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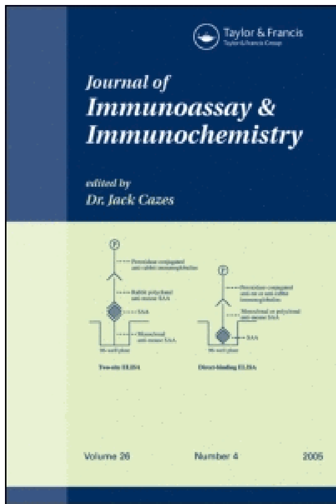

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VALIDITY AND USE OF A NON-PARALLEL INSULIN ASSAY FOR PHARMACOKINETIC STUDIES OF THE RAPID-ACTING INSULIN ANALOGUE, INSULIN ASPART

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ABSTRACT

A radioimmunoassay (RIA) for insulin was validated for reliable measurement of the human insulin analogue, insulin aspart, by correction of non-linear measurements. Specificity was equivalent for several species of insulin, except insulin aspart. A non-linear hyperbolic model fitted insulin aspart with a correction formula for non-linearity of: $z = 1503y/(1398 - y)$, where y denotes measured concentration and z denotes true concentration.

Matrix-effects were insignificant for human, porcine, and canine heparin-plasma and for human and porcine serum. The coefficient of variation was below 15% for 80-800 pmol/L human and porcine insulin and for 80-600 pmol/L insulin aspart. The limit of detection for insulin aspart was 11.5 pmol/L with a lower limit of quantification of 17.5 pmol/L. Dilution of serum with Pharmacia dilution media introduced no significant error. In conclusion, this paper demon-

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strates that a non-parallel radioimmunoassay can be used to estimate accurate concentrations of insulin aspart.

INTRODUCTION

The application of DNA technology allows for the production of insulin analogues in which one or more amino acids in the original sequence are replaced. One such analogue is insulin aspart, in which the proline at position 28 of the B chain is replaced by aspartic acid. This transition results in a protein molecule with a lower tendency to form hexamers.(1) The formation of hexamers is a problem with unmodified human insulin, as it delays absorption into the circulation after subcutaneous injection and it reduces the efficacy in maintaining glucose homeostasis.(2) Comparison of the pharmacokinetics of insulin aspart and unmodified human insulin in preclinical studies requires that the concentrations of both insulins can be measured separately and accurately.

Until recently, it was not possible to produce a monoclonal antibody that binds specifically to insulin aspart and not to unmodified insulin. Evaluation of several commercially available radioimmunoassays (RIA), which have been used to measure insulin aspart as well as unmodified insulin, showed that estimated insulin aspart concentrations were too low and disproportionate compared with the known concentrations of insulin aspart. Thus, quantification of insulin aspart could only be performed if a reliable correction formula was found, which converts the measured RIA result to a quantitative concentration.

The Pharmacia insulin RIA 100 kit was chosen as a reliable assay, which was not going to be modified for at least five years.(3) The RIA 100 kit uses an insulin-specific antibody produced in guinea pigs by immunization with porcine insulin. This assay will measure concentrations of human, dog, or porcine insulin, but insulin aspart concentrations are measured non-linearly, compared to known concentrations.

The correction formula for insulin aspart quantification requires that the endogenous insulin concentration be subtracted from the total insulin measured by the RIA 100. In clinical trials, it is possible to estimate endogenous human insulin, based on the measured C-peptide concentration.(4) This approach is precluded in many pre-clinical studies, due to the lack of specific assays for animal insulins or C-peptide. Using fasted animals with a low level of endogenous insulin reduces the error, as does the injection of comparatively large amounts of insulin aspart. Neither of these approaches is feasible for pharmacokinetic studies.

One solution for these experiments is to subtract the measured pre-dose endogenous insulin level (i.e., the total insulin present before dosing with

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insulin aspart) from the measured RIA results after dosing, before correction, for non-linear insulin aspart measurements. Alternatively, the measured total insulin concentration can be corrected without subtraction of endogenous insulin. Which approach introduces less bias can be validated using insulin aspart dosed pigs and an enzyme-linked immunosorbent assay (ELISA), which cross-reacts with pig insulin, but not with insulin aspart.(5)

The aim of this study was to prepare a formulation which can be used to accurately determine insulin aspart concentrations, based on measurements with the insulin RIA 100 kit. The specificity, linearity, precision, and sensitivity of the method were determined, as well as the effect of the matrix to which insulin aspart was added.

EXPERIMENTAL

Peptides

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Insulin aspart, human insulin, and porcine insulin were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). All peptides were mono-component with a purity of >98%. Both insulin aspart and human insulin peptides were recombinant, whereas the porcine insulin peptide was obtained from the pancreas.

Assay Procedure

The Pharmacia insulin RIA 100® kit (Pharmacia Diagnostics AB, Uppsala, Sweden) was used in according to the enclosed directions for use.(3) After counting the ^{125}I-labeled antibody-bound insulin, the data were automatically transferred to the MultiCalc® data-handling program (version 2.50, Wallac Oy, Turku, Finland), which calculated the calibration curve fitting (using a four-parameter logistic function), as well as the insulin concentration (pmol/L).

The DAKO insulin ELISA® kit (DAKO Diagnostics Ltd., Denmark House, Ely, Cambridgeshire, UK) was used according to the enclosed directions for use.(6) The spectrophotometric readings were automatically transferred to the MultiCalc® program as described above.

Serum and Plasma

Pools of serum and heparinized plasma from humans (five individuals), pigs (two animals), and dogs (five animals) were used for specificity

or matrix interference studies. All the pools were depleted of their natural insulin content with a murine monoclonal antibody (HUI-001 specific for human and animal insulin; Novo Nordisk) conjugated to Sepharose® (0.05mg MCA per mg Sepharose®).

Human serum samples from five individuals, with their natural insulin content, were used for analyses of dilution linearity.

Heparinized plasma samples were taken at various time intervals before and after insulin aspart injection to analyse the precision of the different methods used to approximate endogenous insulin concentrations. These samples were taken from six female pigs, which received an intravenous injection of insulin aspart (0.025 U/kg)

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Spiking of Samples

Spiking of serum and plasma with human insulin, insulin aspart, and porcine insulin was performed using insulin preparations with internationally standardized concentration units, defined as 1.00 IU/6 nmol/L.(7) All volumes used in the preparation of spiked samples were accurately weighed and the spiked concentrations were calculated using the formula: $\text{concentration} = [C_1 \times (W_1/d_1) + C_2 \times (W_2/d_2)]/[(W_1/d_1) + (W_2/d_2)]$; where C_1 is the standard insulin concentration with volume weight W_1 and density d_1, and C_2 is the concentration in the sample before spiking with volume weight W_2 and density d_2. Each spiked sample was divided into suitable portions and stored at $-18°C$ until assay.

Specificity and Matrix Interference

The insulin-depleted human serum pool was used for the specificity studies. Spiked samples were prepared with human or porcine insulin or with insulin aspart at low, medium, and high concentrations in the assay range (0–1440 pmol/L). Each spiked sample was divided into five separate identical samples and stored at $-18°C$. The insulin concentration at each concentration level was analysed four times in duplicate. This was repeated in five independent assays.

The insulin-depleted pools of serum and heparinized plasma (human, porcine, and canine) were used for matrix interference studies. Each matrix was spiked with a low, medium, or high concentration of insulin aspart.

Each spiked sample was divided into two parts and stored at $-18^{\circ}C$ until analysis. The samples were analysed in duplicate.

Dilution Linearity

Two sets of five individual human serum samples were spiked with human insulin and insulin aspart, respectively, with concentrations in the upper end of the assay range. On the day of analysis, the spiked samples were measured in duplicate, either undiluted or diluted (2-, 4-, 8-, and 16-fold) with Pharmacia dilution buffer.

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Imprecision and Sensitivity

Five sets of four concentrations each of human insulin and insulin aspart were analysed four times, in duplicate, to determine the assay imprecision. The total imprecision of the assay was derived as follows: $y_{ij} = \delta_j + \varepsilon_{ij}$, where y_{ij} is the 'i-th' measurement of the sample in the 'j-th assay'. The parameter δ_j is assumed to have a normal distribution with a mean μ (the true concentration) and a variance σ_1^2 (between-assay). ε_{ij} are independent, normal distributed variables with a mean 0 and a variance σ_0^2 (within-assay). The total assay variance for a given sample is $\sigma_t^2 = \sigma_0^2 + \sigma_1^2$, where σ_t is the standard deviation corresponding to the total imprecision, σ_0 corresponds to within-assay imprecision and σ_1 corresponds to between-assay imprecision. Estimates of imprecision were reported as standard deviations (SD) or as coefficients of variation (CV).

Assay Use for Samples Containing Endogenous Insulin Plus Insulin Aspart

Six female pigs received an intravenous injection of insulin aspart (0.025 U/kg). Blood samples were collected into heparin tubes at various time intervals after injection, and these samples were assayed for total insulin using the Pharmacia RIA 100 kit, and for endogenous porcine insulin using the DAKO insulin ELISA kit.

All statistical analyses were done using SAS® 6.12 statistical procedures.

RESULTS

Assay Validation

Specificity

Measured concentrations of human insulin, insulin aspart, and porcine insulin were compared with known added concentrations within the assay range (Table 1). The insulin-depleted samples were found to contain a residual concentration of insulin (about 10 pmol/L). Clearly, the SD increased with increasing concentration, while the CV showed a minimum at intermediate concentrations. The human insulin results were analysed according to the linear regression model and the insulin aspart results according to a non-linear regression model.

With only four concentration levels, modeling the variation as a function of the mean was unreliable, so a weighted regression approach was used in both cases. The inverse of the empirically determined variance within-concentration level was chosen as the weight factor. The true

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Table 1. Insulin Concentrations Measured in Spiked Serum Samples (N = 20)

Added Insulin (pmol/l)*	Measured Mean (pmol/l) ± SD	CV (%)
Human		
0	11.75 ± 4.14	35.2
~100	84.05 ± 4.80†	5.7
~500	374.20 ± 33.63	9.0
~1000	734.45 ± 53.30	7.3
Insulin aspart		
0	11.11 ± 3.67†	33.0
~100	77.60 ± 5.29	6.8
~500	279.45 ± 26.74	9.6
~1000	435.75 ± 49.35	11.3
Porcine insulin		
0	10.53 ± 4.16†	39.5
~100	85.70 ± 9.16	10.7
~500	404.95 ± 34.06†	8.4
~1000	845.75 ± 101.50	12.0

* The exact concentrations were found for each addition by accurately weighing the volumes of serum and added standard.
† N = 19.

Table 2. Non-linear Regression Analysis Between Measured and True Insulin Aspart Concentrations

Assay Number	N	a (pmol/l)	c (pmol/l)	d (pmol/l)
1	15	2.18 ± 1.23	1647 ± 388	1931 ± 510
	15	$\equiv 0$*	1388 ± 258	1556 ± 320
2	16	3.21 ± 1.05	1456 ± 182	1664 ± 270
	16	$\equiv 0$	1240 ± 136	1304 ± 183
3	16	5.81 ± 1.66	1392 ± 324	1460 ± 437
	16	$\equiv 0$	1021 ± 185	916 ± 226
4	16	-4.46 ± 1.04	1000 ± 65	955 ± 98
	16	$\equiv 0$	1166 ± 129	1241 ± 195
5	16	-1.81 ± 2.45	1066 ± 482	985 ± 646
	16	$\equiv 0$	1536 ± 520	1638 ± 669
All	79	1.47 ± 1.01	1490 ± 166	1645 ± 220
	79	$\equiv 0$	1398 ± 132	1503 ± 163

* Intercept fixed at zero.

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concentration of insulin in insulin-depleted samples was taken into account. When the linear model gave an acceptable fit, the specificity was expressed as 100b%, where b was the slope of the measured versus actual concentrations.

The slope (b) of the weighted linear regression line was 0.892 ± 0.012, which meant that all the known concentrations derived from standard solutions used by Novo Nordisk, which are based on the First International Standard for human insulin,(8) with 1.00 IU = 6.00 nmol,(7) could be converted to the WHO units used by Pharmacia(9,10) by multiplying by 0.892. The equation for the hyperbolic regression model is: $y = a + dz/(c + z)$, where 'a' is the residual insulin concentration, 'y' is the measured concentration and 'z' is the true concentration (in Pharmacia RIA units). Parameters 'a', 'c', and 'd' are given in Table 2. Small negative or positive values were obtained for 'a', which can be assumed to be equal to 0 in the mean. The parameters 'c' and 'd' varied between assays, but the mean estimates based on all results were taken as the best value, which provided a correction equation, $z = 1503y/(1398 - y)$, when $a = 0$. The concentration z and y had to be expressed in pmol/L.

The goodness of fit between the measured insulin aspart and the model based on the weighted non-linear regression analysis showed that the hyperbolic relationship was acceptable for use in the conversion of the measured concentrations into the true concentrations. Figure 1 shows the non-linear

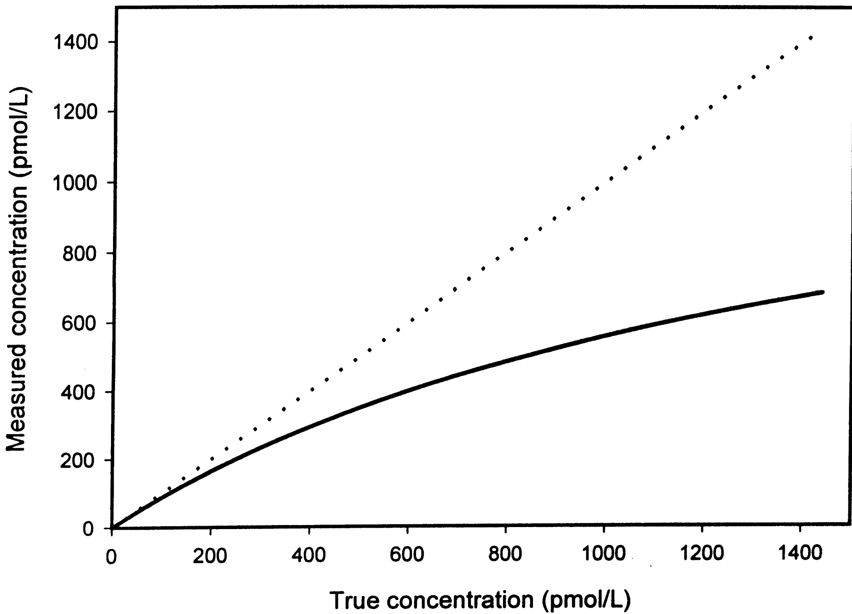

Figure 1. The non-linear relationship between the measured insulin aspart concentration and the true concentration. The dashed line illustrates an accurate and linear correlation.

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relationship between measured insulin aspart concentration and the true concentration.

Matrix Interference

A known amount of insulin aspart added to human, porcine, and canine serum and plasma was assessed with RIA. The measured concentration was compared with the expected concentration, which was calculated by using the insulin aspart hyperbolic correction model. The relative effects of the various matrices are summarized in Table 3. Human serum, human plasma, porcine serum, porcine plasma, and canine plasma all showed acceptable insulin recovery within the 85–115% acceptable range. Canine serum was the only matrix not suitable for insulin determination with the Pharmacia insulin RIA 100, as the mean recovery was 22% higher than the expected concentration.

Table 3. Summary of Matrix Interference

Matrix Species and Type	% Recovery* (±SE†)			
	Low Range	Medium Range	High Range	Mean
Human serum	104.0 (±4.9)	102.10 (±2.1)	90.30 (±4.0)	100.80 (±3.9)
Human plasma	106.00 (±9.4)	100.50 (±3.5)	104.20 (±4.0)	103.60 (±6.3)
Porcine serum	108.00 (±1.6)	92.80 (±5.8)	89.50 (±3.6)	96.80 (±4.1)
Porcine plasma	103.20 (±9.0)	105.50 (±2.8)	105.90 (±3.8)	104.90 (±5.9)
Canine serum	138.80 (±3.6)	117.20 (±5.2)	111.10 (±11.0)	122.40 (±7.4)
Canine plasma	107.10 (±4.2)	100.20 (±4.1)	91.40 (±7.6)	99.50 (±5.5)

* Measured concentration as a percentage of expected concentration.
† Standard error.

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Dilution Linearity

Dilution of human serum samples spiked with human insulin using the Pharmacia dilution media resulted in good linear relationships (correlation coefficients 0.990-0.999). The fact that the imprecision is inversely proportional to the measured concentration could bias the regression lines. Therefore, the measured concentration was multiplied by the dilution factor to obtain variance homogeneity. Table 4 shows the mean recovery, i.e., the mean measured concentration expressed as a percentage of the expected concentration. All mean percentage values of recovery fell within the 85-115% acceptable limits for dilutions up to 16-fold.

Table 4. Recovery* of Insulin by the RIA 100 Assay from Spiked Human Serum Diluted with Pharmacia Diluent (Mean ± SD)

Recovery (%) n = 5	Dilution				
	1:1	1:2	1:4	1:8	1:16
Human insulin	96.46 ± 4.06	104.42 ± 11.82	110.40 ± 6.50	98.92 ± 6.56	89.80 ± 3.14
Insulin aspart	93.98 ± 2.15	106.13 ± 11.95	108.48 ± 3.42	100.16 ± 11.10	96.72 ± 9.43

* True concentration (i.e. corrected measured concentration) as a percentage of expected concentration.

Spiked serum samples with insulin aspart were diluted in a similar way with non-spiked human serum. The insulin aspart fraction of the measured concentrations was found by subtracting the human insulin concentration present in the non-spiked serum from the measured RIA results. The measured insulin aspart fraction was corrected to obtain the true concentration by applying the non-linearity correction equation, $z = 1503y/(1398 - y)$, as described earlier. The true concentration of insulin aspart was multiplied by the dilution factor (to obtain variance homogeneity, as described above) and the percentage of the expected concentration recovered was calculated (Table 4). Again, the mean percentage recovery for all serum samples fell within the acceptable limits.

Imprecision and Sensitivity

The non-spiked samples were analysed separately. The analysis showed a mean concentration of 11.66 pmol/L, with within-assay SD 2.52 pmol/l, between-assay SD 2.41 pmol/L and total-assay SD 3.49 pmol/L (Table 5).

The limit of detection (LOD) was calculated as $3.3 \times SD_0$ where SD_0 is the SD in the region of the detection limit, which was found to be 3.49 pmol/L at 11.66 pmol/L. The LOD was 11.5 pmol/L, while the lower limit of

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Table 5. Imprecision of Measured Insulin Concentration in pmol/L (N = 20)

Insulin Type	Measured Mean	Within Assay SD	Between Assay SD	Total Assay SD
Human	11.7*	2.52	2.41	3.49
	84.1	4.80	0.00	4.80
	374	33.63	0.00	33.63
	734	53.30	0.00	53.30
Porcine	85.7	6.93	6.52	9.52
	405†	22.15	27.69	35.46
	846	101.50	0.00	101.50
Aspart	77.6	5.29	0.00	5.29
	279	26.74	0.00	26.74
	436	49.35	0.00	49.35

* N = 98.
† N = 19.

Table 6. Accuracy and Imprecision of True Insulin Concentrations in pmol/L (N = 20)

Insulin Type	Spiked Concentration	Mean Concentration	% Inaccuracy	Total CV %
Human	0	11.7*	—	30
	88.6	84.1	−5.1	5.4
	356	374	5.1	9.4
	708	734	3.6	7.5
Porcine	96.5	85.7	−11.2	9.9
	403	405†	0.5	8.8
	792	946	6.8	12.8
Aspart	89.6	88.4‡	−1.3	7.2
	358	376.6‡	5.2	12.3
	704	686.5‡	−2.5	17.6

* N = 98.
† N = 19.
‡ True concentration (i.e. corrected measured concentration).

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quantification (i.e., the lowest concentration that can be assessed with an imprecision of 20%, or $5 \times SD_0$) was 17.5 pmol/L.

Table 5 shows the calculation of the within-assay, between-assay, and total-assay imprecision in human serum with human insulin, porcine insulin, and insulin aspart at the different concentration levels. It appears that the imprecision was approximately proportional to the measured concentration, independent of the type of insulin in the serum. The between-assay variance component SD was not detectable (≤ 0) in 7 cases out of 10 measurements. This indicates that the between-assay error was insignificant, compared to the within-assay error.

Table 6 shows the mean %-inaccuracy (percentage difference between the measured mean value and the known concentration) and the total CV for human insulin and porcine insulin measurements, and also the mean %-accuracy and the total CV for the true insulin aspart concentration (i.e., corrected measured concentration) at low, medium, and high concentrations. All %-inaccuracies fell within the (15% acceptable limits. The total CV was less than the 15% acceptable limit, except for the non-spiked sample with human insulin (30% CV) and the highest insulin aspart concentration (17.6% CV). However, the 30% CV for the non-spiked sample with 11.7 pmol/L was below the lower limit of quantification, and the 17.6% CV for the highest insulin aspart concentration (686.5 pmol/L) was due to the

non-linear correction, which magnified the analytical error at high insulin aspart concentrations.

Assay Use

As outlined in the Introduction, several options exist regarding the method of correction for endogenous insulin. The aim was to find the approach that would lead to the smallest error. The DAKO insulin ELISA cross-reacts with pig insulin, 300% compared to human insulin,(5) but did not show any effect on the insulin measurements if the samples contain less than 10000 pmol/L insulin aspart (data not shown). Therefore, this assay was used to describe the true endogenous insulin concentration in samples from pigs dosed with insulin aspart.

Hence, the endogenous insulin concentration measured by RIA (e) could be determined by the formula: $e = p + d/3$, where d is insulin concentration measured by the DAKO insulin ELISA and p is additional immunoreactivity measured by Pharmacia insulin RIA but not by DAKO insulin ELISA. The additional immunoreactivity (p) may result from partly degraded insulin in the circulation,(11) which is measured by the one-sided RIA, but not by the two-sided ELISA.(5,12) Thus, with DAKO insulin ELISA, it was possible to estimate the true endogenous pig insulin (d/3) and calculate the true insulin aspart concentration.

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The results obtained with the different correction methods for endogenous insulin were compared with the true total concentration in samples containing both insulin aspart and porcine insulin. Heparinized plasma samples were taken from 5 individual pigs at several time points (-15 min, pre-dose, 5, 7, 9, 12, 15, 18, 21, 25, 30, 40, 50, 60, 70, 80, 90, and 120 min) in relation to the dosing time for insulin aspart, which was injected intravenously (150 pmol/kg). These samples were analysed with Pharmacia Insulin RIA (P) and DAKO Insulin ELISA (D). The measured range of P was 24-381pmol/L. The mean values of endogenous insulin prior to dosing (-15 min and pre-dose) measured with RIA were for the 5 individual pigs (P_p): 123, 46, 51.6, 72.5, and 43.1pmol/L, respectively. The total insulin, after non-linear correction, was found as follows:

1. Non-linear correction without considering endogenous insulin concentration (Method A) $T_A = 1503 \times P/(1398 - P)$
2. Non-linear correction considering the individual endogenous insulin prior dose (Method B) $T_B = P_p + [1503 \times (P - P_p)/(1398 + P_p - P)]$

3. True correction $T_T = E + [1503 \times (P - E)/(1398 + E - P)]$. E is the true endogenous insulin concentration determined according to $E = (P_p - D_p) + D$; where D_p is the mean concentration measured by ELISA prior to dosing for each individual pig.

The %-difference was calculated between methods A and B, relative to method T. As expected, the method A, which does not take the endogenous insulin into consideration, gave a high overestimation (10.2%; 95% confidence limits ±0.62%) of the total insulin concentration in the samples compared with method T. On the other hand, method B, which uses the individual mean of endogenous insulin concentration prior to dosing, gave a slight underestimation (−1.5%; 95% confidence limits ±0.40%) of the true total insulin concentration.

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DISCUSSION

It was not possible to find a commercial insulin assay which could be used to measure both human insulin and insulin aspart with equal accuracy. However, to maintain a rapid development of insulin aspart, it was important to use a commercially produced assay. That assay should remain unchanged for several years until an insulin aspart specific assay could be developed.

For this study, the Pharmacia Insulin RIA 100 kit was chosen. This assay will measure concentrations of human, dog, or porcine insulin,(3) but insulin aspart concentrations are measured non-linearly, compared to known concentrations. Most likely, this is due to the anti-insulin guinea pig antibody used in the assay, which contains clones of antibody with different affinities for the two insulin types. Affinity purification of the antibody used in the RIA kit was not feasible; nor was it practical to use specially prepared insulin aspart or human insulin calibrators dependent on the type of insulin to be analysed, as most experimental studies would be blinded. Therefore, a correction formula was required which would convert measured RIA data into true insulin aspart concentrations. Such a formula could be applied automatically after the data in the database were released. This formula had to be suitable for measuring insulin aspart in human and animal blood samples.

The samples from the experimental studies contained unknown concentrations of both insulin aspart and endogenous human or animal insulin. Therefore, it was necessary to estimate the insulin aspart fraction in the measured RIA data, and to convert this fraction into accurate insulin aspart concentrations.

The mathematical function describing this conversion was determined by repeated measurements of known insulin aspart concentrations within a relevant concentration range. Four levels of insulin aspart concentrations were chosen and found to be sufficient to establish a reliable correlation between measured and known concentration. A pool of sera from different individuals was used to minimize possible bias due to matrix differences.

The serum pool depleted of insulin still contained a residual amount of 11.66 pmol/L human insulin. This residual concentration was the mean of 98 measurements and had an insignificant error. The accuracy experiment for measuring human insulin showed linear recovery within the analytical assay range, but was 89.2% of the expected standard concentration. This apparent inaccuracy is probably due to the different strengths between the international standard used to calibrate the RIA kit (Research standard A (66/304) for human insulin from WHO,(9,10), which was established in 1974, and the international standard(8) used to calibrate the human insulin used for the spiking experiment, established in 1987. An inaccuracy around 11%, compared to the most recent human insulin standard, was found to be acceptable, as the performance of insulin aspart in the experimental trials was compared with human insulin using the same RIA kit.

The correlation between measured (y) and known concentrations (z) of insulin aspart could be described by a hyperbolic model according to $z = 1503y/(1398 - y)$. This model indicates that the error in the true concentration will increase with increasing measured concentration. Samples with high measured concentrations (above 647 pmol/L) will require dilution and re-analysis in order to keep the CV of the true concentration below the upper limit of quantification (20%).

Substances contained in the serum or plasma from different species might interfere with the performance of the assay. This problem would arise when the samples contain insulin dissolved in media of a different composition than the solvent used for the RIA-kit calibrators. Therefore, matrix interference was thoroughly investigated in this study, using insulin aspart-spiked samples of serum and heparin plasma from the relevant species (human, porcine, and canine). The measured concentrations were compared with the expected concentration using the hyperbolic conversion formula described above. The recovery values showed that the insulin aspart recovery for all tested matrices was within the acceptable range of $100 \pm 15\%$(13) for concentrations within the analytical range (75-896 pmol/L), with the exception of canine serum. Thus, the canine serum matrix should be avoided when measuring insulin aspart concentrations using the Pharmacia insulin RIA 100 kit. The matrix experiments confirmed that the non-linear correction formula was valid for insulin aspart, not only in human serum but also in porcine serum and in heparinized human, porcine, and canine plasma.

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Dilution linearity of the Pharmacia insulin RIA 100 was shown to be sufficient, as serum samples containing human insulin or insulin aspart are measured with acceptable accuracy for dilutions up to 16-fold using Pharmacia diluent.(13) This confirms the validity of the correction formula, as the insulin aspart-spiked samples used in this investigation were not the same as the samples used to find the hyperbolic correction formula.

The within-assay SD increased considerably with increasing concentrations of insulin aspart, and was mostly higher than the between-assay error. The total SD in samples with low concentration of human insulin (mean 11.66 pmol/L) was 3.49 pmol/L, indicating that the limit of detection is 11.5 pmol/L (3.3×3.49 pmol/L), assuming a constant SD in the low assay range around 0 pmol/L, and a lower limit of quantification of 17.5 pmol/L (5×3.49 pmol/L). The total CV% was below 15% for human insulin, porcine insulin, and insulin aspart in the analysed concentration range, which is acceptable for immunoassays.(13) The only exceptions were the concentrations outside the limits of quantification, which were the lowest human insulin concentration and the highest insulin aspart concentration in this study.

As porcine insulin has the same amino-acid composition as canine insulin, and no significant differences in the relevant matrices, it can be expected that the total CV% for canine insulin will also be below the acceptable limit of 15% for a similar concentration range. All three insulin types were measured linearly, compared to the insulin calibrator in the kit, with only small acceptable inaccuracies in the range of 80-800 pmol/L.

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In human blood, the steady state ratio between human insulin and human C-peptide is assumed to be constant for a non-diabetic person. Hence, the endogenous human insulin concentration can be described as $\text{insulin}_{\text{endogenous}} = \text{C-peptide}_{\text{measured}} \times \text{insulin}_{\text{predose}}/\text{C-peptide}_{\text{predose}}$. The insulin aspart fraction (IAsp_{f}) of the measured concentration to be described as $\text{IAsp}_{\text{f}} = \text{insulin}_{\text{measured}} - \text{insulin}_{\text{endogenous}}$. Finally, the corrected total insulin concentration can be calculated using the formula $\text{insulin}_{\text{total}} = \text{insulin}_{\text{endogenous}} + (1503 \times \text{IAsp}_{\text{f}})/(1398 - \text{IAsp}_{\text{f}})$. For measurements in Type I diabetes patients with insignificant concentrations of C-peptide, care must be taken to avoid the presence of protracted insulin in the circulation, as this will violate the insulin aspart quantification.

If there is no available assay to measure endogenous insulin in preclinical studies, it is not possible to quantify the concentration of insulin aspart directly. Instead, certain approximations of endogenous insulin concentrations can be made to calculate the total insulin concentration. This approach is feasible in studies in which pharmacokinetic parameters of insulin aspart are compared with other insulin types. If the correction formula is applied without subtracting endogenous insulin, the degree of

overestimation is greater, compared with the method in which the pre-dose endogenous insulin concentration is subtracted from the total before the correction is applied. The method in which the total mean of the pre-dose endogenous insulin concentration is subtracted before applying the correction formula would appear to be more suitable in animals with small volumes of blood (e.g., mice and rats), as several animals will be used to construct a single pharmacokinetic profile of the dosed insulin. For all three approximations, it should be noted that samples containing less insulin than the mean pre-dose endogenous insulin concentration should not be corrected and that the animals must not be given food during the test period.

The validity of the correction method for insulin aspart measurements in human serum using the Pharmacia insulin RIA 100 was also compared with a newly developed ELISA specific for insulin aspart.(14) The comparison showed a mean difference of 3.4% (95% confidence interval: 1.4-5.4%), which indicates an insignificant bias. This result also confirms that the correction method outlined in this paper is valid and that the procedures chosen to find this formula were sufficient.

In conclusion, non-linear assays can be used to estimate accurate concentrations if linear assays are not available. In this paper, this was shown for insulin aspart, which could be accurately measured with the non-linear Pharmacia insulin RIA 100. However, precautions must be taken to carefully estimate a correction formula using spiked samples, without cross-reacting components. When using the assay, care must be taken to adjust for concentrations of cross-reacting substances.

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