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Total error profiling of a proinsulin time-resolved fluorescence immunoassay☆

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ABSTRACT

We applied total error profiling to evaluate the conversion of a known proinsulin (PI) enzyme-linked immunosorbent assay (ELISA) into a time-resolved fluorescence immunoassay (TRFIA). The formula and acceptance criteria proposed by the Ligand Binding Assay Bioanalytical Focus Group (LBABFG) of the American Association of Pharmaceutical Scientists (AAPS) were applied. We found that the expected dynamic range enlargement with TRFIA compared to ELISA ([0.5–240] versus resp. [0.7–98] pmol/L) is limited by an interference of C-peptide when present in the sample at high concentrations (>7000 pmol/L).

1. Introduction

The standard curve of most enzyme-linked immunosorbent assays (ELISAs) is sigmoidal, and the dynamic range therefore typically comprises only two orders of magnitude. Conversion to TRFIA may lead to a broader dynamic range and better signal to noise ratios [1]. When the frequently applied biotin-streptavidin system is used to amplify the ELISA detection signal, conversion into TRFIA can easily be accomplished by replacing the enzyme-conjugated streptavidin with commercially available Europium labeled streptavidin [2]. This way, the assay conditions including the utilized capture and detecting monoclonal antibodies remain the same, and only the detection system is changed. We applied this adaptation to the proinsulin ELISA method of Kjems et al. [3] in the perspective of broadening the dynamic range of the immunoassay. We therefore set out to look for tools to document and validate this broadening, and decided to apply the recommendations of the Food and Drug Administration (FDA) and the American Association of Pharmaceutical Scientists (AAPS) [4,5] to use the concept of total error (including variability and bias) profiling [6]. We applied the actually prevailing FDA/AAPS formula to investigate if a diminished lower limit of quantitation (LLOQ) and a higher upper limit of quantitation (ULOQ) in proinsulin measurement can be obtained with TRFIA compared to ELISA. We further aimed to evaluate the practical pros and cons of this formula and the related validation criteria. For this purpose, we compared this new experience with previous work [7,8], where an earlier formulation of the total error profiling and criteria for immunoassays [8,9] was used.

2. Experimental

2.1. Sample preparation

Pancreatic hormone-free plasma (PHFP) was prepared from whole blood of three insulin autoantibody (IAA) negative type 1 diabetic patients (20 mL each) and collected in S5-Monovettes® from Sarstedt (Darmstadt, Germany) supplemented with Trasylol from Bayer (Brussels, Belgium) containing 600 kallikrein inactivator units/mL aprotinin and 1.6 mg/mL di-potassium ethylenediaminetetraacetic acid (K_2-EDTA). After centrifugation at 1600 × *g* during 15 min, the obtained plasma was pooled, mixed and subdivided in 300 μL aliquots, which were stored in safe-lock Eppendorf tubes at −80 °C until analyzed. To obtain plasma samples with known pancreatic hormone levels, PHFP was spiked with recombinant human proinsulin (rhPI) [10], calibrated towards the World Health Organization (WHO) International Reference Reagent (IRR) 84/611 from the National Institute for Biological Standards and Control (NIBSC) (South Mimms, UK), in a range of 0.3–910 pmol/L proinsulin, without or with synthetic C-peptide from Bachem (Weil am Rhein, Germany), calibrated against the WHO IRR 84/510 for

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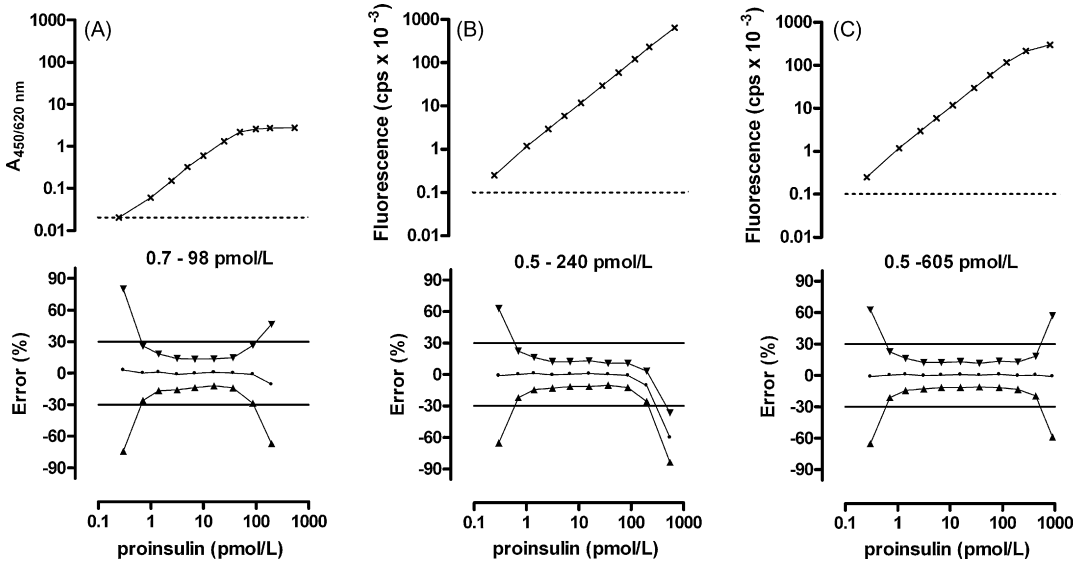

Fig. 1. Standard curves and accuracy profiles for proinsulin measurement with (A) ELISA, (B) TRFIA with proinsulin calibrator only in the standard solutions and (C) TRFIA with PI and Cp calibrators in the standard solutions (overall PI/C ratio = 2.44%). The accuracy profiles show the mean bias (●) and its 90% expectation tolerance limits (▼ and ▲) plotted versus the nominal proinsulin concentration.

human C-peptide. In the latter case, a constant proinsulin over C-peptide ratio (PI/C ratio = 100 × [PI]/[C-peptide]) of 2.44% was achieved at all levels, corresponding to the median value previously observed in healthy subjects [11].

2.2. Analysis

Proinsulin standard solutions were prepared from IRR 84/611 calibrated rhPI [10] in "Diluent II" from PerkinElmer (Zaventem, Belgium), in a range of 1.0–650 pmol/L proinsulin (n = 10 standard points). In one experiment the synthetic C-peptide was also added to the standard solutions to obtain a PI/C ratio of 2.44% at all points of the standard curve. For proinsulin ELISA, the two-step protocol described by Kjems et al. [3] was followed, but 4-parameter logistic regression was performed instead of reading unknown sample concentrations on the standard curve. The proinsulin TRFIA was based on this ELISA, but low fluorescence background Nunc Yellow strip microtiter plates from PerkinElmer were used for coating the capture antibody PEP-001 from DAKO Cytomation (Glostrup, Denmark). First incubation buffer was 40 mmol/L Na-phosphate, 30 g/L purified bovine serum albumin (BSA), "Probumin" from Millipore (Brussels, Belgium), 1 g/L bovine gamma globulin (BGG) from Sigma–Aldrich (Bornem, Belgium), 60 g/L NaCl, 0.5 g/L NaN_3, 5% (v/v) fetal bovine serum from Invitrogen (Paisley, UK) and 1 ml/L Tween 20 from Merck (Darmstadt, Germany), pH 7.4 at 20 °C. One hundred μL of this buffer and 25 μL sample or standard were incubated overnight at 20 °C (the plate was covered with a plastic seal) under continuous horizontal shaking with a PerkinElmer DELFIA 1296 plateshaker at 50% of maximum speed. After four washings on a PerkinElmer DELFIA 1296 Platewasher provided with washing solution (a 1:25 dilution in H_2O of "Wash concentrate", PerkinElmer), each well was filled with 125 μL of second incubation solution consisting of assay buffer (50 mmol/L Tris–HCl, 9 g/L NaCl, 0.5 g/L NaN_3, 0.5 g/L BGG, 5 g/L purified BSA, 7.44 mg/L Na_2EDTA and 0.1 mL/L Tween 20, pH 7.75 at 20 °C), and to which 100 μg/L of biotinylated detection mAb HUI-001 from NOVO Nordisk (Bagsværd, Norway) was added. The microtiter plate was shaken overnight at 20 °C, and after washing, 125 μL per well of a 120 mg/mL solution of Europium labeled streptavidin (PerkinElmer) in incubation buffer was added and incubated by shaking during 60 min at 20 °C. After a third washing, "Enhancement solution" (140 μL/well) from PerkinElmer was added followed by 5 min of shaking. The Europium time-resolved fluorescence was measured on a Victor^2 apparatus (PerkinElmer). Regression analysis and calculation of results for PI was performed with the smoothed spline algorithm of the Multicalc© 120 Multicalc V 2.6 software, as recommended earlier [8]. Because of the high cross-reactivity with conversion intermediates [74% for split(32–33)PI, 65% for des(31,32)PI, 78% for split(65–66)PI and 99% for des(64,65)PI] [3], the TRFIA was considered to measure total immunoreactive proinsulin material.

2.3. Total error profiling

The experimental setup is based on the recommendations of the AAPS for the development and validation of ligand-binding assays [5], in which formulae and criteria are summarized. ELISA and TRFIAs were performed on proinsulin and C-peptide spiked PHFP (n = 10 concentration levels ranging between 0.3 and 910 pmol/L proinsulin with a constant PI/C ratio of 2.44%). Ten assay runs (i.e. microtiter plates) were performed on separate days with three duplicates per microtiter plate for each concentration level. The two-sided 90% expectation tolerance interval for relative error was calculated according to the recommended formula [6], considering the Sattertwaite's approximation of degrees of freedom [12] and using Grubbs' outlier rule for replicate acceptance [13].

3. Results

Standard curves and related total error profiling graphs are shown in Fig. 1. Standard solutions containing calibrated rhPI without C-peptide were used for the proinsulin ELISA (Fig. 1A), and for the proinsulin TRFIA (Fig. 1B), whereas in Fig. 1C, C-peptide with a PI/C ratio = 2.44% was added to the proinsulin calibrators. In order to mimic the naturally occurring situation in samples, PHFP was spiked with both proinsulin and C-peptide (PI/C ratio = 2.44%) at different concentrations (n = 10) and each concentration was repeatedly measured (see Section 2.3) for total error profiling of the three assay formats (lower graphs in Fig. 1).

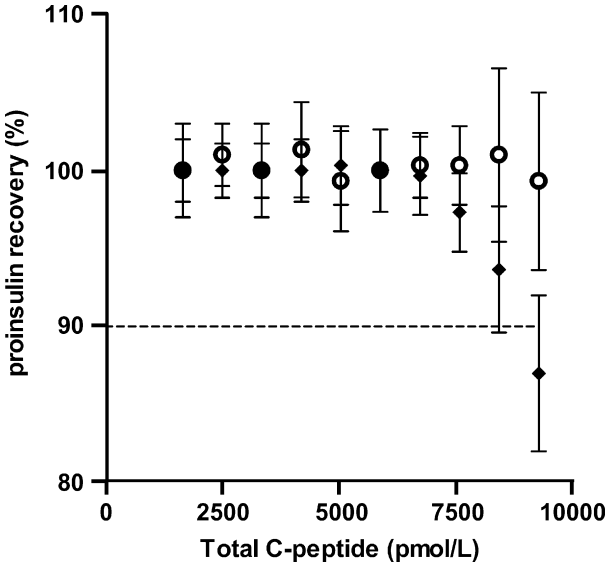

Fig. 2. Proinsulin recovery with TRFIA as a function of C-peptide. A hormone-free plasma was spiked with different concentrations of proinsulin and C-peptide, but with a fixed PI/C ratio = 2.44%. TRFIA was performed on these samples with proinsulin only (♦) or with proinsulin and C-peptide (○, PI/C = 2.44%) in the calibrators. Averages of recoveries in three independent experiments are shown with error bars representing ± S.D. (%).

The obtained sigmoidal proinsulin ELISA standard curve results in an analytical range of 0.7–98 pmol/L, essentially limited by the *variability* outside of this range (Fig. 1A). The TRFIA standard curve does not flatten at higher concentrations, but the consequently expected range broadening was limited to 0.5–240 pmol/L. A negative *bias* was observed at concentrations ≥ 200 pmol/L proinsulin (Fig. 1B). This bias could be tempered by adding C-peptide also to the proinsulin calibrator solutions, but with a consequent flattening of the standard curve (Fig. 1C). A range of 0.5–605 pmol/L was obtained, and the *variability* turned again to be the essential limiting factor at the ULOQ.

Fig. 2 shows the proinsulin recovery as a function of C-peptide present in the samples (PHFP with a PI/C ratio of 2.44%), using calibrators with or without C-peptide. In the latter case, the recovery is lowered to 90% at 8600 pmol/L C-peptide (209.8 pmol/L proinsulin) and the variability becomes more important (S.D. > 5%). We also tested the recovery of 10 times less proinsulin (20.98 pmol/L) at this C-peptide level (8600 pmol/L), and this resulted in 96% recovery with an S.D. = 7.2% (not shown). The addition of C-peptide to the calibrators results in better proinsulin recoveries at high C-peptide sample concentrations, but imprecision remains high (S.D. > 5%).

4. Discussion

This study aimed to apply total error (including bias and variability) profiling according to the most recent guidelines [5] to evaluate the conversion of a proinsulin ELISA into a proinsulin TRFIA. Development and evaluation of other validation parameters of the latter assay were previously published [8].

Specifically for ligand-binding assays, recommendations for method validation were published by the American Association of Pharmaceutical Scientists (AAPS) [5], including formulae and criteria for total error profiling. This approach is based on the β expectation tolerance interval theory [6], and was proposed earlier by Findlay et al. for pre-study validation of immunoassays [9]. They suggested that the two-sided 90% confidence limits of the total error should not exceed 25% (greater limits were deemed permissible if a scientific rationale exists). The actually prevailing formula only differs from Findlay's one in the sense that sample means can be weighed, and the related acceptance criteria for total error are suggested to be less severe: the 90% confidence limits should not exceed 30% (40% at the limits of quantification) [5].

Now that a consensus is achieved about the formula and the criteria, there is a need for field tests. Earlier publications by our group [7,8] were based on Findlay's formulation and criteria [8,9]. We experienced that in order to obtain a greater control of risk, a rather cumbersome formulation including Sattertwaite's approximation of the degrees of freedom [12] had to be applied. On the other hand, total error profiling could reveal bias and imprecision problems independently over the whole studied concentration range, and the latter feature led us to consider this approach as the best choice for evaluating the conversion of an ELISA into a TRFIA. Indeed, the core aim of such a conversion is to broaden the analytical range while keeping at least an equal total error at all points. We decided to use the FDA/AAPS formula (5, 6), and to stay in line with the 4-6-30 rule applied in our laboratory for acceptance of in-study runs, so we applied the acceptance criterion of $|\text{total error}| \leq 30\%$ for all concentrations.

The obtained data show that conversion of the proinsulin ELISA to TRFIA enlarges the dynamic range only to a certain extent because a bias is observed which results in a relatively small enhancement of the ULOQ, whereas a gain in analytical range in order of a magnitude was expected. This observation can be explained by saturation of the capture antibody PEP-001. Indeed, PEP-001 is raised against a C-peptide epitope of proinsulin [3], and the binding of proinsulin molecules might be partially obstructed in the presence of high C-peptide levels. This hypothesis is in line with the 90% recovery of 209.8 pmol/L proinsulin and 96% recovery of 20.98 pmol/L proinsulin at C-peptide concentrations of 8600 pmol/L. Adding C-peptide to the proinsulin calibrators eliminates the observed bias, but this addition leads to more variability at high PI concentrations due to the flattening of the standard curve. This addition also has only a limited practical use, because real plasma samples vary in PI/C ratio [11], whereas this experiment considers a fixed PI/C ratio. Based on these observations we would suggest expressing the ULOQ of the proinsulin TRFIA in terms of C-peptide instead of proinsulin. The binding capacity of the capture antibody is the limiting factor, and variation in microtiter plate coating efficiency should therefore be taken into account.

Albeit relatively small, a range broadening was obtained with ELISA to TRFIA conversion, and this can be considered as sufficient because less than 0.05% of all C-peptide analysis performed in our routine laboratory during the year 2007 (n = 12,420) resulted in concentrations higher than 7000 pmol/L. Furthermore, in case of doubt, a repeat on a diluted sample can always reveal the correct proinsulin content of a sample.

We experienced that total error profiling is a powerful tool for pre-study validation of immunoassays. On behalf of more flexibility in the experimental settings, (mean weighing is possible in case of outliers), the actual formula is rather complex. The development of corresponding software including calculation facilities would certainly enhance its user-friendliness and widespread availability.

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References

[1] I. Hemmilä, Clin. Chem. 31 (1985) 359.
[2] P. Dahlen, Anal. Biochem. 164 (1987) 78.
[3] L.L. Kjems, M.E. Røder, B. Dinesen, S.G. Hartling, P.N. Jørgensen, C. Binder, Clin. Chem. 39 (1993) 2146.
[4] Guidance for Industry: Bioanalytical Method validation of the Food and Drug Administration, Rockville, 2001. http://www.fda.gov/cder/guidance/index.htm.
[5] B. DeSilva, W. Smith, R. Weiner, M. Kelley, J. Smolec, B. Lee, M. Khan, R. Tacey, H. Hill, A. Celniker, Pharm. Res. 20 (2003) 1885.
[6] R.W. Mee, Technometrics 26 (1984) 251.
[7] M. Rui, C.S. Hampe, C. Wang, Z. Ling, F.K. Gorus, A. Lernmark, D.G. Pipeleers, P.E. De Pauw, J. Immunol. Methods 319 (2006) 133.
[8] P.E. De Pauw, I. Vermeulen, O.C. Ubani, I. Truyen, E.M. Vekens, F.T. van Genderen, J.W. De Grijse, D.G. Pipeleers, C. Van Schravendijk, F.K. Gorus, Clin. Chem., 2008 (Epub ahead of print as doi:10.1373/clinchem.2008.109710).
[9] J.W. Findlay, W.C. Smith, J.W. Lee, G.D. Nordblom, I. Das, B.S. DeSilva, M.N. Khan, R.R. Bowsher, J. Pharm. Biomed. Anal. 21 (2000) 1249.
[10] R.B. Mackin, M.H. Choquette, Protein Express. Purif. 27 (2003) 210.
[11] I. Truyen, P. De Pauw, P.N. Jorgensen, C. Van Schravendijk, O. Ubani, K. Decochez, E. Vandemeulebroucke, I. Weets, R. Mao, D.G. Pipeleers, F.K. Gorus, Diabetologia 48 (2005) 2322.
[12] F.E. Satterthwaite, Biometrics Bull. 2 (1946) 110.
[13] F. Grubbs, Technometrics 11 (1969) 1.