Journal of Pharmaceutical and Biomedical Analysis 162 (2019) 179–184

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of a double-antibody sandwich ELISA for rapid detection to C-peptide in human urine

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ARTICLE INFO

Article history:
Received 22 March 2018
Received in revised form 18 July 2018
Accepted 29 July 2018
Available online 2 August 2018

Keywords:
C-peptide
Competitive enzyme-linked immunosorbent assay
Double antibody sandwich enzyme-linked immunosorbent assay
Urine

ABSTRACT

C-peptide level is recognized as an important indicator of diabetes diagnosis. A sensitive and specific double-antibody sandwich enzyme-linked immunosorbent assay for the detection of C-peptide based on double antibody sandwich method was studied in this paper. The rabbit and hen were innunized with PLL-C-peptide and BSA-C-peptide respectively to obtain specific Yolk antibody (IgY) and polyclonal antibody used to construct the sandwich ELISA for the measurement of C-peptide. The limit of detection was 0.51 μg/mL and the half maximal inhibitory concentration (IC50) was 3.26 μg/mL. The method developed in the study showed no evident cross-reactivity with other similar analogs. The detection standard curve of C-peptide exhibited a good linearity (R^2 = 0.9896, n = 15). 17 types of the urine of diabetes patients on c-peptide levels compared with the hospital type of diabetes information, with a conclusion of a high consistent rate. Therefore, the methods could be selectively used for rapid screening of C-peptide in human urine, and the type of diabetes has some referential significance.

1. Introduction

Diabetes mellitus is a group of insulin secretion caused by low absolute or relative metabolic syndrome characterized by chronic hyperglycemia [1]. A new study suggests that the disease in the adult population in China has about 92.4 million cases, accounting for 9.7% of the total population. In addition, other 14.82 million adults with prediabetes symptoms, account for about 1.55% of the population [2]. In current clinical diagnostic screening, serum C-peptide and insulin levels for the classification of diabetesdiagnosis, treatmentoptions and efficacy monitoring have important clinical referential value as the main routine screening for the diagnosis of diabetes patients. In addition, they also have important referential value for the diagnosis of insulin tumor [3]. In the body, a molecule of proinsulin is digested and decomposed into insulin and C-peptide, so C-peptide values can be used to evaluate the endogenous insulin secretion capacity. C-peptides are not degraded by the liver, whether they are coexisting in the secretory granules or are simultaneously released in the capillary loop. And the half-life of the C-peptide is longer than insulin [4], so the C-peptide has a wider clinical application [5]. For the patients who have been treated with exogenous insulin or have already produced insulin resistance with antibody, C-peptide pancreatic/island element ratio in the peripheral blood is used for evaluation of insulin in the liver clearance; Determination of C-peptide can also be used to distinguish between type I and type II diabetes. Whether typeII diabetes need insulin treatment [6], C-peptide measurement can also be used for evaluation of islet function in patients with diabetic ketoacidosis. Various causes of hypoglycemia are identified to monitor the endocrine function of the pancreas 66 graft, monitoring of kidney function not congruent [7].

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is a specific, sensitive, and convenient method for measuring macromolecular protein, polysaccharide and bacteria [8]. The method is precise and reproducible and employs stable reagents and inexpensive equipment. Therefore, DAS-ELISA is applicable in the routine detection of C-peptide in clinical and research laboratories. Recently, the eggs of immunized hens have been used as convenient, non-invasive and inexpensive source for polyclonal antibodies compared with mammals [9]. DAS-ELISA is

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https://doi.org/10.1016/j.jpba.2018.07.049
0731-7085/

Table 1
Percentage of cross-reactivity of C-peptide and analogs measured by sandwich ELISA.

number	analogue	$OD_{450\,(n=3)}$	P/N	
123456	C-peptide	1.003	14.97	+
	A strand for insulin	0.103	1.84	–
	B strand for insulin	0.097	1.75	–
	S o m a t o s t a tin	0.094	1.40	–
	G l u ca go n	0.085	1.27	–
	negative control	0.067	1.43	–

*P/N value (>2.1) was determined to be positive results (+).

widely used in determination of protein, antibody, or hormones in liquid sample immune analysis technology. A novel sandwich ELISA utilizing an anti metatype antibody has been developed and successfully applied in the quantification of the matter [10]. The chicken yolk immunoglobulin (IgY) as the detective antibody has shown effective as an alternative agent for haptens detection by ELISA [11]. Additionally, IgY technology is non-invasive, following the precepts of 3Rs (replacement, reduction, and refinement). Each egg contains 50–100 mg of total IgY (comparable with IgG in 10 mL of rabbit serum) [12], and the antigen-specific antibody accounts for 2–10% of total IgY. Furthermore, IgY antibodies do not cross-react with a series of influencing factors, such as mammalian IgG, rheumatoid factor, protein A, and protein G in immunoassays [13], making it an ideal candidate to be used in immunodiagnostics.

Various methods of C-peptide estimation have been advocated, which are summarized in Table 1. Urinary C-peptide (UCP) is a non-invasive test, which can be performed in an outpatient setting. When being collected in 88 boric acid UCP is stable at room temperature for up to 3 days. In patients with normal renal function, UCP quantity is reflective of 5–10% of total C-peptide secreted by the pancreas [14]. The 24 h Urinary C-peptide sample collection (24 h UCP) is a more time-consuming method, which is inconvenient for the patient, making it a less attractive option than spot UCP. In subjects with normal glucose tolerance Urinary C-peptide to Creatinine Ratio (UCP/CR) has been shown to correlate well with 24 h Urinary C-peptide [15]. This suggests that UCP/CR might be a simple, reliable, and convenient method of estimating c-peptide.

DAS-ELISA has an antigen with high sensitivity, high specificity without prior purification [16]. In this study, a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was presented for the determination of C-peptide based on the polyclonal antibody and IgY highly specific against C-peptide. This method was successfully applied for the analysis of C-peptide in human urine. The validation of these assays with spiked samples was discussed in detail. Our results suggested that the value of C-peptide can be used to distinguish type I and type II diabetes.

2. Material and methods

2.1. Materials and reagents

All reagents were of analytical grade unless otherwise specified. The BSA/PLL-C-peptide were purchased from Guangzhou jinde biotechnology co. (Guangzhou, China). Bovine serum albumin (BSA) and 3,3′,5,5′–tetramethylbenzidine (TMB) were obtained from Guangzhou Guoao Shengwu Co.Ltd (Guangzhou, China). Polylysine (PLL), C-peptide, Freund's complete adjuvant (FA) and Freund's incomplete adjuvant (FIA) were obtained from Sigma (St Louis, USA). Goat anti-rabbit IgG-HRP and Goat anti-rabbit IgG-HR were purchased from Wuhan Boster Biological Technology Co.Ltd (Wuhan, China). Other reagents were purchased from Guangzhou Huaxin Technology Co. Ltd (Guangzhou, China). ELISA plates were purchased from Xiamen Yunpeng Technology Co. Ltd (Xiamen, China). Centrifugation (HC-3018R Anhui USTC Zonkia Scientific instruments, China). New Zealand white rabbits (1.5–2.3 kg) were purchased from the Animal center of Guangdong province. The ELISA results were measured at 450 with an RT-200C microplate reader (Rayto, China). In this work, ultrapure water was used throughout the experiments.

Phosphate-buffered saline (PBS, pH = 7.4, 0.01 M); carbonate buffer saline (CBS, pH = 9.6, 0.1 M); the substrate solution was prepared by mixing 500 mL of TMB solution (10 mg TMB in 5 mL of ethanol) with 9.5 mL of citrate buffer (pH = 5.0, 0.1 M, 3.6 g $Na_2HPO_4.12H_2O$, 1.02 g $C_6H_8O_7.H_2O$ in 0.2 L of distilled water) and 10 mL of 30% H_2O_2. 10 mM PBST solution phosphate buffer saline (PBS, pH = 7.4, containing 0.1% Tween-20) was used for washing plates.

2.2. Production of polyclonal antibodies

New Zealand female rabbits were immunized with PLL-C-peptide conjugate at multiple sites according to the reported method with some modifications [17]. Routinely, PLL-C-peptide(1 mg/mL)was emulsified with Freund's complete adjuvant 1 : 1 (v/v) at the first injection. One month later, five subsequent booster doses emulsified with equal amount of Freund's incomplete adjuvant were injected at two-week intervals. Ten days after the fifth immunisation, serum titres were detected by indirect ELISA. Fifteen days after the final injection, serum was collected and stored at −20 °C. The serum was purified through the saturated ammonium sulfate precipitation method [18]. The titre of polyclonal antibodies was determined by direct ELISA [19].

2.3. Production of anti-C-peptide IgY

White hens were intramuscularly injected with BSA-C-peptide which emulsified with the same amount of complete Freud's adjuvant. Four subsequent booster injections were given on the days of 21, 35, 49, and 63 after the first immunization. Eggs were collected daily and stored at 4 °C until they were purified by PEG-6000 as described before [20].

In brief, the yolk of each egg was diluted 1:2 with sterile PBS. The PEG-6000 was added at the concentration of 3.5% (w/v) and subjected to gentle shaking at room temperature for 20 min. Following centrifugation at 12,000 rpm for 20 min, the supernatant was collected and then mixed with PEG-6000 to a final concentration of 12% (w/v). The mixtures were thoroughly stirred and centrifuged at 12,000 rpm for 20 min. Then the precipitate was dissolved in PBS; the final concentration of 12% (w/v) PEG-6000 was added and centrifuged as described above. Finally, the precipitate was dissolved and dialyzed with PBS for overnight at 4 °C. The crude extract with PBS dissolved was added in the solid ammonium sulfate to the concentration of 50% (v/v) and subjected to gentle shaking at room temperature for 30 min and put at 4 °C overnight. After centrifugation at 8000 rpm for 20 min, the precipitate was collected and then mixed with PBS and solid ammonium sulfate to a final concentration of 33% (v/v). Finally, C-peptide IgY was obtained, centrifuged, lyophilized and stored at −20 °C. The titre of IgY was determined by direct Enzyme-Linked Immunosorbent Assay [21].

2.4. Establishment and optimization of DAS - ELISA

DAS - ELISA was carried out as follows: (1) the coating conjugate rabbit anti-C-peptide pAbs diluted with the coating buffer at an optimum concentration were added into a microtitre plate (100 μL/well) and incubated at 37 °C overnight. (2) Plates were washed three times with the washing buffer and then 100 u L of C-peptide diluted with PBS (0.001, 0.01, 0.1, 1, 10, 100, 1000ug/mL) was added to each well. (3) After incubation for 30 min at 37 °C, the unbound compounds were washed away. 100 u L of anti-C-peptide