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Comparison of a Luminescent Oxygen Channeling Immunoassay and an ELISA for detecting Insulin Aspart in human serum

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ABSTRACT

The study was a comparison between a Luminescent Oxygen Channeling Immunoassay (LOCI™) and an enzyme-linked immunosorbent assay (ELISA) for quantification of Insulin Aspart (IAsp) in human serum. The advantage of LOCI™ compared to ELISA is reduced workload and higher throughput.

The ELISA assay was performed as published (Andersen et al., 2000 [5]). The LOCI™ followed a 2-step reaction. First, the sample was incubated for 1 h with a mixture of biotinylated antibody specific for IAsp and beads coated with insulin-detecting antibody. This step was followed by a 30-min incubation with beads covalently coated with streptavidin. When the beads were brought in proximity through binding of IAsp, light was generated from a chemiluminescent reaction in the beads. This light was measured and quantified.

Spiked samples with different concentrations of IAsp were prepared in human serum to compare ELISA and LOCI™. Human serum samples ($n = 510$) from a pilot study with healthy subjects receiving IAsp were also analysed and compared in the two assays.

Higher precision, improved accuracy and a wider analytical range were found using LOCI™ compared to ELISA. However, sample haemolysis interfered more when using LOCI™ than ELISA. The IAsp concentrations determined in the human serum samples from the pilot study gave a good correlation between the two assays.

In conclusion, LOCI™ can determine IAsp in human serum just as well as ELISA. Using LOCI™ reduces the workload, which is particularly useful when handling large sample sizes.

1. Introduction

Insulin Aspart (IAsp) is a well-known insulin analogue used in the treatment of diabetes. It is a rapid-acting analogue used as meal time insulin or as basal-bolus insulin in Continuous Subcutaneous Insulin Infusion (CSII) [1–4]. Large sample sizes are often produced when using the analogue in research or clinical development. This process creates a considerable workload which can be reduced with the shorter analysis time and higher throughput provided by LOCI™ compared to ELISA. Accordingly, LOCI™ has the potential to be a good alternative to the currently used ELISA for determination of IAsp [5–7]. LOCI™ is already being used to analyse research samples from pigs, dogs and rats for determination of IAsp internally at Novo Nordisk A/S, Denmark.

LOCI™ is a homogenous bead-based assay, in which the associated wash steps in the ELISA method are eliminated, as separation of bound from free analytes is not necessary in LOCI™ [6]. It reduces the number of steps in the assay procedure. In the specific IAsp ELISA method, the total incubation time is 20–24 h. With LOCI™ this time period is reduced to 1.5 h incubation. Another advantage is that LOCI™ has been optimised for 384-well plates, whereas 96-well plates are used for the ELISA today; contributing to a higher throughput. LOCI™ is faster and can analyse many samples at the same time. With large sample sizes, these advantages are considerable compared to the ELISA [5,6].

Furthermore, LOCI™ often has a higher sensitivity, a potentially lower quantification limit and a wider working range than many other immunoassays such as the ELISA [6].

Human samples from clinical trials with subjects receiving IAsp have been analysed in ELISA [3,8,9]. As mentioned, the method for measuring IAsp using LOCI™ was developed to analyse samples from animals, such as pigs, dogs and rats. Recently, a method for detecting human insulin in human plasma has been published using the LOCI™ technology [10]. Thus, it should be possible to analyse IAsp in human samples using the LOCI™ technology.

The present study compared the performance of the IAsp ELISA to the IAsp LOCI™ with regard to precision, accuracy, range,

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stability and interference from haemolysis of spiked human serum samples.

Finally, serum samples from a pilot study including healthy volunteers receiving IAsp were analysed using both assays and the IAsp concentrations were compared.

2. Materials and methods

2.1. ELISA

2.1.1. Antibodies

Two different monoclonal antibodies from mice were used. The antibodies were X14-6F34 (detecting antibody) and HUI-018 (insulin catching antibody), where X14-6F34 is specific for IAsp (Novo Nordisk A/S, Denmark) [5]. Biotinylation of X14-6F34 was performed essentially as described by Berger et al. [11] with 60 μL Biotin-NHS (Sigma H1759, MO, USA) in DMF (Merck 103053, Germany) per 1 mg antibody.

2.1.2. Assay plates

Microtiter wells (Nunc F96 Maxisorb, Thermo Fisher Scientific, Denmark) were coated with 100 μL per well of PBS buffer containing 10 μg/mL HUI-018 antibody. The plates were incubated at room temperature (RT) (20–25 °C) during the night. The coated wells were washed 3 times with washing buffer (0.5%, v/v Tween 20 (Merck 822184, Germany) in PBS), 400 μL in each well for every wash.

Hereafter 200 μL blocking buffer (5 g/L bovine serum albumin (BSA; Sigma A-7888, MO, USA) and 0.05%, v/v sodium-azide (Bie & Bertsen, LAB 52300, Denmark) in PBS) were added to each well. The plates were incubated for 1 h at RT and then stored at 1–9 °C until use.

2.1.3. Assay procedure

The ELISA was a sandwich ELISA. The procedure was performed as described in the literature by Andersen et al. [5]. Buffers and chemicals are described previously [12]. The validated range was 11.5–800 pM and the lower limit of quantification (LOQ) was 13 pM in the published study [5].

2.1.4. Calibration

Calibrators were prepared by gravimetric spiking in a human serum pool (H/S Blodbank, Denmark) with an IAsp standard of 609 μM (Novo Nordisk A/S, Denmark). Nominal concentrations were calculated with respect to densities of analyte and serum. Ten calibrators were spiked at the following approximate concentration levels; 5000, 2500, 1000, 600, 300, 75, 40, 10, 5 and 0 pM. The concentrations exceeded the range of the validated assay. However, in order to compare ELISA and LOCI™ technology at the same concentrations, the above described calibrators were chosen as LOCI™ had an expected range of 5–5000 pM.

The calibration data was fitted to a 4-parameter logistic function by weighted least-squares curve-fitting. The squared errors were weighted by the reciprocal of the signal raised to the second power at each concentration level as described for the validated assay.

2.1.5. Controls

Controls were also prepared by gravimetric spiking using the same IAsp standard and human serum pool as for the calibrators. Nominal concentrations were calculated with respect to densities of analyte and serum. A total of nine controls were spiked with the approximate IAsp concentration levels; 5000, 3045, 1000, 700, 250, 30, 10, 5 and 2 pM. On a routine basis three controls were used at high, medium and low concentration levels (700, 250 and 30 pM). Extra controls were made to cover the extended calibration range described above and to determine the lower and upper limit of quantification.

2.2. LOCI™

2.2.1. Antibodies

The same antibodies (but different batches) as described for the ELISA method were used. The biotinylation was performed according to standard procedures, essentially as described [11] using Biotin-X-NHS (Calbiochem 203188, Germany).

2.2.2. Beads

Unconjugated Eu-acceptor beads (A beads) (EUROP050, PerkinElmer, USA) and streptavidin coated donor beads (SA-D beads) (6760002B, PerkinElmer, USA) were used.

2.2.3. Coupling of antibody to beads

HUI-018 antibody was coupled to A beads as described by the manufacturer with the exception, that the antibody was in 0.1 M sodium phosphate (pH 8.0) and the amount of antibody used was 0.6 mg per 1 mg beads.

2.2.4. Assay plates

Low cross-talk 384-well Optiplates (PPN6005359, PerkinElmer, USA).

2.2.5. Assay procedure

In the LOCI™ assay, a bead-aggregate-immunecomplex was formed, which combines the three reactants with analyte. The SA-D beads captured the biotinylated X14-6F34 antibody and together with the HUI-018 antibody coupled A beads they were brought in proximity through the binding of IAsp. Illumination of the complex released singlet oxygen from the SA-D beads, which travelled to the nearby A beads and triggered chemiluminescence that was read on an EnVision plate reader. The amount of light generated was proportional to the concentration of IAsp.

The assay buffer contained 25 mM Hepes (H3375, Sigma–Aldrich, Germany), 50 mM NaCl, 10 mM K-EDTA, 2 mg/mL Dextran (GE Healthcare 17-0320-01, Uppsala, Sweden), 0.5% BSA (A-7888, Sigma, MO, USA), 0.1% BGG (G-5009, Sigma, MO, USA), 0.2 mg/mL HBR1 (Scantibodies Laboratories Inc. 3KC533, CA, USA), 0.1% Tween 20 (822185 E. Merck, Germany), 0.01% Proclin 300 (48912-U, Supelco, USA), 0.01% gentamycin (03-035-1C Biological Industries, Israel) and was adjusted to pH 7.4 by 1 M NaOH.

The assay was performed in 384-well plates. 1 μL sample was mixed with 15 μL mixture of biotinylated antibody and antibody coated A beads in assay buffer. The sample was dispensed by Biomek FX (Beckmann Coulter Inc., CA, USA) and the mixture was dispensed by FlexDrop IV (PerkinElmer, USA). In the 15 μL mixture the A beads were diluted to 66.7 μg/mL, and the biotinylated antibody was added to a concentration of 3.1 nM. The mixture and sample were incubated 1 h at RT and then 30 μL steptavidin coated D beads were added. Steptavidin coated D beads were diluted in assay buffer to 66.7 μg/mL, which was prepared in green light. The wells were incubated for 30 min at RT and then read on an EnVision Turbo Alpha (PerkinElmer, USA). The filter used, had a bandwidth of 520–645 nm and the complex was excitated by the 680 nm laser. The total measurement time per well was 210 ms including a 70 ms excitation time. The total reading time for 384 wells was 2.75 min. The LOCI™ technology used was described by Ullman et al. [6].

2.2.6. Calibration

The same calibrators as described for the ELISA assay were used. The two calibrators at 600 and 10 pM IAsp were left out of the calibration curve due to a limitation of 8 calibrators in the calculation

program (Software ELISA Calculator, Novo Nordisk, Denmark) in the LOCI™ setup.

The calibration data was fitted to a 5-parameter logistics function by weighted least-squares curve-fitting. As a standard procedure, the squared errors were weighted by the reciprocal of the signal raised to the second power at each concentration level.

2.2.7. Controls

The same controls as described for the ELISA assay were used.

Calibrators and controls were stored at −18 °C. Hereafter, the spiked samples were applied to the LOCI™ plates and then frozen (for maximum 14 days) until assay. With regard to the ELISA assay samples, controls and calibrators were thawed and frozen (for maximum 8 days) once until assay. This step was implemented to mimic the same step in LOCI™ and as such standardise the two assay procedures.

2.3. Validation parameters

The validation parameters were precision, accuracy, stability and haemolysis.

Specificity was not included in this study as it has previously been shown, that human insulin, porcine insulin, human proinsulin and human c-peptide do not cross-react when using the same pair of antibodies. Furthermore, dilutional linearity was not tested as the expected ULOQ was above the concentration level expected for human samples with IAsp.

2.3.1. Assay precision and accuracy

Human serum from a serum pool (H/S blodbank, Denmark) and human serum from six individuals (Bioreclamation Inc., USA) were used. The controls spiked in the human serum pool were used to determine accuracy, precision, LOQ and the upper limit of quantification (ULOQ). The individual sera were spiked to the following approximate concentration levels; 700, 250, 30 and 0 pM. All samples were analysed twice in double determination per plate. Samples in pooled serum were analysed on seven ELISA plates and four LOCI™ plates. This choice was made due to the number of samples analysed in total, and since the LOCI™ plates contained more wells, the number of plates was smaller than for ELISA. Samples in individual sera were analysed on one assay plate per technology.

2.3.2. Stability

The stability of spiked human serum samples at RT and after repeated freezing and thawing was tested. RT stability was tested at time 0, 1, 4 and 24 h with IAsp concentrations at 700 and 30 pM (control samples). The same samples were tested with up to 5 cycles of freezing and thawing (freezing time 20–24 h). All samples were analysed twice in double determination on each assay technology. Two sets of samples were analysed with regard to RT stability.

2.3.3. Haemolysis

Spiked human serum samples at IAsp concentration levels 250 and 30 pM (control samples) were prepared with erythrocytes to determine the influence of haemolysis. The packed erythrocytes were produced from sedimentation of whole blood (Novo Nordisk A/S Donor Corps, Denmark) and hereafter centrifuged. Serum was removed and cell lysis was introduced by three steps of freezing and thawing of the packed erythrocytes. The lysed erythrocytes were added to the spiked samples (approximately final concentration 250 and 30 pM) with a dilution factor of 9 and 90, where the samples with dilution factor 9 were the most haemolysed. The same samples were made without erythrocytes and used as a reference. The spiked samples were frozen at −80 °C to burst the erythrocytes overnight. All samples were analysed twice in double determination on each assay technology.

Content of haemoglobin in the samples was measured by Medonic CA620 VET (Boule Medical AB; Stockholm, Sweden).

2.4. Assay comparison using human serum samples from a pilot study

Human serum samples from a pilot study with healthy volunteers receiving IAsp were analysed in both ELISA and LOCI™. 510 human serum samples were analysed in double determination. The samples were from four different subjects entering a pilot study with two study periods and a minimum wash-out period of one week. They received IAsp by CSII with a rate of 0.5 and 0.75 U/h. In contrast to the LOCI™ assay procedure described above, the calibrators and controls used for the LOCI™ assay of the pilot study samples were spiked in porcine plasma. Because the routine LOCI™ assay procedure used porcine plasma as matrix. The ELISA assay procedure used for analysis of the pilot study samples was the same as previously described [5].

2.4.1. Method, LOCI™

The calibrators and controls were prepared in a pool of porcine EDTA plasma (Kraeber GMBH & Co., Germany) instead of human serum. The porcine EDTA plasma was centrifuged for 30 min at 4 °C, 10,000 × *g* followed by filtration to remove possible precipitate.

The calibrators were spiked volumetrically in the porcine EDTA plasma in the approximate IAsp concentrations of 5000, 2500, 1000, 300, 75, 20, 5 and 0 pM and the controls 4000, 100 and 2 pM. The assay procedure was the same as described above.

Each assay plate had human serum calibrators and controls placed as samples. This was done in order to compare the calibrators to the porcine calibrators. The human serum calibrators had the following approximate IAsp concentrations (pM): 1000, 800, 600, 400, 200, 75, 40 and 10. The human serum controls had concentrations at the approximate IAsp concentration levels (pM); 700, 230 and 30. This was the concentrations used in the validated ELISA. The human serum samples from the pilot study were analysed before the comparison described above. Thus the analytical range from the validated ELISA was used. The human serum calibrators and controls were prepared by gravimetric spiking in a human serum pool as described in Sections 2.1.4 and 2.1.5. The controls were analysed twice in double determination on each plate.

3. Results

3.1. Calibration and acceptance criteria

A calibration curve was run on each ELISA and LOCI™ plate, and all samples on the plate were measured against the corresponding calibration curve. In total, seven ELISA plates and four LOCI™ were analysed with spiked samples, each point in double determination. Table 1 shows the mean results and %CV for the calibration curves on the seven and four plates. The table also gives the percent relative error %RE, which is the ratio between the difference of the measured and the expected value, given as Eq. (1).

$$\%\mathrm{RE} = \left(\frac{|\mathrm{measured} - \mathrm{expected}|}{\mathrm{expected}}\right) \times 100 \quad (1)$$

No calibration points were excluded from the calibration curve. Thereby, the two methods were compared with all points taken into account. During routine analysis, the acceptance criteria were as follows: at least 75% of the calibrator points (mean of duplicate) must have maximum 20%RE and %CV and 25% at LOQ and ULOQ. These acceptance criteria were fulfilled fully for LOCI™ but not for ELISA. Here, the lowest calibrator had a %CV above 25% (Table 1).

Table 1
IAsp calibrators measured in ELISA and LOCI™. Results from seven ELISA plates and four LOCI™ plates.

Nominal concentration (pM)	ELISA		LOCI™	
	Mean pM (%CV)	%RE	Mean pM (%CV)	%RE
5656	6135 (12.3)	13.6	5670 (2.84)	2.30
2875	2965 (6.82)	5.45	2860 (0.86)	0.78
1143	1134 (7.59)	6.88	1188 (1.74)	3.89
681	641 (1.79)	5.92	708 (1.97)	4.00
335	337 (2.84)	2.30	339 (1.20)	1.19
86	87.7 (5.09)	4.30	82.9 (1.53)	3.66
45	45.3 (4.43)	3.29	42.8 (1.16)	4.89
11	10.4 (13.0)	7.09	10.8 (1.90)	2.05
5	5.85 (44.4)	28.5	5.48 (2.25)	9.65
0	Min	Min	Min	Min

%RE was given as mean values. At individual level the highest calibrator on Plate 1 in ELISA had a %RE at 26%. On Plate 7 in ELISA the two lowest calibrator points were above 25%RE.

3.2. Analytical precision and accuracy

Samples at nine concentration levels in pooled human serum were analysed twice in double determination on seven ELISA plates and four LOCI™ plates. From Table 2, it is apparent that the %CV of the ELISA ranged from 3.8 to 32.2 whereas the range in the LOCI™ assay was only 3.42–10.9. Inter-assay %CV ranged from 2.35 to 17.7 for ELISA and from 1.58 to 7.1 for LOCI™. Intra-assay %CV range was larger for both assays. ELISA; 2.42–35.9 and LOCI™; 3.71–11.7 (Table 2). Inter- and intra-assay %CV was calculated in Excel (2003) by ANOVA, single factor ($\alpha = 0.05$) for every concentration level.

Accuracy reflected as %RE ranged from 3.95 to 44.6 in ELISA and from 4.02 to 13.9 in LOCI™. The 44.6%RE in ELISA was for the lowest concentration (2 pM) and the same concerned %RE of 13.9 for LOCI™. When excluding the lowest concentration the range for ELISA was 3.95–21.1 and for LOCI™ 4.02–12.8. Table 2 also shows that %RE was better for LOCI™ in the low and high range whereas they were very similar in the middle.

Fig. 1 depicts the precision profile for ELISA and LOCI™. The concentration and the corresponding %CV used in Fig. 1, were results from the spiked samples (also illustrated in Table 2). The figure shows data with two excluded double determinations. LOQ and ULOQ could be determined by projecting %CV at 25% down to the *x*-axis. However, the lowest/highest control values with a %CV below 25% were used to determine LOQ and ULOQ since accuracy data for these samples was known.

For LOCI™ all control samples had a %CV below 25%. LOQ was determined to 2.28 pM and the ULOQ to 5131 pM as these controls were the lowest and highest. Also, the accuracy of these samples was within acceptable limits: the %RE was 13.9 at LOQ and 7.19 at ULOQ. The lowest calibrator had a value of 5.48 pM and the highest calibrator point had a value of 5670 pM (%RE and %CV acceptable as previously mentioned). As LOQ and ULOQ determined from the controls could not be lower or higher than the lowest or highest calibrator point. Therefore, LOQ was set to 5.48 pM and ULOQ to 5131 pM.

The ELISA was different; the lowest and highest control sample had %CV above 25%. The following values were determined as LOQ and ULOQ: 6.04 and 3310 pM as the accuracy and %CV was within acceptable limits: %RE 12.2 and 12.5 (two double determinations near LOQ were excluded as described in Table 2). Including the results from the calibrators, LOQ will be 10.4 pM and ULOQ 3310 pM as it was below the highest calibrator point. This range was wider than the published range of 11.5–800 pM [5].

Table 2
IAsp controls measured in ELISA and LOCI™. Results from seven ELISA plates and four LOCI™ plates. Each plate contained two double determinations.

Nominal concentration pM (N)	Mean pM (%CV)	Inter-assay %CV	Intra-assay %CV	%RE
ELISA				
5424 (14)	5373 (25.5)[a]	14.4	20.6	21.1
3423 (14)	3310 (17.9)	17.7	5.50	12.5
1100 (14)	1107 (8.08)	4.94	9.37	5.69
787 (14)	750 (3.80)	3.05	2.42	4.64
283 (14)	279 (7.75)	7.11	3.66	6.20
35 (14)	35.7 (4.78)	2.35	4.21	3.95
12 (12)	13.6 (17,3)[b]	15.3	9.27	15.3
6 (14)	6.04 (17.8)[c]	8.40	15.8	12.2
2 (12)	2.81 (32.2)[d]	15.2	35.9	44.6
LOCI™				
5424 (8)	5131 (7.77)	7.10	10.2	7.19
3423 (8)	3248 (5.33)	2.60	5.84	5.13
1100 (8)	1168 (3.60)	2.55	4.30	6.14
787 (8)	768 (4.27)	3.49	5.36	4.02
283 (8)	261 (4.15)	3.01	5.00	7.64
35 (8)	31.9 (3.49)	2.52	4.20	8.93
12 (8)	11.5 (3.42)	1.58	3.71	4.58
6 (8)	5.44 (10.9)	4.52	11.7	12.8
2 (8)	2.28 (5.39)	3.32	4.43	13.9

The validated range for ELISA was 13–800 pM [5].

[a] 14 double determinations, where results from two single values in two double determinations were out of analytical range.

[b] Two double determinations were excluded from ELISA run 7. One double determination was excluded due to a large %CV. Hereafter performing an outlier test (www.graphpad.com/quickcalcs/Grubbs1.cfm ($\alpha = 0.05$)), gave an outlier in the same ELISA run, which was excluded.

[c] 14 double determinations, where the result from one single value in one double determination was out of analytical range.

[d] 12 double determinations, where two double determinations were out of analytical range.

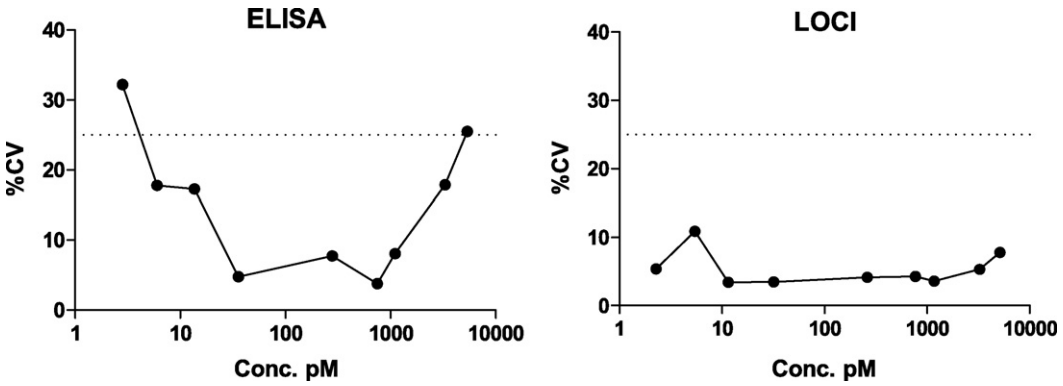

Fig. 1. Precision profile of spiked IAsp samples, ELISA and LOCI™. Two double determinations were excluded in the ELISA profile.

The results from the six individual serum samples are displayed in Table 3. All individual samples tested in LOCI™ had a %RE below 20. This was not the case for ELISA, where ID5 had the highest %RE of the six individuals. Three other individuals (ID1, ID2 and ID3) had one sample per individual with a %RE above 20. The non-spiked sample for ID2 was measured to 11.2 pM. This is below the published LOQ (ELISA) (11.5 pM) but not LOQ determined here (10.4 pM), although it was very close. This was a unique case as no response was seen in the pre-dose samples from the human pilot study data (see Section 3.5). Furthermore, response in pre-dose samples was not observed in the published ELISA study or in the clinical studies using the ELISA assay [5,8,9]. The high %RE was also not seen in the original paper addressing the IAsp ELISA [5].

3.3. Stability

Table 4a illustrates the results from the stability tested at RT. %CV was low both in ELISA and LOCI™ whereas %RE for ELISA generally was higher than the corresponding LOCI™ results. Seen over the 24-h period, %RE for all samples analysed in both ELISA and LOCI™ was below 20%.

In Table 4b, the results from the freeze/thaw stability are presented. Samples at two concentration levels were frozen and thawed 5 times in total. There was not a large difference in the results between the two assays. Both assays showed a %RE below 20.

3.4. Haemolysis

The amount of haemoglobin was increasing with the increasing amount of erythrocytes. The content of haemoglobin in the most haemolysed samples was 3.14 g/dL (approximately concentration level 250 pM) and 3.96 g/dL (approximately concentration level 30 pM) whereas the content was 0.99 g/dL (approximately concentration level 250 pM) and 0.83 g/dL (approximately concentration level 30 pM) in the samples with the lowest content of lysed erythrocytes. The reference samples had a haemoglobin content of 0.17 and 0.33 g/dL. Visually, the samples with most erythrocytes

Table 3
Spiked individual data of IAsp concentration.

	Nominal concentration (pM)	ELISA		LOCI™	
		Mean pM (%CV)	%RE	Mean pM (%CV)	%RE
ID1	859	904 (1.19)	5.22	969 (3.58)	12.8
	278	299 (2.63)	7.40	292 (2.91)	5.04
	29	37.1 (2.93)	27.8	30.0 (1.18)	3.28
	0	BLOQ	BLOQ	–	–
ID2	838	966 (2.55)	15.3	935 (0.76)	11.6
	273	321 (0.13)	17.7	287 (1.48)	5.13
	45	64.0 (0.81)	42.2	47.7 (4.45)	6.00
	0	11.2 (25.3)	(*11.2*)	–	–
ID3	841	1064 (5.65)	26.6	945 (1.27)	12.3
	277	313 (1.07)	12.9	298 (1.90)	7.58
	37	43.9 (5.12)	18.6	38.8 (0.18)	4.73
	0	BLOQ	BLOQ	–	–
ID4	802	807 (3.71)	0.59	895 (1.90)	11.6
	275	279 (4.81)	1.59	276 (3.34)	0.18
	47	46.2 (0.58)	1.63	39.5 (1.43)	16.0
	0	BLOQ	BLOQ	–	–
ID5	808	1120 (0.51)	38.6	915 (2.47)	13.2
	275	393 (0.60)	42.8	290 (1.95)	5.45
	42	72.6 (1.94)	72.8	44.3 (1.60)	5.48
	0	BLOQ	BLOQ	–	–
ID6	777	768 (2.32)	1.12	870 (2.20)	11.9
	277	291 (3.25)	5.14	291 (1.46)	5.05
	46	49.2 (1.40)	6.94	45.9 (0.15)	0.33
	0	BLOQ	BLOQ	–	–

BLOQ—below LOQ.
Two double determinations for each result.

Table 4a
Stability at RT of spiked IAsp concentration.

Hours at RT	Nominal concentration (pM)	ELISA		LOCI™	
		Mean pM (%CV)	%RE	Mean pM (%CV)	%RE
0 h	787	711 (2.69)	9.63	783 (2.90)	0.51
	35	32.0 (1.88)	8.66	31.8 (2.66)	9.07
1 h	787	692 (1.77)	12.1	772 (1.54)	1.87
	35	30.4 (3.79)	13.1	31.9 (4.31)	8.86
4 h	787	700 (3.40)	11.0	760 (1.40)	3.46
	35	30.7 (5.12)	12.3	30.8 (1.65)	12.1
24 h	787	678 (2.17)	13.8	746 (0.71)	5.21
	35	30.9 (6.17)	11.8	30.0 (2.74)	14.4

Four double determinations for each result.

Table 4b
Freeze/thaw stability of spiked IAsp samples.

Freeze/thaw	Nominal concentration (pM)	ELISA		LOCI™	
		Mean pM (%CV)	%RE	Mean pM (%CV)	%RE
1th	787	742 (1.32)	5.74	778 (0.09)	1.21
	35	33.2 (5.59)	5.14	32.4 (0.66)	7.57
2th	787	726 (5.40)	7.79	769 (1.29)	2.29
	35	37.1 (3.64)	6.12	33.2 (1.07)	5.29
3th	787	736 (0.11)	6.47	755 (3.09)	4.13
	35	37.5 (0.45)	7.14	32.6 (0.65)	7.00
4th	787	730 (3.74)	7.24	759 (0.37)	3.56
	35	35.8 (0.47)	2.36	33.2 (4.05)	5.29
5th	787	773 (0.05)	1.83	768 (1.29)	2.41
	35	35.0 (0.96)	0.03	34.3 (2.47)	2.00

Two double determinations for each result.

looked like whole blood, and the samples with the lowest content looked very haemolysed.

%RE was higher for the LOCI™ assay compared to the ELISA assay when lysed erythrocytes were added. For both analyses, %RE was highest for the samples, to which the largest amount of lysed erythrocytes was added. The haemolysis had an effect in LOCI™ whether the smaller amount or the larger amount of erythrocytes was added. In the ELISA assay, the haemolysis only had an effect on the most haemolysed samples. Data is presented in Table 5.

3.5. Human samples from a pilot study

The human serum controls (used for both ELISA and LOCI™) were on LOCI™ measured by use of the human serum calibration curve as well as the porcine plasma calibration curve in LOCI™. As it was done in order to compare the method by which the human samples were measured to the rest of the data presented in this manuscript.

Table 6 demonstrates the results of the same controls when a porcine plasma or human serum calibration curve was used. The results obtained with the porcine calibrator were comparable to the results obtained with a human serum calibrator. The human serum results are presented in Section 3.2. The porcine plasma results gave a %CV range from 3.14 to 10.3. The inter-assay range was from 1.31 to 5.83, and the intra-assay range was from 3.66 to 11.6, which was matching the LOCI™ (human serum). %RE had a range of 4.02–13.9 with the human serum calibrators, whereas the range was 3.64–17.4 with the porcine plasma calibrators. In all, LOCI™ results obtained with human serum calibrators were comparable to the results obtained with porcine plasma calibrators.

Serum samples from the human pilot study were analysed with porcine plasma reagents as standard procedure. To every assay plate human serum calibrators and controls were applied to ensure that similar results were obtained. The human serum calibrators followed the porcine plasma calibrator. The acceptance criteria for the controls were that 4 out of 6 double determinations were within ±15% of the nominal concentration (data not shown). The results of the 510 human samples (strongly haemolysed samples were excluded) analysed in both LOCI™ and ELISA are shown as an x–y plot, with the ELISA data on the y-axis and the LOCI™ data on the

Table 5
Haemolysis of spiked IAsp samples, by added erythrocytes.

Erythocytes	Nominal concentration (pM)	ELISA		LOCI™	
		Mean (%CV)	%RE	Mean (%CV)	%RE
Diluted 9 times	261	104 (8.49)	60.3	35.9 (6.51)	86.2
	31	13.2 (19.1)	57.6	2.95 (7.39)	90.5
Diluted 90 times	261	224 (1.16)	12.5	167 (1.87)	35.0
	31	32.6 (2.73)	5.23	21.5 (2.02)	30.6
None	261	228 (1.92)	11.1	230 (0.82)	10.1
	31	27.2 (4.41)	12.1	28.8 (1.64)	7.18

Four double determinations for each result.

Table 6
IAsp controls measured in LOCI™ with human serum calibrators and porcine plasma calibrators.

Mean pM (%CV)	Inter-assay %CV	Intra-assay %CV	%RE
LOCI™ (human serum)			
5131 (7.77)	7.10	10.2	7.19
3248 (5.33)	2.60	5.84	5.13
1168 (3.60)	2.55	4.30	6.14
768 (4.27)	3.49	5.36	4.02
261 (4.15)	3.01	5.00	7.64
31.9 (3.49)	2.52	4.20	8.93
11.5 (3.42)	1.58	3.71	4.58
5.44 (10.9)	4.52	11.7	12.8
2.28 (5.39)	3.32	4.43	13.9
LOCI™ (porcine plasma)			
4479 (6.67)	5.64	8.74	17.4
2973 (5.43)	1.63	5.63	13.2
1128 (3.73)	1.31	3.92	3.64
735 (4.21)	3.43	5.28	6.64
252 (4.09)	2.91	4.90	11.1
31.0 (3.40)	2.63	4.18	11.5
11.2 (3.14)	2.04	3.66	14.4
5.27 (10.3)	5.83	11.6	6.56
2.18 (5.38)	2.90	4.66	8.94

Nominal concentration given in Table 2.

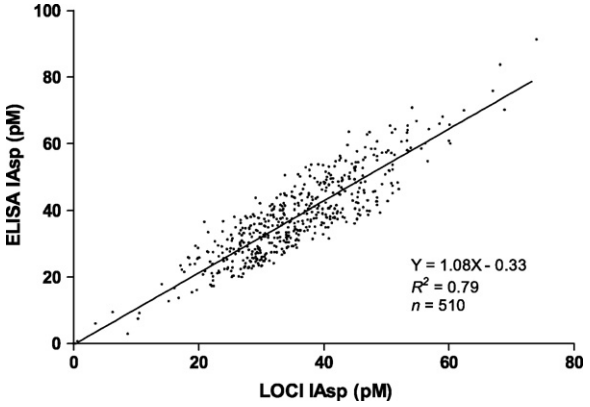

Fig. 2. *x*–*y* plot from human pilot study with healthy volunteers received IAsp, ELISA data on the *y*-axis and LOCI™ data on the *x*-axis.

x-axis (Fig. 2). Regression analysis was performed and gave the line $y = 1.08x - 0.33$ with $R^2 = 0.79$.

Table 7 shows regression analysis of the same human data from the four subjects (two study periods per subject) as individual data and also the pooled data. The individual R^2 value was better than the pooled data except for one individual in one of the study periods.

The human samples were also plotted in a Bland–Altman plot (Fig. 3). The bias was 2.45 and the 95% confidence interval was −9.33 to 14.2.

Table 7
Regression analysis, individual data from human pilot study with healthy volunteers.

	n	Slope	Intercept	R^2
1A	62	0.88	5.93	0.70
1B	61	1.26	−2.16	0.91
2A	64	1.08	4.58	0.82
2B	65	1.07	2.25	0.90
3A	65	0.96	−0.93	0.96
3B	65	0.92	−2.04	0.97
4A	64	0.93	2.12	0.93
4B	64	1.16	0.62	0.97
Pooled	510	1.08	−0.33	0.79

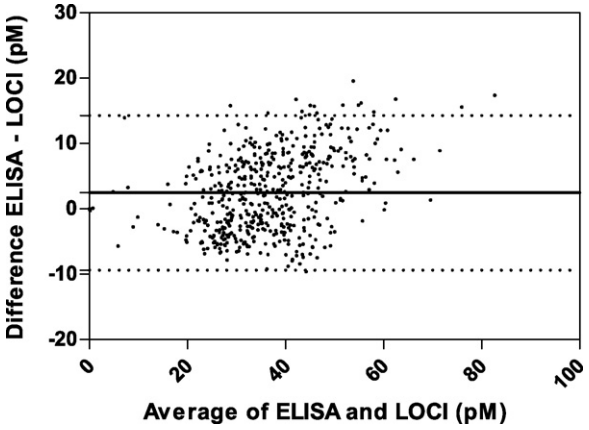

Fig. 3. Bland–Altman plot of ELISA and LOCI™ data for human pilot study with healthy volunteers received IAsp.

Like Table 7, Table 8 illustrates the individual data of a Bland–Altman plot. The individual data showed that two of the confidence intervals did not include zero. The remaining six individuals and the pooled data did.

The eight individual pre-dose samples were all analysed in both assays and no IAsp was measured. One pre-dose sample was excluded due to strong haemolysis.

4. Discussion

This study compared the published ELISA assay for detecting IAsp in human serum with a homogenous chemiluminescent sandwich immunoassay called LOCI™. The precision profile showed that LOCI™ had a wider assay range than the ELISA assay as well as better precision and accuracy, i.e. lower %CV and %RE. Even though LOCI™ demonstrated greater accuracy and precision over a broader range compared to ELISA in this study, it is worth noticing that it was compared for a broader range than the validated ELISA. Both assays proved to fulfil the precision and accuracy criteria, which are generally used as acceptance criteria for ligand binding assays used for clinical bioanalysis, i.e. 20% (25% at LOQ) for both %CV and %RE [13]. In this study, the LOQ (ELISA) based on results from the controls and calibrators was 10.4 pM, whereas the published value was 11.5 pM [5] correlating well with each other. However, LOQ (LOCI™) in the present study was determined to be lower than the LOQ (ELISA) (5.48 pM LOCI™). ULOQ was determined to be higher for LOCI™ than ELISA. The precision profile for LOCI™ did not cross the line at 25%CV at any time. In the ELISA, 25%CV crossed the line close to 5373 pM with an accuracy above 20%RE compared to 12.5%RE at 3310 pM. These results showed, that it may be possible to determine a higher ULOQ with more controls made in the high range for both assays. However, this might be at the expense of a higher %RE. The same can be argued for LOQ (ELISA and LOCI™).

Table 8
Bland–Altman plot, individual data.

	Bias	Confidence interval
Sub. 1A	1.74	−10.8; 14.3
Sub. 1B	7.20	−2.13; 16.5
Sub. 2A	7.78	−1.67; 17.2
Sub. 2B	4.42	−5.41; 14.2
Sub. 3A	−2.38	−6.31; 1.55
Sub. 3B	−4.89	−8.35; −1.43
Sub. 4A	−0.43	−4.85; 3.98
Sub. 4B	6.52	0.40; 12.6
Pooled	2.45	−9.33; 14.2

The six individual serum samples showed acceptable results when measured in LOCI™. %RE and %CV were below 20% for all the spiked samples. The same was not observed in ELISA. ID5 had a very high %RE for all the samples, and %RE for ID1 and 2 was high at the lowest concentration level and for ID3 %RE was high for the highest concentration level. The non-spiked sample from ID2 gave a signal very close to LOQ (10.4 pM) in this study. This was not seen in any of the pre-dose samples from the human pilot study or in the published ELISA study [5]. Furthermore, it has not described in previously published studies using the IAsp ELISA [5,8,9]. The same applies to the high %RE. There was no direct explanation of the cause with regard to the high %RE for ID5, solo samples from ID1, 2 and 3 or the non-spiked sample for ID2. Since this has not been described before, it must be a special case for the individual sera used and analysed in ELISA.

Both ELISA and LOCI™ showed acceptable stability. It was possible to freeze and thaw samples at least 5 times and leave the samples at RT for 24 h. Although %CV increased slightly with time for both assays, the %CV and %RE were never above 20%.

Although LOCI™ had a wider analytical range and better precision and accuracy than ELISA, the latter performed better with regard to haemolysis. The comparison showed, that haemolysis had a greater influence on the LOCI™ assay. The lysed erythrocytes released haemoglobin, which interfered with the light emission in the LOCI™ assay in such a way, that the signal was inhibited/quenched [10]. Furthermore, a lower signal in haemolysed samples containing insulin, regardless of the method of analysis, could be due to insulin degradation from the release of insulin degrading enzyme from lysed cells [14].

Since the LOCI™ assay is a homogenous assay, it is more sensitive to interfering components from for example serum than the heterogenic ELISA assay. The latter method includes several wash steps, washing the interfering components away. The haemolysed samples were all observed to be very haemolysed. It would be expected that less haemolysed samples would have a better accuracy (lower %RE), because the interference in LOCI™ decreased with decreasing haemolysis.

Human serum samples from a pilot study were analysed in the two assays. The LOCI™ calibrators and controls used to analyse these samples were different from the calibrators and controls used to analyse the spiked samples. The calibrators were spiked in porcine plasma since the LOCI™ assay used porcine calibrators as routine. When using human serum calibrators or porcine plasma calibrators the controls gave almost the same (described in Section 3.5). Thus, it made no difference that the pilot study samples were measured using a porcine plasma calibrator. The same matrix would be preferred and thereby also fulfil the regulatory guidelines.

The LOCI™ results from the human pilot study using porcine plasma calibrators were compared with ELISA in an *x*–*y* plot and in a Bland–Altman plot. The results showed good correlation between the two assays, indicating that both assays could be used. Individual data gave better correlation than the pooled data. The caused may be the use of calibrators made in porcine plasma—a better correlation could be expected if the same set of calibrators were used for both assays. Pooling data in itself will give a worse correlation compared to the individual data. The good correlation with a slope and an intercept very near 1 and 0 indicates, that there was a random pattern in the noise of the plot. In the Bland–Altman plot no pattern could also be seen. Individual data of the Bland–Altman plot showed that two of the confidence intervals did not include zero. As described above with the *x*–*y* plot, the same (concerning pooled and individual data) could be taken into consideration. It is therefore important not to switch between assays in studies. A switch could give skewed results and thereby affect the conclusion of a study. In all, the correlation between the two assays for determination of IAsp was good in the measured range of the human serum samples from the pilot study—up to approximately 80 pM. Above 80 pM, it could be speculated if the correlation would be as good. It would have to be tested with human samples from the clinic, although the spiked samples indicate a good correlation within the higher range.

5. Conclusion

In conclusion, LOCI™ could determine IAsp in human serum just as well as ELISA, and calibrators could be prepared in either human serum or porcine plasma for the LOCI™ assay. Using the LOCI™ technology could be an advantage, since it has a shorter analysis time and fewer analytical steps decreasing the workload. Therefore, a higher throughput can be achieved and as such LOCI™ could be preferred for large sample sizes such as in research and clinical trials.

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References

[1] A. Lindholm, L.V. Jacobsen, Clinical pharmacokinetics and pharmacodynamics of insulin aspart, Clin. Pharmacokinet. 40 (2001) 641–659.
[2] P.D. Home, A. Lindholm, B. Hylleberg, P. Round, Improved glycemic control with insulin aspart: a multicenter randomized double-blind crossover trial in type 1 diabetic patients. UK Insulin Aspart Study Group, Diabetes Care 21 (1998) 1904–1909.
[3] A.M. Rosenfalck, P. Thorsby, L. Kjems, K. Birkeland, A. Dejgaard, K.F. Hanssen, S. Madsbad, Improved postprandial glycaemic control with insulin Aspart in type 2 diabetic patients treated with insulin, Acta Diabetol. 37 (2000) 41–46.
[4] B.W. Bode, P. Strange, Efficacy, safety, and pump compatibility of insulin aspart used in continuous subcutaneous insulin infusion therapy in patients with type 1 diabetes, Diabetes Care 24 (2001) 69–72.
[5] L. Andersen, P.N. Jorgensen, L.B. Jensen, D. Walsh, A new insulin immunoassay specific for the rapid-acting insulin analog, insulin aspart, suitable for bioavailability, bioequivalence, and pharmacokinetic studies, Clin. Biochem. 33 (2000) 627–633.
[6] E.F. Ullman, H. Kirakossian, A.C. Switchenko, J. Ishkanian, M. Ericson, C.A. Wartchow, M. Pirio, J. Pease, B.R. Irvin, S. Singh, R. Singh, R. Patel, A. Dafforn, D. Davalian, C. Skold, N. Kurn, D.B. Wagner, Luminescent oxygen channeling assay (LOCI): sensitive, broadly applicable homogeneous immunoassay method, Clin. Chem. 42 (1996) 1518–1526.
[7] E.F. Ullman, H. Kirakossian, S. Singh, Z.P. Wu, B.R. Irvin, J.S. Pease, A.C. Switchenko, J.D. Irvine, A. Dafforn, Luminescent oxygen channeling immunoassay: measurement of particle binding kinetics by chemiluminescence, Proc. Natl. Acad. Sci. USA 91 (1994) 5426–5430.
[8] K. Hermansen, M. Colombo, H. Storgaard, A. Ostergaard, K. Kolendorf, S. Madsbad, Improved postprandial glycemic control with biphasic Insulin Aspart relative to biphasic Insulin Lispro and biphasic Human Insulin in patients with Type 2 diabetes, Diabetes Care 25 (2002) 883–888.
[9] P.T. McSorley, P.M. Bell, L.V. Jacobsen, A. Kristensen, A. Lindholm, Twice-daily biphasic insulin aspart 30 versus biphasic human insulin 30: a double-blind crossover study in adults with type 2 diabetes mellitus, Clin. Ther. 24 (2002) 530–539.
[10] F. Poulsen, K.B. Jensen, A luminescent oxygen channeling immunoassay for the determination of insulin in human plasma, J. Biomol. Screen. 12 (2007) 240–247.
[11] M. Berger, T.A. Gaither, R.M. Cole, T.M. Chused, C.H. Hammer, M.M. Frank, Biotinylation of human C3, Mol. Immunol. 19 (1982) 857–864.
[12] L. Andersen, B. Dinesen, P.N. Jorgensen, F. Poulsen, M.E. Roder, Enzyme immunoassay for intact human insulin in serum or plasma, Clin. Chem. 39 (1993) 578–582.
[13] B. DeSilva, W. Smith, R. Weiner, M. Kelley, J.M. Smolec, B. Lee, M. Khan, R. Tacey, H. Hill, A. Celniker, Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules, Pharm. Res. 20 (2003) 1885–1900.
[14] R. Sapin, J.C. Ongagna, F. Gasser, D. Grucker, Insulin measurements in haemolysed serum: influence of insulinase inhibitors, Clin. Chim. Acta 274 (1998) 111–117.