural homology with insulin-like growth factor I. *Horm. Res.* 4(Suppl. 2), 93–96 (1994).
- 60 Brange J. The new era of biotech insulin analogs. *Diabetologia* 40, S48–S53 (1997).
- 61 Lee WL, Zinman B. From insulin to insulin analogs. progress in the treatment of type 1 diabetes. *Diabetes Rev.* 6(2), 73–88 (1998).
- 62 Bolli GB, DiMarchi RD, Park GD, Pramming S, Koivisto VA. Insulin analogs and their potential in the management of diabetes mellitus. *Diabetologia* 42, 1151–1167 (1999).
- 63 Brange J, Volund A. Insulin analogs with improved pharmacokinetic profiles. *Adv. Drug Del. Rev.* 35, 307–335 (1999).
- 64 Roskamp RH, Park G. Long-acting insulin analogs. *Diabetes Care* 22(Suppl. 2), B109–B113 (1999).
- 65 Vajo Z, Duckworth WC. Genetically engineered insulin analogs. diabetes in the new millennium. *Pharmacol. Rev.* 52(1), 1–9 (2000).
- 66 Vajo Z, Fawcett J, Duckworth WC. Recombinant DNA technology in the treatment of diabetes. insulin analogs. *Endocr. Rev.* 22, 706–717 (2001).
- 67 Simpson SH, Toth EL. Expanding the options in insulin therapy. a review of insulin analogs. *Can. J. Diabetes* 26(3), 195–205 (2002).
- 68 Zinman B. Effective use of insulin therapy in Type 2 diabetes. *Adv. Stud. Med.* 2(26), 954–960 (2002).
- 69 Owens DR, Bolli GB. Beyond the era of NPH insulin – long-acting insulin analogs. chemistry, comparative pharmacology, and clinical application. *Diabetes Technol. Therap.* 10(8), 333–349 (2008).
- 70 Roach P. New insulin analogs and routes of delivery. Pharmacodynamic and clinical considerations. *Clin. Pharmacokinet.* 47(9), 595–610 (2008).
- 71 Galloway JA. Chemistry and clinical use of insulin, Chapter 7. In. *Diabetes Mellitus, 9th Edition.* Galloway JA, Potvin JH, Shuman CR (Eds), Lilly Research Lab., IN, USA 106–133 (1988).
- 72 Heller S, Kozlovski P, Kurtzhals P. Insulin's 85th anniversary – an enduring medical miracle. *Diabet. Res. Clin. Prac.* 78, 149–158 (2007).
- 73 Laube BL. Treating diabetes with aerosolized insulin. *Chest* 120(3 Suppl.), 99S–106S (2001).
- 74 Owens DR, Zinman B, Bolli G. Alternative routes of insulin delivery. *Diabet. Med.* 20(11), 886–898 (2003).
- 75 Howey DC, Bowsher RR, Brunelle RL, Woodworth JR. [Lys(B28),Pro(B29)]-human insulin. A rapidly absorbed analog of human insulin. *Diabetes* 43, 396–402 (1994).
- 76 Holleman F, Hoekstra JB. Insulin lispro. *NEJM* 337(3), 176–183 (1997).
- 77 Chance RE, Frank BH, Radziuk JM, DiMarchi RD. Discovery and development of insulin lispro. *Drugs Today* 34(Suppl. C), 1–9 (1998).
- 78 Heinemann L, Heise T, Jorgensen LN, Starke AA. Action profile of the rapid acting insulin analog. Human insulin B28Asp. *Diabet. Med.* 10(6), 535–539 (1993).
- 79 Simpson KL, Spencer CM. Insulin Aspart. *Drugs* 57(5), 759–765 (1999).
- 80 Garg SK, Ellis SL, Ulrich H. Insulin glulisine. A new rapid-acting insulin analog for the treatment of diabetes. *Expert Opin. Pharmacother.* 6(4), 643–651 (2005).
- 81 Garnock-Jones KP, Plosker GL. Insulin glulisine. A review of its use in the management of diabetes mellitus. *Drugs* 69(8), 1035–1057 (2009).
- 82 Wang F, Carabino JM, Vergara CM. Insulin glargine. A systematic review of a long-acting insulin analog. *Clin. Ther.* 25(6), 1541–1577 (2003).
- 83 Gillies PS, Figgitt DP, Lamb HM. Insulin glargine. *Drugs* 59(2), 253–260 (2000).
- 84 Bolli GB, Owens DR. Insulin glargine. *Lancet* 356(9228), 443–445 (2000).
- 85 Heinemann L, Sinha K, Weyer C *et al.* Time-action profile of the soluble, fatty acid acylated, long-acting insulin analog NN304. *Diabet. Med.* 16(4), 332–338 (1999).
- 86 Dea MK, Hamilton-Wessler M, Ader M *et al.* Albumin binding of acylated insulin (NN304) does not deter action to stimulate glucose uptake. *Diabetes* 51, 762–769 (2002).
- 87 Home P, Kurtzhals P. Insulin detemir. From concept to clinical experience. *Expert Opin. Pharmacother.* 7(3), 325–343 (2006).
- 88 Sirkka-Liisa K, Melamies L, Kostamo E, Poyry K. Problems in the determination of short-acting insulin analog by commercial insulin kits. *Diabetes* 44(Suppl. 1), 229A (1995).
- 89 Sapin R, LeGaludec V, Gasser F, Pinget M, Grucker D. Elecsys insulin assay. Free insulin determination and the absence of cross-reactivity with insulin lispro. *Clin. Chem.* 47(3), 620–605 (2001).

- 90 Sapin R. Insulin assays. Previously known and new analytical features. *Clin. Lab.* 49(3–4), 113–121 (2003).
- 91 Owen WE, Roberts WL. Cross-reactivity of three recombinant insulin analogs with five commercial insulin immunoassays. *Clin. Chem.* 50(1), 257–259 (2004).
- 92 Moriyama M, Nobuhide H, Ohyabu C *et al.* Performance evaluation and cross-reactivity from insulin analogs with the ARCHITECT insulin assay. *Clin. Chem.* 52(7), 1423–1426 (2006).
- 93 Heald AH, Bhattacharya B, Cooper H *et al.* Most commercial insulin assays fail to detect recombinant insulin analogs. *Ann. Clin. Biochem.* 43(Pt 4), 306–308 (2006).
- 94 Song D, Davidson J. Cross-reactivity of Actrapid® and three insulin analogs in the Abbot IMX insulin immunoassay. *Ann. Clin. Biochem.* 44(Pt 2), 197–198 (2007).
- 95 Neal JM, Han W. Insulin immunoassays in the detection of insulin analogs in factitious hypoglycemia. *Endocr. Pract.* 14(8), 1006–1010 (2008).
- 96 Bowsher RR, Lynch RA, Brown-Augsburger P *et al.* Sensitive RIA for the specific determination of insulin lispro. *Clin. Chem.* 45(1), 104–110 (1999).
- **First description of an insulin analog assay.**
- 97 Andersen L, Jorgensen PN, Jensen LB, Walsh D. A new insulin immunoassay specific for the rapid-acting insulin analog, insulin aspart, suitable for bioavailability, bioequivalence and pharmacokinetic studies. *Clin. Biochem.* 33(8), 627–633 (2000).
- 98 Holloway DL, Santa PF, Compton JA, Bowsher RR. Humalog, a rapidly absorbed analog of human insulin, determined with Coat-A-Count insulin radioimmunoassay. *Clin. Chem.* 41(12 Pt 1), 1777–1778 (1995).
- 99 Andersen L, Volund A, Olsen KJ, Plum A, Walsh D. Validity and use of a non-parallel insulin assay for pharmacokinetic studies of the rapid-acting insulin analog, insulin aspart. *J. Immunoassay Immunochem.* 22(2), 147–163 (2001).
- 100 Chance RE, Root MA, Galloway JA. The immunogenicity of insulin preparations. *Acta Endocrinol. Suppl. (Copenh.)* 205, 185–198 (1976).
- 101 Barcinski MA, Rosenthal AS. Immune response gene control of determinant selection. I. Intramolecular mapping of the immunogenic sites on insulin recognized by guinea pig T and B cells. *J. Exptl Med.* 145, 726–742 (1977).
- 102 Rosenthal AS, Rosenwasser LJ, Baskin BL *et al.* Genetic control of the immune response to insulin. Its dependence upon a macrophage mediated selection of distinct antigenic sites. *Adv. Exp. Med. Biol.* 98, 447–458 (1978).
- 103 Schroer JA, Bender T, Feldman RJ, Kim KJ. Mapping epitopes on the insulin molecule using monoclonal antibodies. *Eur. J. Immunol.* 13(9), 693–700 (1983).
- 104 Marks A, Yip C, Wilson S. Characterization of two epitopes on insulin using monoclonal antibodies. *Mol. Immunol.* 22(3), 285–290 (1985).
- 105 Storch M-J, Petersen K-G, Licht T, Kerp L. Recognition of human insulin and proinsulin by monoclonal antibodies. *Diabetes* 34, 808–811 (1985).
- 106 Rathjen DA, Underwood PA. Identification of antigenic determinants on insulin recognized by monoclonal antibodies. *Mol. Immunol.* 23(4), 441–450 (1986).
- 107 Storch M-J, Licht T, Petersen K-G, Obermeier R, Kerp L. Specificity of monoclonal anti-human insulin antibodies. *Diabetes* 36, 1005–1009 (1987).
- 108 Ottesen JL, Nilsson P, Jami J *et al.* The potential immunogenicity of human insulin and insulin analogs evaluated in a transgenic mouse model. *Diabetologia* 37, 1178–1185 (1994).
- 109 Neubauer HP, Obermeier R, Schnorr G. Immunological properties and biological effectiveness of insulin analogs substituted at position B30. *Diabetologia* 27, 129–131 (1984).
- 110 Blundell T, Dodson G, Hodgkin D, Mercola D. Insulin. The structure in the crystal and its reflection in chemistry and biology. *Adv. Protein Chem.* 26, 279–402 (1972).
- 111 Nell LJ, Virta VJ, Thomas JW. Recognition of human insulin *in vitro* by T cells from subjects treated with animal insulins. *J. Clin. Invest.* 76, 2070–2077 (1985).
- 112 Glimcher LH, Schroer JA, Chan C, Shevach EM. Fine specificity of cloned insulin-specific T cell hybridomas. Evidence supporting a role for tertiary conformation. *J. Immunol.* 131(6), 2868–2874 (1983).
- 113 Hallden G, Gafvelin C, Mutt V, Jornvall H. Characterization of cat insulin. *Arch Biochem. Biophys.* 247(1), 20–27 (1986).
- 114 Seino S, Steiner DF, Bell GI. Sequence of a new world primate insulin having low biological potency and immunoreactivity. *Proc. Natl Acad. Sci. USA* 84, 7423–7427 (1987).
- 115 Gennaro WD, Van Norman JD. Quantitation of free, total and antibody-bound insulin in insulin-treated diabetics. *Clin. Chem.* 21(7), 873–879 (1975).
- 116 Armitage M, Wilkin T, Wood P, Casey C, Loveless R. Insulin autoantibodies and insulin assay. *Diabetes* 37, 1392–1396 (1988).
- 117 Carter P. Preparation of ligand-free human serum for RIA by adsorption on activated charcoal. *Clin. Chem.* 24(2), 362–264 (1978).
- 118 Haffner SM, Mykkanen L, Valdez RA *et al.* Disproportionately increased proinsulin levels are associated with the insulin resistance syndrome. *J. Clin. Endocrinol. Metab.* 79(6), 1806–1810 (1994).
- 119 Nagi DK, Knowler WC, Mohamed-Ali V, Bennett PH, Yudkin JS. Intact proinsulin, des 31,32 proinsulin, and specific insulin concentrations among nondiabetic and diabetic subjects in populations at varying risk of type 2 diabetes. *Diabetes Care* 21(7), 127–133 (1998).
- 120 Duckworth WC, Bennett RG, Hamel FG. Insulin degradation. progress and potential. *Endocrin. Rev.* 19(5), 608–624 (1998).
- 121 Moore JA, Wroblewski VJ. Pharmacokinetics and metabolism of protein hormones. In: *Protein Pharmacokinetics and Metabolism, Pharmaceutical Biotechnology (Volume 1)*. Ferraiolo BL, Mohler MA, Gloff CA (Eds). Plenum Press, NY, USA 94–98 (1992).
- 122 Walters E, Henley R, Barnes I. Stability of insulin in normal whole blood. *Clin. Chem.* 32(1 Pt. 1), 224 (1986).
- 123 Nowatzke WL, Delhaye R, Reinacher S *et al.* Stability of insulin in human serum and plasma. Presented at: *The AAPS National Biotech Conference*. Boston MA, USA, 18–21 June 2006.
- 124 Hamel FG, Siford GL, Fawcett J *et al.* Differences in the cellular processing of AspB10 human insulin compared with human insulin and LysB28ProB29 human insulin. *Metab.* 48(5), 611–617 (1999).
- 125 Wang F, Carabino JM, Vergara CM. Insulin glargine. A systematic review of a long-acting insulin analog. *Clin. Ther.* 25(6), 1541–1577 (2003).
- 126 Duckworth W, Davis SN. Comparison of insulin glargine and NPH insulin in the treatment of Type 2 diabetes. A review of clinical studies. *J. Diabetes Complications* 21(3), 196–204 (2007).
- 127 Again A, Jeandidier N, Gasser F, Grucker D, Sapin R. Glargine blood biotransformation. *in vitro* appraisal with human insulin immunoassay. *Diabetes Metab.* 33(3), 205–212 (2007).

- 128 Ibrahim F, Bagnard G, Boudou P. Differences in circulating insulin levels following glargine administration. *Clin. Biochem.* 41(6), 429–431 (2008).
- 129 Kuerzel GU, Shukla U, Scholtz HE *et al.* Biotransformation of insulin glargine after subcutaneous injection in healthy adults. *Curr. Med. Res. Opin.* 19(1), 34–40 (2003).
- 130 Kuerzel G, Sandow J, Seipke G *et al.* Kinetic and metabolite profile of insulin glargine. *Diabetologia* 44(Suppl. 1), 798 (2001).
- 131 DeSilva B, Bowsher RR. Validation of ligand binding assays to support pharmacokinetic assessments of biotherapeutics. In: *Ligand-Binding Assays. Development, Validation, and Implementation in the Drug Development Arena*. Khan MN, Findlay JW (Eds). John Wiley & Sons Inc, 81–90 (2010).
- 132 Thway TM, Macaraeg CR, Calamba D *et al.* Bioanalytical method requirements and statistical considerations in incurred sample reanalysis for macromolecules. *Bioanalysis* 2(9), 1587–1596 (2010).
- 133 Fast DM, Kelley M, Viswanathan CT *et al.* Workshop report and follow-up – AAPS Workshop on Current Topics in GLP Bioanalysis. assay reproducibility for incurred samples – implications of Crystal City recommendations. *AAPS J.* 11(2), 238–241 (2009).
- 134 Burtis CA, Ashwood ER. *Tietz Textbook of Clinical Chemistry, 2nd Edition*. W.B. Saunders Co., USA. (1986).
- 135 Kratz A, Ferraro M, Sluss PM *et al.* Case records of the mass. General hospital. Laboratory reference values. *NEJM* 352, 1548–1563 (2004).
- 136 Gennaro WD, Van Norman JD. Quantitation of free, total and antibody-bound insulin in insulin-treated diabetics. *Clin. Chem.* 21(7), 873–879 (1975).
- 137 Steffends AB. Plasma insulin content in relation to blood glucose level and meal pattern in the normal and hypothalamic hyperphagic rat. *Physiol. Behav.* 5(2), 147–151 (1967).
- 138 Martin GB, O'Brien JF, Best R *et al.* Insulin and glucose levels during CPR in the canine model. *Annals Emer. Med.* 14(4), 293–297 (1985).
- 139 APIDRA package insert (2009).
- 140 LEVEMIR package insert (2005).
- 205 Guidance for industry on bioanalytical method validation. Federal Register 66, 28526–28527 (2001). www.fda.gov/OHRMS/DOCKETS/98fr/052301b.pdf
- 206 European Medicines Agency Draft Guideline on validation of bioanalytical methods. EMA/CHMP/EWP/192217/2009. www.ema.europa.eu/pdfs/human/ewp/19221709en.pdf
- 207 US Pharmacopeia. Insulin human. www.pharmacopeia.cn/v29240/usp29nf24s0_m40600.html
- 208 sanofi-aventis. Lantus™ (insulin glargine [rDNA origin] injection). Prescribing Information. <http://products.sanofi-aventis.us/Lantus/Lantus.html#section-12>
- 209 Diabetes Forecast. 2008 ADA Resource Guide. http://forecast.diabetes.org/webfm_send/6
- 210 Tanyolac S, Goldfine ID, Kroon L. Insulin pharmacology, types of regimens and adjustments (2008). www.endotext.org/diabetes/diabetes17/diabetesframe17.htm
- 211 USP on-line catalog and US Pharmacopeia [11061-68-0 and 133107-64-9]. www.usp.org
- 212 Human Insulin ELISA kit Package Insert, Catalog # KA0921 V.01 www.abnova.com
- 213 Human Insulin ELISA Package Insert, Catalog # EIA-1825 V6.0 www.drg-international.com

■ Websites

- 201 International Diabetes Federation. www.diabetesatlas.org/content/diabetes-and-impaired-glucose-tolerance
- 202 21 CFR Part 58. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=58&showfr=1.
- 203 International Conference on Harmonisation. ICH Q2A. Text on Validation of Analytical Procedures. Federal Register 60 FR 11260 (1995). www.fda.gov/cder/guidance/ichq2a.pdf
- 204 International Conference on Harmonisation. ICH Q2B. Validation of Analytical Procedures. Methodology. Federal Register 62 FR 27463 (1997). www.fda.gov/cder/guidance/1320fnl.pdf