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Development of an improved time-resolved fluoroimmunoassay for simultaneous quantification of C-peptide and insulin in human serum ☆

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ABSTRACT

Objectives: Diabetes mellitus is a chronic disease affecting millions of people globally and resulting in significant death rates each year. A fast, inexpensive alternative to traditional testing and monitoring techniques is desirable, since secretion of insulin and C-peptide is impaired in diabetes mellitus.

Design and methods: A highly sensitive immunoassay was developed for the simultaneous measurement of C-peptide and insulin levels in human serum, utilizing dual-label time-resolved fluoroimmunoassay (TRFIA) and magnetic particle technologies. This assay was characteristic for a single-step sandwich-type immunoassay, wherein antibody-coated magnetic particles were used as the solid phase and Eu^{3+} and Sm^{3+} chelate labels were used for detection.

Results: Antibody-coated magnetic particles in a TRFIA format performed well in addressing a number of quantitative needs.

Conclusions: The results of this assay correlated well with commercial chemiluminescence assays and provided a number a advantages, including reduced sample volume, reduced reagent and personnel costs and reduced assay time, while maintaining the required clinical sensitivity.

Introduction

Diabetes mellitus is a chronic disease affecting about 347 million people in the world [1]. It is of greatest burden in low- and middle-income countries, accounting for approximately 80% of diabetes-related deaths [2,3]. In healthy lean individuals circulating fasting insulin and C-peptide concentrations are considered to be about 3 to 15 mIU/L and 260 pmol/L and 1030 pmol/L, respectively [4–7]. People who have diabetes mellitus may stay out of these ranges. Insulin and C-peptide are closely associated with diabetes and are secreted in equimolar amounts into the bloodstream. Insulin is known to be a significant marker of diabetes risk in the diagnosis and monitoring of diabetes [8–10]. Conventional insulin assays are particularly problematic when monitoring diabetes in patients undergoing insulin therapy, as they fail to discriminate between endogenous insulin secreted by β-cells and exogenous insulin injected [11]. As endogenous insulin production correlates well with serum C-peptide levels, quantization of the latter is a useful alternative to traditional insulin assays. Additionally, C-peptide levels are independent of exogenous insulin administration and not affected by insulin autoantibodies induced by insulin therapy [12]. Because C-peptides do not undergo significant hepatic metabolism, their circulating half-life is between two and five times longer than that of insulin [13]. Furthermore, a low C-peptide concentration is expected if insulin secretion is diminished as in insulin-dependent diabetes. Similarly, an increase in β-cell activity as observed for hyperinsulinism or insulinoma results in a corresponding increase in C-peptide concentration [14–17]. C-peptide levels more accurately reflect pancreatic insulin secretion rates and are helpful for diabetes classification and choice of treatment [12]. C-peptide is a stable indicator of insulin secretion while the insulin levels change rapidly [16]. Hence, the combined determination of blood insulin and C-peptide levels is invaluable for differentiating between Type 1 and Type 2 diabetes, selecting the best treatment in patients with Type 2 diabetes and identifying when it is appropriate to discontinue insulin therapy, especially in obese subjects.

To date, multi-immunolabeling techniques hold great promise for use in immunoassays, in part because of the lower volume of analyte required, shorter analysis time, less labor cost, and higher sample throughput [18]. At present, only a few efficient dual-label technologies used in TRFIA have been reported in the literature [19]. In the current study, we developed a double-antibody sandwich assay for the simultaneous detection of C-peptide and insulin in serum using dual-label time-resolved fluoroimmunoassay (TRFIA) to overcome the limit of detection and decrease the sample volume assayed.

For decades now, much attention has been focused on TRFIA using lanthanide chelate labels. The chelates act as a fluorophore, replacing the need for enzymes, isotopes, or chemiluminescent substances that have typically played an important role in labeling biological materials

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[20–23]. The main fluorescent lanthanides (Eu^{3+}, Tb^{3+}, Sm^{3+}, and Dy^{3+}) are ideal candidates for double-label or even multiple-parameter assays because of their characteristic narrow band emission and large Stokes' shift [24,25]. The long emission lifetimes are also efficient in reducing background fluorescence and increasing assay sensitivity [26–28]. The Eu^{3+} and Sm^{3+} configuration was chosen for the current study.

Our previous work showed that multiple-label immunoassays were feasible; however, the sensitivity, linear range, minimum detection limits and assay time might have room for improvement [29]. A major feature of our current research was the use of magnetic particles instead of the traditional 96-well plate as the solid-phase carrier. Greater surface area increased the potential to capture a greater number of target agents and the antibody coated micro-particle suspension greatly reduced the diffusion distances. During separation, the antibody-coated magnetic particles were attracted to the bottom of the well by magnets. Any unwanted materials were simply removed from the wells by decanting and the remaining magnetic particles resuspended in the liquid phase for further analysis [30–32]. Combined with the advantages of the magnetic particles and the time-resolved fluorescence of the lanthanide chelates, the main advantage of this method is the potential for ultrasensitive measurements within a very short reaction time. It is suitable for application in dual-label or multi-analyte immunoassays [33].

Materials and methods

Reagents and buffers

Reagents were of analytical grade, and ultrapure MilliQ water (Millipore) was used throughout. All solutions were equilibrated to 25 °C prior to use. Anti-C-peptide MAb (9013, E54094M) and anti-insulin MAb (E86306M, E86802M) were purchased from Meridian Life Science (Memphis, USA). Recombinant human C-peptide and insulin were obtained from Sigma. Magnetic particles (1101GA-03) were obtained from JSR Life Sciences (Tokyo, Japan). The enhancement solution was from PerkinElmer (Turku, Finland). Bovine serum albumin (BSA), 4-morpholineethanesulfonic acid (MES), N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Tween-20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). DTTA-Eu/Sm (N^1-[p-isothiocyannatobenzyl]-diethylene-triamine-N^1,N^2,N^3,N^4-tetraacetate-Eu^{3+}/Sm^{3+}) was obtained from PerkinElmer Life (Waltham, USA). Eu^{3+} and Sm^{3+} labeling kits, and commercially available Chemiluminescence immunoassay kits for C-peptide and insulin, were obtained from PerkinElmer Wallac (Turku, Finland).

Buffer solutions were as follows; coating buffer (0.1 mol/L MES, pH 5.0), labeling buffer (50 mmol/L Na_2CO_3–$NaHCO_3$, 0.9% NaCl, pH 9.0), assay buffer A (25 mmol/L Tris–HCl, 0.9% NaCl and 0.05% Tween-20, pH 7.2), assay buffer B (50 mmol/L Tris–HCl, 0.02% BSA, 0.09% NaCl, 0.05% Tween-20 and 0.05% NaN_3, pH 7.8), elution buffer (50 mmol/L Tris–HCl, 0.9% NaCl, pH 7.8), washing buffer (0.05 mmol/L Tris–HCl, 0.9% NaCl, 0.2% Tween-20 and 0.05% NaN_3, pH 7.8), and blocking buffer (5% BSA, pH 7.0). All solutions were freshly prepared prior to use.

Instrumentation

Spectral analyses of the fluorescent chelates were performed on a Victor^3 ™1420 Multilabel Counter purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). The shaker incubator was from Thermo Scientific (Shaanxi, China). The 96 wells Microtiter Plates were purchased from Shanghai Yiji Industries Co., Ltd(Shanghai, China). Sephadex G-50 was obtained from GE Healthcare (Freiburg, Germany). A NanoDrop 2000 was purchased from Thermo Fisher Scientific Inc(Mississauga, Canada). ELx50 Microplate Strip Washer for washing of the magnetic particle was obtained from Bio Tek (Göttingen, Germany).

Coating conjugate of magnetic particles

Covalent conjugates of magnetic particles and anti-C-peptide antibody were prepared as outlined below and as described in Fig. 1 [34,35]. All incubation steps were performed at room temperature unless stated otherwise. First, 100 μL of magnetic particles (100 mg/L, 2.0×10^9 magnetic particles/L in H_2O) were suspended in 900 μL coating buffer, to which 25 μL EDC (10 mg/mL) and 40 μL NHS (10 mg/mL) were added. The mixtures were incubated for 30 min with constant shaking to activate the carboxylic acid groups on the surface of the magnetic particles, after which the activated magnetic particles were magnetically separated from the supernatant. After washing with coating buffer four times, 100 μg capture anti-C-peptide antibody in 1 mL coating buffer was added, and the mixture incubated with gentle stirring for 18 h. Assay buffer A was added to the mixtures, and magnetic separation used four times to remove any unbound antibody. The magnetic particle–antibody conjugates were incubated in 1 mL blocking buffer with shaking for 3 h to eliminate nonspecific binding and for the blocking of any remaining active groups. After washing with assay buffer A, the magnetic particle–antibody conjugates were resuspended in assay buffer A and stored at 4 °C until required.

The magnetic particle–anti-insulin antibody conjugates were produced using the same method as outlined above for the magnetic particle–anti-C-peptide conjugates.

Labeling of antibody with Eu^{3+} and Sm^{3+} chelate

Sm^{3+} chelate labeling of anti-C-peptide MAb and Eu^{3+} chelate labeling of anti-insulin MAb were as described below [34,35]. All incubation steps were performed at room temperature unless stated otherwise. Anti-C-peptide antibody was dialyzed against labeling buffer overnight. The antibody (0.2 mg) was diluted in labeling buffer to a final concentration of 0.5 mg/mL, 0.5 mg Sm^{3+}-DTTA was added, and the solution was stirred thoroughly and incubated for 18 h with gentle shaking. Sm^{3+}-labeled anti-C-peptide was purified from free Sm^{3+}-DTTA and aggregated antibodies on a Sephadex G-50 column (1.5 cm × 40 cm), and eluted from the column with elution buffer. The concentration of Sm^{3+}-labeled anti-C-peptide antibody was determined spectrophotometrically at λ280 nm and calculated using the equation: $c\ (mg/mL) = \{A280 - (0.008 \times [Eu^{3+}])\} / 1.43$. Eu^{3+} labeling of anti-insulin was similar to the protocol described for Sm^{3+} labeling of anti-C-peptide, with the exception that Sm^{3+}-DTTA was replaced by 0.2 mg Eu^{3+}-DTTA. Finally, a filler protein (0.2% BSA) was added and the labeled antibodies stored at 4 °C for up to 12 months.

Preparation of antigen samples

Standard dilution series were constructed for recombinant human C-peptide antigen (0, 158, 1974, 5636, 9300 pmol/L) and insulin antigen (0, 3.1, 13.6, 73, 180 mIU/L); diluted in assay buffer B. Test samples were also prepared from these standards. The concentrations of all samples were verified using NanoDrop 2000 and stored at 4 °C.

Preparation of serum samples

One hundred one anonymous surplus serum samples collected from various stages of disease progression in patients suffering from diabetes were randomly obtained from Nanfang Hospital (Guangdong, China). In addition, according to procedures supervised by local authorities responsible for ethical research, the protocols were conducted with full respect on the individuals' rights to confidentiality. For the method comparisons, no attention was given to the glycemic state of the patient