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Comparative study between liquid phase and solid phase radioimmunoassay system for determination of C-pipted in human serum

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ABSTRACT

This study aimed to examine the benefits and drawbacks of both solid phase and liquid phase radioimmunoassay (RIA) systems used to quantify C-peptide in human serum. The fundamental reagents were produced. The first RIA was ^{125}I-C-peptide tracer. An indirect tracer preparation utilized chloramine-T and tyrosine methyl ester (TME). Using HPLC column, the tracer was separated from the reaction mixture. The second reagent was the polyclonal antibody which was produced previously in another work. The coated tube was carried out using a highly purified polyclonal antibody. A set of C-peptide standards was created using highly purified C-peptide. A liquid phase system radioimmunoassay (RIA) was created to measure the C-peptide levels in human serum. Numerous parameters were investigated to perform this study including cross reaction, specific and nonspecific binding, sample volume, temperature influence, incubation time, and the valid period. Different samples of human serum were examined using both solid and liquid phases. The statistical study showed a strong relation between the outcomes of the two procedures with minor differences. The solid phase has many advantages over the liquid phase. A high nonspecific binding and a low specific binding percent were observed. On the contrary a high binding percent and the low cost are the main advantages in Liquid phase system.

INTRODUCTION

In order to determine the concentration of chemicals of interest in human body fluids (like vitamins, drugs, medicine and etc), radioimmunoassay (RIA) is the most typically used with human plasma or serum. RIA is a technique in which no radiation is given to the human. However, there are other factors that contribute to its widespread use. It forms the basis for very sensitive and accurate diagnostic techniques, and its use in contemporary medicine led to a virtual explosion of knowledge on the pathophysiology of many diseases [1-4].

Pro-insulin was discovered by Steiner and his coworkers [5, 6]. The method of insulin manufacture is explained by the discovery of pro-insulin, which also sparked the growth of the pro-hormone theory. It was crucial to understanding the production of proteins. Given that the research was conducted on the full parent molecule of pro-insulin as well as its "offspring," the linking peptide or C-peptide, the structure of pro-insulin was discovered. The direction of pro-insulin research was upstream in the direction of the precursor, downstream in the direction of conversion, and wide. From in vitro studies on the subatomic scale to cell and tissue studies to in vivo tests on animals, every level of experimental work was carried out. The investigations' attention swiftly turned to man.

In diabetes research and clinics, several studies involving healthy individuals and patients are becoming more and more crucial. C-peptide as a stand-alone substance and C-peptide as a constituent of pro-insulin were the two features. The C-peptide started its own, increasingly independent life not long after the initial biochemical studies. C-peptide as a bioactive molecule and C-peptide as a marker for diabetes mellitus diagnosis were the two main research areas once more. In-vivo study in animals as well as synthetic chemistry scale to

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cells and tissues were added to these investigations based on the native molecule (s). The focus of the inquiries immediately changed [5, 6].

Between 1973 and 1976, the study of immunology dramatically increased. Chemically generated C-peptides, their derivatives, and fragments are useful tools for investigating the immunological properties of human, porcine, and bovine C-peptides [5-7]. The development of immunoassays, particularly RIAs, as tools for in-vitro investigations obtains evaluations under normal/disease situations as well as tests for antigenicity through the synthesis of numerous specialized antibodies. The C-attachment peptide's to the tyrosine methyl ester makes it possible to later label the synthetic ^{125}I-Tyr-Cpeptide tracer with ^{125}I, making it a unique tracer molecule. In particular, significant antibody titers are produced after immunizing with human carbobenzoxy (Z)-C-peptide, which is the N-terminal derivative of albumin-conjugated antigen. [7-10].

In this study, RIA kit was created in both solid phase & liquid phase systems to measure the presence of c-peptide in body fluids (human serum). Comparative analyses of these aspects were conducted, and the benefits were registered.

MATERIALS AND METHODS

For construction of solid phase and double phase RIA systems for measurement of c-peptide in human sera, numerous reagents were needed. The following chemicals were bought from Sigma Co., USA: C-peptide, chloramine-T (Ch-T), sodium metabisulphite (MBS), and tyrosine methyl ester (TME). The Institute of Isotopes Co., Ltd. in Budapest provided the radioactive iodine in form sodium iodide - 125 (5 mCi/50 ul) (185 MBq), pH 7-11, and IZOTOP RIA kit. Analytical grade chemical reagents of all other kinds were bought from reputable producers.

The following preparations allowed the research strategy to be accomplished:

^{125}I-TME-C-peptide conjugate tracer:

TME was first activated before being combined with the c-peptide. 35 mg of TME were dissolved in 3.2 ml of anhydrous dimethyl formamide were added to 25 µl of tri-n-butyl amine about five minutes with continuous stirring at 10 ºC. Then 25 µl of isobutyl chloroformate were added. Approximately 60 minutes at 10 ºC spent maintaining the mixture after adding [7]. The conjugation was done as follows: 35 mg of C-peptied were given to the activated TME product, suspended in 2.9 ml of dimethyl formamide. The pH was then brought down to 8.8 with NaOH and the mixture was continually agitated for 24 hours at 25 ºC. After the combination was added to 3.5 ml of distilled water, the result was extracted four times with 3.0 ml of ethyl acetate. Every extract was gathered and dried. The item was segregated. Utilizing Ch-T, It was radiolabeled according to Hunter and Greenwood [8] and Karir et al., [9]. 20 µl of ethanol were used to dissolve the 3.0 g of TME conjugate, and the vapour was then vented under nitrogen gas. The precipitate was dissolved in 15 µl of pH 7.4 0.5M phosphate buffer after first being dissolved in 10 µl of ethyl acetate. 10 µl of Na ^{125}I was then added to the mixture (1.0 mCi, 37.0 MBq). Starting the reaction required 25 µg/10 µl of Ch-T. After incubating for 5 minutes, the reaction was halted by adding 30 g/ 10 ml of sodium metabisulphite. 40 µl of potassium iodide, 0.5 mg/ml, was added to the reaction mixture as a carrier. The reaction mixture was injected into an HPLC column (ODS-H-OL5-27883, Capital Analytical LTD, England). To obtain 50 000 cpm/100 µl, the pure tracer was diluted in 0.05M phosphate buffer pH 7.4. The immunoreactivity percent, radiochemical yield percent, and radiochemical purity % were examined in all the prepared tracer.

Production of polyclonal antibody:

According to Katagiri et al.,[10], three male adult white New Zealand rabbits were injected with the previously described [11,12]. C-peptide:BSA combination in order to prepare polyclonal anti-C-peptide. The sodium sulfate/ammonium sulphate precipitation approach was used to purify a chosen pool of anti-C-peptide antibodies with a greater titre [12-14].

C-Peptide Standards:

A stock solution (5.0 mg/ml) was created by dissolving 5.0 mg of highly purified C-peptide antigen in 1.0 ml of methanol. The buffer (0.05M phosphate buffer, pH 7.4, 0.1% BSA, and 0.1 mg sodium azide) was prepared to generate a high concentration solution (1 mg/ml). The daily routine labour required the preparation and maintenance of the working standards (0.1, 0.5, 2.0, 10, 25, 50, and 100 µg/dl) at 4 ºC [13-15].

Liquid-phase RIA improvement:

The construction of a standard curve and investigating the immunoreactivity of the tracer and antibody was carried out to maximize the performance of the liquid-phase RIA system. It was investigated how the reaction parameters may be improved to achieve a high binding percentage.

Preparation of Coated Tube:

The plane tubes were coated into surface using purified antibody according to previous protocol [16]

Optimization of solid-phase RIA system:

Utilizing coated tubes, the standard curve was constructed. Reaction immunoassay optimization was researched in order to provide a high binding percent and displacement.

RESULTS AND DISCUSSION

To create a radioimmunoassay system, three fundamental components were developed. These components are polyclonal antibodies, ^{125}I-C-peptide tracer, and C-peptide standards. In this study, double phase and solid phase coated tubes were prepared, and the findings were presented as follows:

^{125}I-C-peptide tracer:

Using an HPLC-chart, the radiochemical yield percentage of the ^{125}I-C-peptide tracer was estimated and equaled 72%, as shown in Fig (1). Paper electrophoresis was used to determine the ^{125}I-C-peptide tracer's purification degree as shown in Fig.(2). The radiochemical purity percentage was 96%.. The data were used to compute the tracer's specific activity (44.9 Ci/g). By using the liquid phase RIA system, the maximum binding percent (% Bo) and non-specific binding (% NSB) were 32.3% and 3.3%, respectively.

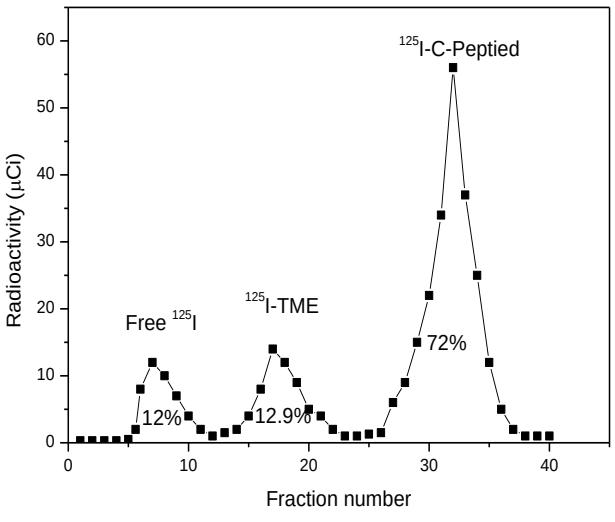

Fig. (1): HPLC-based radiochemical yield% of ^{125}I-C-Peptide tracer

In the current investigation, the labelling settings were improved to provide a higher efficiency of ^{125}I incorporation and a lower degree of radioiodination damage.

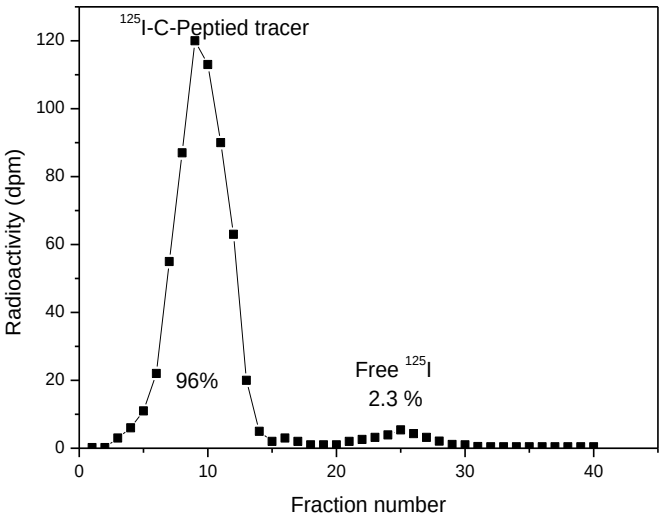

Fig. (2): An electrophoretic purification process for radiochemistry

Liquid phase RIA system

To produce a trustworthy assay, it is necessary to adhere to the fundamental ideas of assay optimization. 100 µl of the purified anti-C-peptide with an initial dilution of 1:8000 (R1), 100 µl of the C-peptide standard or sample, and 100 µl of the ^{125}I-C-peptide tracer were used in the test. The assay tubes' contents were combined, and then incubated at 37 ^{o}C overnight. All of the reagents are in the non-specific binding (NSB) tubes, with the exception of the first anti-body. With the exception of the tubes marked with a (TA), second antibodies (100 µl goat anti-rabbit IgG 1/80), normal rabbit serum 1/200, and 500 µl of PEG-8000 (12%) were added to each tube and incubated for 30 min at room temperature following the separation agent second antibodies. Centrifugation of the tubes was place for 10 min. at 4 ^{o}C and 4000 rpm. The tubes were decanted and counted using a multi-crystal gamma counter. The optimized standard curve was drawn as shown in Fig. (3).

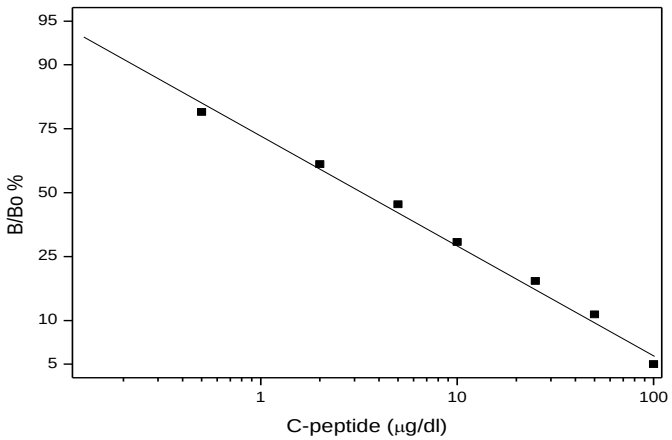

Fig. (3): Calibration curve of double phase RIA system for C-peptide

The design of the solid phase RIA test:

Coated tubes were put to the test using the RIA method. The assay can be summed up as follows: Except for total counts tubes, the matching coated tubes had 50 ul of C-peptide standards pipetted into them. All test tubes were then filled with 250 ul of each ^{125}I-C-peptide. The tubes were combined and incubated for 3 hours at 37 ^{o}C. Following the completion of the incubation period, all test tubes, aside from the total count tubes, were decanted and washed with 2 mL of distilled water. The decanting step was done with the tubes' contents and calibration curve of C-peptide is shown in Fig. (4) after they counted in a gamma counter

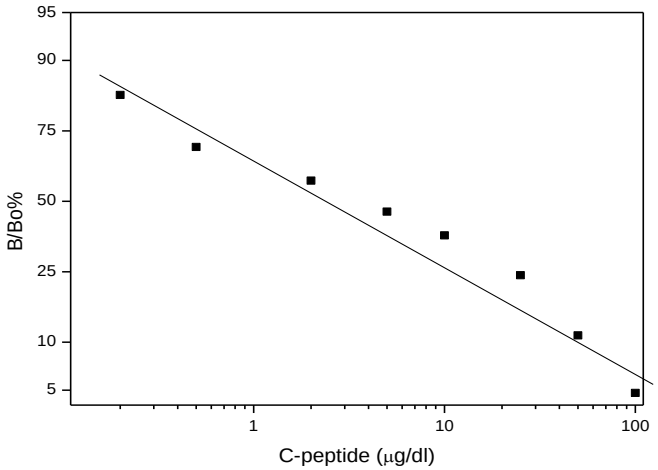

Fig. (4): Calibration curve of solid phase RIA system for C-peptide

The binding percentage and nonspecific binding were higher than in a solid phase, as shown in Table (1). The displacement percentage, however, was larger than the liquid phase. The findings are consistent with earlier research. [13-17]

Table (1)

	Bo%	NSB%	Displacement percent
Liquid phase	**48**	**3.9**	**50**
Solid phase	**26**	**1.8**	**60**
Reference Kit	**30**	**0.8**	**70**

The incubation period and sample volume were longer than in a solid phase, as shown in Table (2). However, compared to the liquid phase validation duration, the solid phase validation period was greater. The findings are consistent with earlier research [18-23].

Table (2)

	Incubation time (hr)	Validity period (d)	Sample volumes (ul)
Liquid phase	**3**	**180**	**100**
Solid phase	**2**	**270**	**25**
Referance Kit	**1**	**360**	**25**

Liquid phase method comparison:

To compare the C-peptide results of 50 distinct human serum samples acquired using the commercially available kit (IZOTOP, RIA-coated tube, CT) (Isotope Institute, Budapest) to those obtained using the two current technologies, statistical analyses were conducted. The reference values for serum C-peptide are 2-18 g/dL in the afternoon and 7-28 g/dL in the morning. One hundred individual human serum samples were split into 50 with normal C-peptide levels and 50 with problematic cases. The methods used now make it possible to distinguish between typical and abnormal subjects in a reasonable and precise manner. There was a significant correlation (r = 0.9989) between the results from the local systems and the commercially available kit, as shown in Fig. (5).

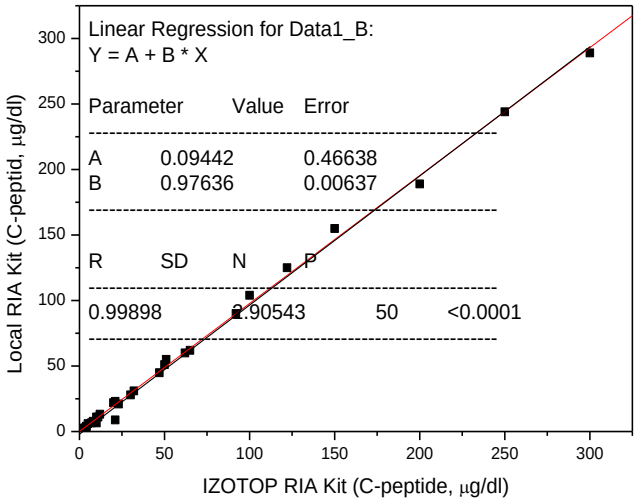

Fig. (5): Comparison of the c-peptide levels in fifty samples of human serum to determine the correlation coefficient (r) between the liquid phase kit and the IZOTOP kit

Solid phase method comparison:

Statistics were used to compare the C-peptide results of 48 different human serum samples acquired using the current system to those obtained using a commercially available kit (IZOTOP, RIA-coated tube, CT) (Isotope

Institute, Budapest). The reference values for serum C-peptide are 2-18 g/dL in the afternoon and 7-28 g/dL in the morning. The present methods provide a decent and precise differentiation between typical and atypical people. The results from the regional systems and the commercially available kit had a high correlation (r = 0.9987), as shown in Fig. 6.

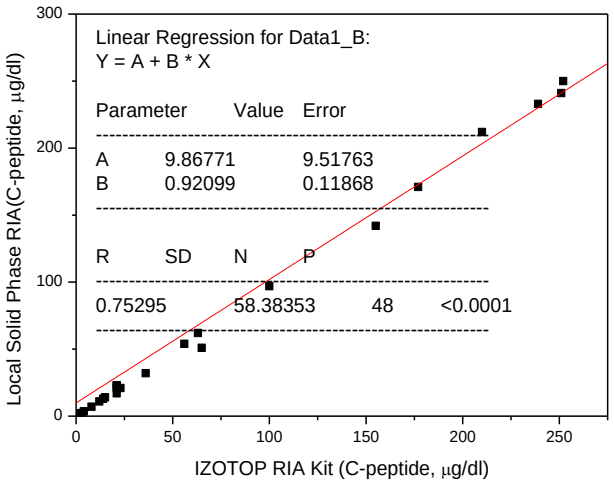

Fig. (6): C-peptide levels in 48 samples of human serum were analyzed to determine the correlation coefficient (r) between the solid phase kit and the IZOTOP kit

CONCLUSION

In summary, the solid phase radioimmunoassay method provides greater benefits than the liquid phase method. High sensitivity, low nonspecific binding, ease of separating free and bound antigens, and a brief incubation period are these advantages. On the other hand, the liquid phase has few advantages, such as a high binding percentage.

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