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A sensitive chemiluminescent enzyme immunoassay for the bioanalysis of carboxyl-terminal B-chain analogues of human insulin

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Abstract

Quantification of analogues of human insulin in biological matrices is complicated by differences in their immunoreactivity and the presence of both the analogue and endogenous concentrations of insulin in test samples. To facilitate pharmacokinetic comparisons of carboxyl-terminal B-chain analogues of human insulin, we undertook development of a sensitive ELISA. The ELISA detection method was optimized systematically to permit routine analysis of 10-μl serum samples. Accordingly, a noncompetitive 'sandwich' chemiluminescent ELISA was validated for the quantification of carboxyl-terminal B-chain insulin analogues in human serum over a concentration range from 5 to 3125 pM. The mean bias (RE%) within the validated range varied from −10.3 to 4.3%, with an intermediate precision (inter-assay CV%) from 4.2 to 11.5%. The two-sided 90% expectation tolerance interval for total measurement error was within ±25% of the nominal concentration for all levels of validation samples. Insulin lispro, human insulin, proinsulin, despentapeptide insulin (DPI) and porcine insulin displayed comparable crossreactivity in the ELISA. Potential utility of the new assay for insulin bioanalysis in nonhuman species was investigated by assessing the pharmacokinetic profile of DPI in rats following administration of a single subcutaneous dose. The sensitive chemiluminescent detection method is simple to perform and should be readily adaptable for ELISAs of other therapeutic proteins.

Keywords: Insulin; Analogues; Immunoassay; Validation; Diabetes; Chemiluminescence; Pharmacokinetics; ELISA

1. Introduction

Modern biotechnology has spurred research in the development of insulin analogues with improved pharmacokinetics and pharmacodyamics relative to subcutaneously administered human insulin [1,2]. For conventional regular insulin, the

Abbreviations: BSA, bovine serum albumin; CL, Chemiluminescence; DPI, Des(B26-B30)human insulin; ELISA, enzyme-linked immunosorbent assay; MoAb, monoclonal antibody; PBS, phosphate buffered saline; SA-ALP, streptavidin-alkaline phosphatase.

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onset of action after subcutaneous administration is too slow, and the duration of action is too long to mimic the pattern of insulin secretion in a normal healthy individual [1–3]. Therefore, the goal has been to design new insulins that offer patients with Diabetes Mellitus improved therapeutic options to achieve sustained glycemic control.

The carboxyl-terminus of the B-chain is known to be important for the dimerization of insulin [2,4]. Systematic alteration of the primary amino acid sequence in this region is one successful strategy that has been used to develop several short-acting insulin analogues [1–3]. For example, insulin lispro is a fully potent analogue in which the natural amino acid sequence of the B-chain at the positions 28 and 29 has been inverted [5–7]. This change results in an insulin molecule with a reduced capacity for self-association [1,8,9]. Another approach to reduce self-association has been to replace the proline at position B28 with a negatively charged aspartic acid residue [1,2,10]. Alteration of the primary structure at the carboxyl-terminus of the B-chain has also been attempted for the purpose of designing new long-acting analogues [1,2,11] and other novel insulins [1]. Thus, the carboxyl-terminus of the B-chain is a region that has been attractive to alter in attempts to develop new and improved insulin analogues.

Assessment of the pharmacokinetics of carboxyl-terminal B-chain analogues is complicated by the fact that these insulins often differ in their immunoreactivity. This is because the carboxyl-terminal of the B-chain region is an antigenic determinant [12–14], and insulin antisera are often capable of detecting slight structural modifications in this region [14,15]. The current study was undertaken with the goal of developing a sensitive ELISA that would be suitable for the bioanalysis of carboxyl-terminal B-chain analogues of human insulin. DPI (Fig. 1), a monomeric insulin analogue that lacks the 5-terminal amino acids at the carboxyl-terminus of the B-chain [16], was used to screen for monoclonal antibodies with the desired selectivity and affinity.

In this study chemiluminescence was investigated as the ELISA detection method, because one goal was to use only a 10-μL serum sample to facilitate pharmacokinetic screening of insulin analogues in multiple species. Even though chemiluminescence is used widely as a highly sensitive immunoassay detection method [17,18], limited information is available regarding its potential applicability in ELISAs for the bioanalysis of therapeutic proteins within the pharmaceutical industry [19]. Therefore, a systematic optimization of chemiluminescent detection was conducted from the perspective of maximizing ELISA performance for pharmacokinetic assessments of therapeutic proteins.

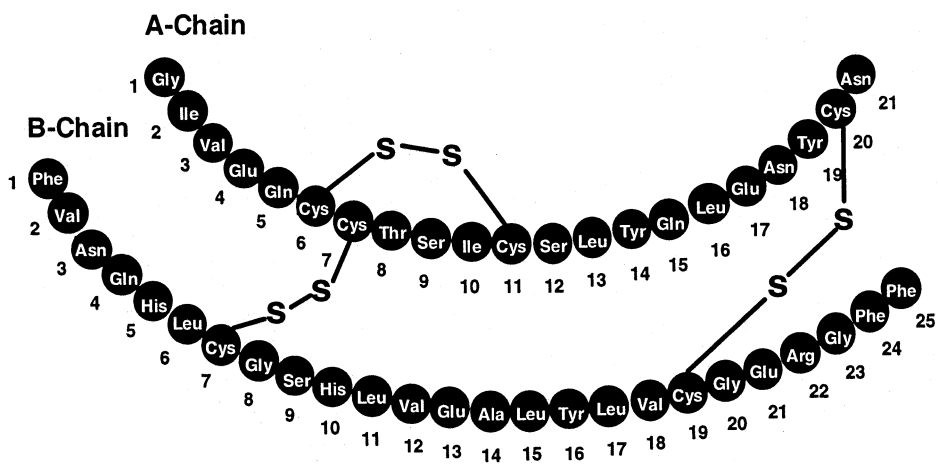

Fig. 1. Primary structure of despentapeptide human insulin, DPI

2. Experimental

2.1. Materials

Despentapeptide insulin (DPI, MW 5216.9, Fig. 1), human insulin, rat insulin, porcine insulin, human C-peptide, human proinsulin, and insulin lispro were prepared at the Lilly Research Laboratories (Indianapolis, IN). Murine monoclonal antibodies, D6C4 and D3E7, were purchased from Research and Diagnostic Inc. (Flander, NJ). The chemiluminescent substrate CDP-star with Sapphire II™ was obtained from Tropix Inc. (Bedford, MA). Streptavidin-Alkaline phosphatase was purchased from Roche (Indianapolis, IN). Black microtiter plates were obtained from Labsystem Inc. (Franklin, MA). EZ-linked™-Sulfo-NHS-LC-Biotin was obtained from Pierce (Rockford, IL). Human serum albumin and treated human serum (defribrinated, heat-treated, charcoal stripped,) were obtained from SeraCare Inc. (Oceanside, CA). Normal mouse serum was purchased from Jackson Immuno Research Inc. (West Grove, PA). Equine serum was purchased from Hyclone (Logan, Utah). The TopCount•NXT™ microplate scintillation and luminescence counter was obtained from Packard Instrument Company (Meriden, CT).

2.2. Biotinylation of MoAb, D3E7

Different quantities of Sulfo-NHS-LC-Biotin (2 mg/ml) were added to a 1 mg/ml solution of D3E7 in 25 mM sodium phosphate/150 mM sodium chloride, pH 7.4 (PBS) to yield molar ratios from 25:1 to 100:1. The reactions were mixed gently by vortexing and then incubated at ambient temperature. After 60 min, the mixtures were dialyzed against PBS (3×4 l) to remove free biotin. Protein concentrations were determined by UV at 280 nm. Biotinylated antibodies were stored frozen in aliquots at -20°C at a concentration of about 0.1 mg/ml in ELISA blocking buffer.

2.3. ELISA reagents

All solutions, except the stock solutions of DPI, were stored at 4°C. Coating buffer consisted of 0.2 M sodium carbonate buffer, pH 9.4. The ELISA plate wash buffer was 50 mM HEPES/150 mM sodium chloride/0.1% sodium azide/0.1% Tween-20, pH 7.5. Blocking buffer consisted of wash buffer supplemented with 2% human serum albumin, pH 7.5. The assay buffer used for diluting the biotinylated secondary MoAb consisted of wash buffer supplemented with 0.5% mouse serum and 0.5% equine serum, pH 7.5.

Separate vials of lyophilized DPI standard (2.71 mg, 519 nmol) were used for preparing stock solutions for standard curves and validation samples. Lyophilized peptide was reconstituted with 0.519 ml of 0.01 N HCl to yield a 1 mM solution. A 0.5-ml aliquot was then added to 49.5 ml of blocking buffer to yield a stock standard at 10 μM. The stock standard solution was stored frozen in 1-ml aliquots at -20°C.

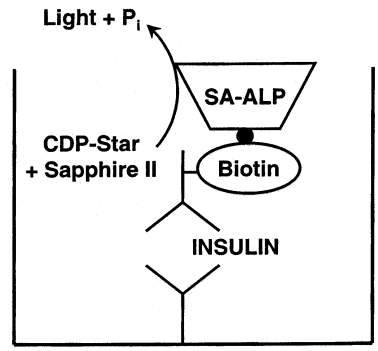

Fig. 2. Schematic representation of the DPI ELISA. The assay was conducted as described in Section 2.4, ELISA procedure. Streptavidin-alkaline phosphatase is abbreviated SA-ALP.

2.4. ELISA procedure

A schematic representation of the assay is shown in Fig. 2. A black microtiter plate was coated with 100 μl of carbonate buffer, pH 9.4 that contained 0.1 μg of MoAb D4C6. After incubation for 16–24 h at 4°C, the plate was washed 3-times with wash buffer. Nonspecific sites were blocked by incubation with blocking

buffer for 1 h at ambient temperature. After washing 3-times and decanting the liquid phase, the plates were sealed and stored dry at 4°C in a humidified chamber for up to 2 weeks prior to use.

To each well was added 100 μl of assay buffer that contained 0.1 μg of the biotinylated secondary MoAb D3E7. This was followed by a 10 μl addition of DPI standard in heat-treated charcoal stripped human serum or serum test sample. The microtiter plate was sealed and incubated with gentle rocking overnight at 4°C. After washing 6-times, 100 μl of Streptavidin-alkaline phosphatase (1:10 000 dilution in blocking buffer) was added to each well, followed by incubation for 30 min at ambient temperature with gentle rocking. The ELISA plate was again washed 6-times and 100 μl of CDP-*star* + sapphire II™ was added to each well. Following incubation for 10 min at ambient temperature with gentle rocking, the plate was read in a Packard TopCount•NXT™ microplate counter (1 s per well) after dark adaptation for 1 min.

A microcomputer software program (StatLIA®; Brendan Scientific, Grosse Pointe Farms, MI) was used to fit ELISA binding data by a weighted 4/5-parameter logistic model [20]. The DPI concentrations in serum test samples were estimated by interpolation of a standard curve of DPI that ranged in concentrations from 0.78 to 7500 pM.

2.5. Assay validation

ELISA validation experiments were conducted according to current bioanalytical recommendations and a recent review publication for validation of immunoassays [21,22]. Inter-assay accuracy (mean bias) and precision were assessed by measuring DPI concentrations in validation samples, which were prepared by adding DPI to heat-treated charcoal stripped human serum at concentrations of 5, 25, 125, 625, and 3,125 pM. Validation samples were stored frozen in 1-mL aliquots at −20°C. Validation experiments were performed by measuring each concentration level four times in triplicate across eight separate assays (2 ELISAs per day × 4 days). For each concentration level, the a priori criteria for method acceptance was that the two-sided 90% expectation tolerance interval for relative total error [23] must have limits within ±25% of the nominal concentration. This criteria was based on the recent recommendations of a SFSTP Commission [24] and a pharmaceutical industry perspective on immunoassay validation [22].

Assay specificity was investigated systematically by comparing the cross-reactivity of DPI with those of a number of structurally related peptides. These included human insulin, rat insulin, porcine insulin, human C-peptide, human proinsulin, and insulin lispro.

2.6. Rat pharmacokinetic study

Potential utility of the new chemiluminescent ELISA for use with nonhuman serum samples was investigated in the rat. A pharmacokinetic study of DPI was conducted in fasted Sprague–Dawley rats (300–350 mg/kg). A 0.5 U/kg (17.4 μg/ml) dose of DPI was administered subcutaneously to each of four rats. Samples of whole blood (200–250 μl) were collected from cannulated tail veins into tubes containing EDTA at pre-dose, and 10, 20, 30, 45, 60, 90, 120, and 180 min following administration of DPI. After centrifugation, plasma samples were stored frozen in sealed vials at −20°C prior to analysis by ELISA.

3. Results

3.1. ELISA specificity

Crossreactivity of a number of structurally related insulins was evaluated in the ELISA. The insulins were either from different species, or analogues of human insulin. Percent crossreactivity was calculated as a ratio of the ED_{50} for DPI to the ED_{50} for each peptide. Most insulin-related peptides, including human insulin, DPI, human proinsulin, insulin lispro and porcine insulin generated similar concentration-response profiles with comparable ED_{50} values (Table 1). Only rat insulin displayed a substantially lower level of crossreactivity. Human C-peptide failed to produce a detectable response in the ELISA.

3.2. Calibration model assessment

Appropriateness of the weighted 4/5-parameter logistic model for fitting the ELISA calibration curves was evaluated by assessing the 'goodness of fit' of back-fitted standard concentrations for the eight validation assays. For standards within the validated assay range, 58 out of 64 back-fitted results (90.6%) were within ± 10% of their nominal concentrations. As reported in Table 2, the mean relative errors (RE%) for these back-fitted standards ranged from − 8.9 to 5.7% with CV% values from 2.5 to 7.6%.

A typical standard curve of DPI in heat-treated charcoal stripped human serum is shown in Fig. 3. For the eight validation runs, the mean values (± S.E.) for the A and D asymptotic parameters were 61 588 ± 4383 cps and 20 680 063 ± 863 741 cps. The slope and ED_{50} values were 1.57 ± 0.10 and 1065 ± 102 pM, respectively.

3.3. Accuracy and precision

Results of assay validation experiments are summarized in Table 3 and depicted in Fig. 4. The validated range of the ELISA was from 5.0 to 3125 pM. In this range, the lower and upper limits of the 90% expectation tolerance interval for relative total error were within ± 25% of the nominal concentration for all validation samples. ELISA accuracy (mean bias, RE%) ranged from − 10.3 to − 1.6%. Intra-assay (repeatability) and inter-assay (intermediate) precision (CV%) ranged from 2.8 to 11.2% and from 4.2 to 11.5%, respectively.

3.4. Rat pharmacokinetic study

The new chemiluminesent ELISA was used to evaluate the pharmacokinetics of DPI after administration of a single subcutaneous dose to fasted rats. Individual plasma concentration-time profiles for the four rats are depicted in Fig. 5. Concentrations of DPI in serum samples collected prior to the administration of DPI were all below the ELISA's lower limit of quantification. In general, the pharmacokinetic profiles were similar with maximal DPI concentrations (C_{max}) of nearly

Table 1
Crossreactivity of structurally-related insulin peptides in the DPI ELISA

Peptide	B26	B27	B28	B29	B30	ED_{50} (pmol/l)	Crossreactivity[a] (%)
DPI[b]	–	–	–	–	–	1010	100
Human insulin	Tyr	Thr	Pro	Lys	Thr	946	107
Insulin lispro	Tyr	Thr	Lys	Pro	Thr	753	134
Porcine insulin	Tyr	Thr	Pro	Lys	Ala	1149	88
Human proinsulin	Tyr	Thr	Pro	Lys	Thr	1000	101
Rat insulin I[c]	Tyr	Thr	Pro	Lys	Ser	6390	16
Rat insulin II[d]	Tyr	Thr	Pro	Met	Ser	N.R.[e]	–
C-peptide			N.A.[f]			N.D.[g]	0

[a] Cross-reactivity was calculated from the ratio of ED_{50} values as follows, [(ED_{50} of DPI/ED_{50} of peptide) × 100]. ED_{50} is the peptide concentration necessary to achieve 50% of the maximal response, the upper asymptote of the 4-PL algorithm.

[b] DPI has the same structure as human insulin, except it lacks the five terminal amino acids at the carboxyl-terminus of the B-chain. See Fig. 1 for the primary structure of DPI.

[c] In addition to differences at the B-chain C-terminus shown above, rat/mouse insulin I also differs from human insulin by having an A4(Asp), a B3(Lys), and a B9(Pro).

[d] In addition to differences at the B-chain C-terminus shown above, rat/mouse insulin II also differs from human insulin by having an A4(Asp) and a B3(Lys).

[e] N.R., no result, rat insulin II was not included the crossreactivity experiments due to its lack of availability. Rat insulin II is anticipated to crossreact in the DPI ELISA at least as well as rat insulin I. Rat insulin II has more sequence homology with human insulin than rat insulin I, except at the B-chain C-terminus which is absent in DPI.

[f] N.A., not applicable, C-peptide has no primary sequence homology with human insulin.

[g] N.D., none detected, C-peptide failed to produce a response above background cps at concentrations as high as 10 000 pM.

Table 2
Assessment of the calibration model 'Goodness of Fit'

Assay run	Back-fitted standard DPI concentrations (pM)										
	7500	3000	1200	480	192	76.8	30.7	12.3	4.92	1.97	0.79
1	4830.5	2676.9	1368.7	512.9	195.8	78.7	26.5	11.1	5.1	3.6	1.8
2	5679.5	2746.7	1294.2	490.4	192.0	78.1	28.5	12.9	4.9	2.1	1.3
3	9579.9	2725.6	1220.7	492.9	185.9	78.9	30.4	11.4	4.5	2.3	1.9
4	6519.8	2792.9	1305.0	481.1	184.7	80.3	29.4	11.6	4.9	0.6	2.3
5	7707.5	2819.8	1165.3	510.0	177.4	80.4	31.3	12.8	4.7	1.4	1.1
6	9278.4	2460.8	1149.8	515.5	179.4	80.3	30.8	12.6	4.7	1.5	1.3
7	4973.2	2824.4	1307.5	508.5	197.7	76.1	27.7	13.0	5.0	2.5	0.3
8	5777.3	2813.4	1336.7	489.8	197.4	75.2	27.7	13.9	5.1	2.1	0.3
Mean	6793.3	2732.5	1268.5	500.1	188.8	78.5	29.0	12.4	4.9	2.0	1.3
SD	1862.8	121.5	80.4	13.0	8.1	2.0	1.7	0.9	0.2	0.9	0.7
CV (%)	27.4	4.4	6.3	2.6	4.3	2.5	5.9	7.6	4.4	44.1	55.3
RE (%)	−9.4	−8.9	5.7	4.2	−1.7	2.2	−5.4	0.9	−0.9	2.9	62.6

1000 pM achieved at ≤ 10 min. Plasma concentrations declined in a monotonic exponential manner with a half-life of about 30 min, and returned to near their pre-dose levels by 3 h. One rat displayed a markedly different pharmacokinetic profile that was characterized by slower absorption and more limited exposure (smaller AUC).

4. Discussion

Analogues of human proteins can present a significant analytical challenge in the validation and application of GLP-compliant immunoassays to support pharmacokinetic and bioequivalence studies. For example, insulin antisera are often capable of detecting minor structural differences between human insulin and an analogue, such as insulin lispro [25–27]. Since insulin is frequently present endogenously at pharmacologically relevant concentrations, the challenge is to validate an immunoassay for an analogue when the native peptide is present in the same biological specimen [28,29].

One successful analytical strategy has been to develop specific immunoassays in which the analogue displays negligible crossreactivity with the endogenous native peptide [28,29]. Even though this approach is highly useful for characterizing the pharmacokinetic behavior of an insulin analogue in clinical studies, the strategy is not broadly applicable in early phase studies to screen analogues. Therefore, the current study was initiated with the goal of developing a versatile ELISA that would be suitable for use in pharmacokinetic studies to compare different carboxyl-terminal B-chain analogues of human insulin.

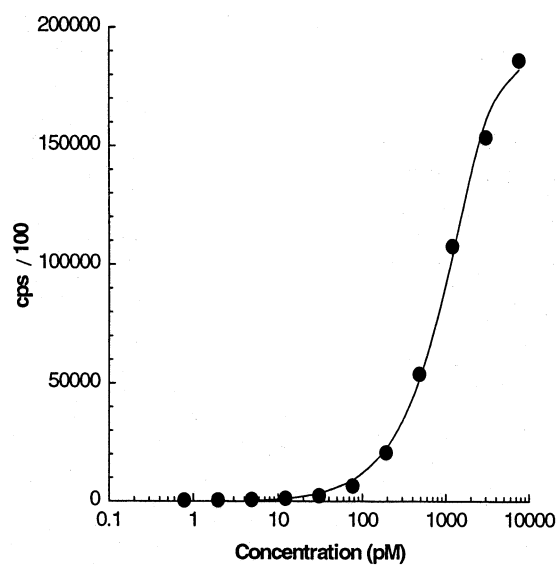

Fig. 3. Representative DPI standard curve.

Table 3
Evaluation of DPI ELISA accuracy and precision

	Statistic	Concentration (pM)				
		5	25	125	625	3125
Bias (RE%)	Mean	4.3	−10.3	−8.9	−2.0	−1.6
Precision (CV%)	Intra-assay	11.2	4.3	2.8	3.0	8.3
	Inter-assay	11.5	6.6	4.2	5.1	9.5
90% Expectation	Lower limit	−15.6	−22.4	−16.6	−11.5	−18.3
Tolerance interval (RE%)	Upper limit	24.2	1.4	−1.3	7.6	15.0

Monoclonal antibodies that detect DPI and insulin in an equivalent manner were hypothesized to be suitable for quantifying carboxyl-terminal B-chain analogues of human insulin. Upon completion of screening experiments, two monoclonal antibodies (clones D6C4 and D3E7) from Research Diagnostic, Inc. (Flanders, NJ) were selected for use in the noncompetitive ELISA. As shown in Table 1, a number of naturally-occurring and synthetic insulins displayed comparable levels of crossreactivity relative to DPI. Thus, we concluded this pair of MoAbs recognizes common insulin epitopes that are not present at or near the carboxyl-terminus of the B-chain. One likely epitope is the immunodominant A-chain loop that includes amino acid residues A8–A10 [30–32].

Of the insulins tested, only rat insulin displayed a reduced level of crossreactivity (Table 1). In rats and mice two distinct circulating forms of insulin (I and II) are expressed as products of different nonallelic preproinsulin genes [33,34]. 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 [33]. Interestingly, the monoclonal antibody pair used in this study was classified by the manufacturer as anti-rat and anti-murine insulin/proinsulin antibodies that also displays crossreactivity with human, bovine, and porcine insulin and proinsulin. To aid interpretation of the results from crossreactivity experiments (Table 1), we requested background information concerning preparation of the monoclonal antibodies. The vendor reported the mice were initially immunized with human insulin. The positive antibody clones were then screened as antibody pairs in a noncompetitive ELISA to detect rat and mouse insulin. Thus the reduced crossreactivity found with rat insulin is understandable, as human insulin was used as the immunogen during preparation of the MoAbs.

A systematic assessment of chemiluminescent detection was conducted to maximize the ELISA's sensitivity and working range. Optimal results were obtained using the glow substrate CDP-*star* with sapphire II™ as the enhancer (Fig. 2) [35]. Assay conditions commonly used for conventional colorimetric ELISAs could not be extrapolated directly to chemiluminescent detection. To achieve the optimal signal to noise ratio, concentrations of both the coating and detection MoAbs were reduced to 1 μg/ml (0.1 μg per well), the

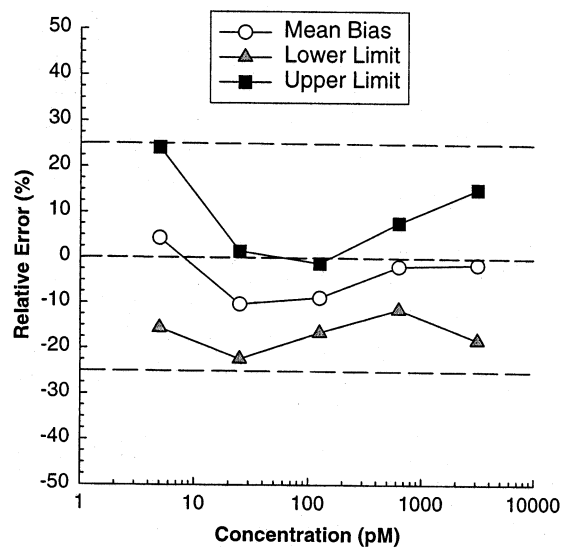

Fig. 4. Estimated mean bias and two-sided 90% expectation tolerance interval (RE%) for DPI ELISA.

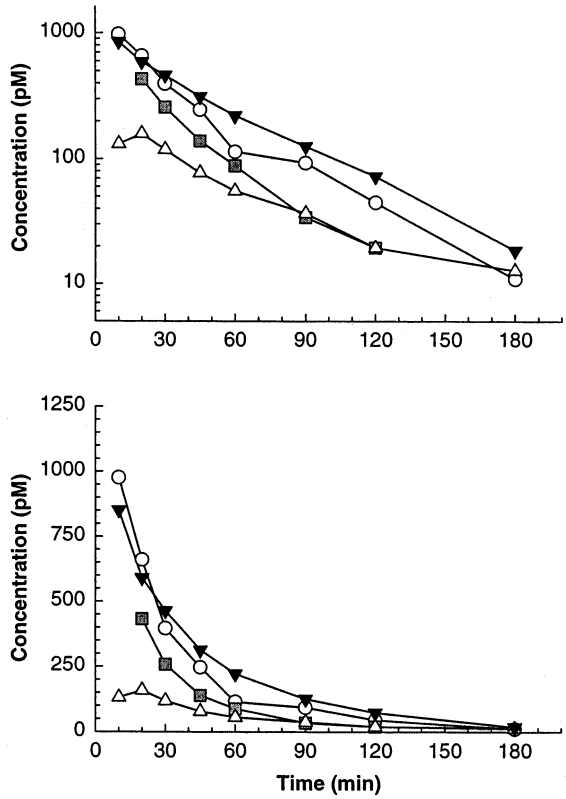

Fig. 5. Plasma concentration-time profile for immunoreactive DPI in Sprague–Dawley rats following administration of a single subcutaneous 0.5 U/kg dose of DPI. Plasma concentration-time data are plotted log-linear in the upper graph and linear–linear in the lower graph.

dilution of the streptavidin-alkaline phosphatase was increased by a factor of about 4, and the incubation time for the alkaline phosphatase reaction was reduced to ≤ 10 min. The inclusion of 0.5% mouse and 0.5% equine sera in the buffer for the biotinylated secondary MoAb was critical for maximizing the assay's signal to noise ratio. Inclusion of nonimmune serum from the same species as the one used for raising the reagent antibodies is a common ELISA strategy to minimize interference from host anti-animal and heterophilic antibodies and the nonspecific association of reagent antibodies [36]. Tween-20 (0.1%) also produced a small, but beneficial effect on the signal to noise ratio. Optimal results were obtained when serum samples were incubated for either 4–6 h at ambient temperature or 16–24 h at 4°C. However, an overnight incubation was chosen to facilitate routine processing of large numbers of test samples.

The type of microtiter plate was critical for assay performance. Black microtiter plates yielded a low background and broad working range. In contrast, white plates provided a maximal signal, but a more limited working range due to a higher background and saturation of the counter's upper dection limit. Conventional clear polystyrene plates could not be used with the microplate counter due to 'cross talk' between wells.

Optimization of the chemiluminescent ELISA resulted in a sensitive assay with a broad validated range from 5 to 3125 pM, corresponding to 0.8–521 μU/ml for human insulin. As reported in Table 3, the values for accuracy (mean bias), intra-assay (repeatability) and inter-assay (intermediate) precision are well within current guidance limits of 15 and 20% for method acceptance [21]. Moreover, the two-sided 90% expectation tolerance interval for the total measurement error (which includes both accuracy and precision) is within $\pm 25\%$ of the nominal concentration for all validation samples (Fig. 4) [22–24]. Satisfaction of this a priori acceptance criterion, in addition to current guidance limits [21], provides greater assurance that future test results will be within $\pm 25\%$ of their true concentration. Two advantages the new chemiluminescent ELISA offers over conventional colorimetric detection are that it requires only a 10 μl serum sample and has a broad validated range with an upper limit of 3125 pM. For pharmacokinetic studies of an insulin analogue, this upper limit of quantification is adequate in most cases to eliminate the need for diluting test samples. To demonstrate the utility of these advantages, a preliminary pharmacokinetic study was conducted that involved administration of a single 0.5 U/kg subcutaneous dose of DPI to four fasted Sprague–Dawley rats (Fig. 5). The small sample volume eliminated the need for sparse-sampling and permitted the use of serial sampling to define the entire concentration-time profile within each rat. This is advantageous in rodent pharmacokinetic studies to screen B-chain analogues of insulin. In all animals, the pre-dose concentration of immunoreactive DPI was found

to be below the assay's lower limit of quantitation. This is likely a consequence of the rats being fasted and the low level of crossreactivity noted for rat insulin (Table 1).

In summary, we developed and validated a sensitive chemiluminescent ELISA for the determination of carboxyl-terminal B-chain analogues of human insulin in human serum. Based on the results from a preliminary pharmacokinetic study in rats, this method should also be adequate for use in preclinical studies to screen the pharmacokinetics of different B-chain analogues of insulin. Following appropriate validation, the new method should be suitable for use in a GLP-compliant manner with sera from nonhuman species. Furthermore, the sensitive chemiluminescent-based detection method described herein is simple to perform and, therefore, should be broadly applicable in ELISAs for other therapeutic proteins.

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