ABBS

Lab Note

A light initiated chemiluminescent immunoassay for procalcitonin

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Human procalcitonin (PCT) is the prohormone form of calcitonin which was first identified in 1981 [1]. It is a glycoprotein composed of 116 amino acids without hormonal activity and is mainly produced by parafollicular cells (C cells) in the thyroid gland. PCT is enzymatically cleaved into lower molecular weight peptides, including the N-terminal with 57 amino acids, C-terminal with 21 amino acids, and calcitonin with 32 amino acids. The concentration of PCT is very low in normal human plasma, but is increased in the plasma of patients with sepsis and infection [2]. Furthermore, PCT is mainly induced during severe systemic inflammation caused by bacterial infections, but not by non-bacterial infection. Therefore, PCT is considered as an important marker of serious systemic infection and septicemia. Although several approaches have been reported for PCT detection and measurement, including enzyme-linked fluorescence, electrochemiluminescence, and colloidal gold labeling [3], some of these methods either lack sensitivity or require complicated operation, including repeated washes and separation, which is time-consuming and not suitable for automation. So it is urgently necessary to establish a simple, rapid, and sensitive method to detect PCT. Here, we described a light initiated chemiluminescent assay (LiCA)-based method [4] to detect PCT and preliminarily evaluated its detection performance.

All blood samples were collected from outpatient and inpatient clinics at the Shandong Rizhao People's Hospital. PCT monoclonal antibodies 44D9 and 18B7 were purchased from Hytest Company (Turku, Finland). Luminescent particles were obtained from Beyond Biotech Company (Shanghai, China). LiCA HT high-throughput homogeneous luminescence immunoassay instrument and LiCA SP automatic pipetting device were purchased from Shanghai Questbiotech Company (Shanghai, China). The NICOMP380 particle sizing system (Particle Sizing Systems, Port Richey, USA) was used to determine the size distribution of colloidal suspensions. A 96-well plate (Corning Life Sciences, Corning, USA) was used together with the LiCA HT high-throughput homogeneous luminescence immunoassay instrument.

Immune luminous particles were prepared as previously reported [5]. Briefly, two tubes of 200 nm luminous particles with a surface of active aldehyde groups were centrifuged. Then, precipitated particles were dispersed in phosphate buffer (pH 7.0; 0.1 M) by sonication. The monoclonal antibody 44D9 was then added to the suspension at a ratio of $10:2$ (w/w). Sodium cyanotrihydridoborate (NaCNBH$_3$, 0.1 μM) was added to the particles at a ratio of $50:1$ (w/w) and the solution was incubated at 37°C for 48 h. Then, the solution was centrifuged and unbounded antibody was removed. Particles were diluted to 10 mg/ml and stored at 4°C until use.

The pH value of the monoclonal antibody 18B7 solution was adjusted to 8.5. Then, biotin was added at the molecular ratio of $1:30$ (antibody to biotin) and the mixture was incubated overnight at 4°C. Then, the unbound biotin was removed by dialysis and the product was diluted to 1 mg/ml and stored at 4°C. The concentration of the PCT stock was determined by the Roche ECL system (Basel, Switzerland), and diluted to 0.2, 0.5, 2, 10, and 100 ng/ml PCT using calf serum. The PCT standard solution was re-standardized by Roche e601, calibration qualified, distributed into 1 ml aliquots, and stored at −20°C. The PCT stock was also diluted to 30 ng/ml (QC H) and 1 ng/ml (QC L) using calf serum, and calibrated by Roche e601 system for each dilution. All qualified samples were distributed into 1 ml aliquots and stored at −20°C.

Twenty-five microliters of the standards, quality controls, or test samples, 25 μl of immune luminous reagent, and 25 μl biotin-labeled reagents were sequentially added to the 96-well plates. The plates were placed in the LICA HT instrument and incubated at 3°C for 20 min. Then, 175 μl of LiCA Common Reagent was added by the automatic pipettor. After being incubated for 15 min at 37°C, the light signal (RLU) was measured by the instrument. According to the EP17-A procedure [6], under the quality control guidelines, the same batch reagent of blank sample or zero calibrator should be run 20 times. The mean value and standard deviation (SD) were calculated. The mean ± SD of blank samples was used to calculate the analytical sensitivity.

Test samples were divided equally into three parts, two of which were mixed in equal volumes with high and low quality control (QC H and QC L) samples, respectively. The measurement of test samples, mixed sample, QC H, and QC L were carried out in triplicate. The mean value was calculated and the recovery rate was obtained according to the formula: the recovery rate (%) = actual detectable concentration/theoretical concentration × 100%. Following the EP5-A2 procedure [7], the same batch of reagent, calibrator, and quality control serum were used as test samples. At least 20 replicates were run at one time and the mean, SD, and co-efficient of variation were calculated. The reagent was stored at 37°C for 7 days, then subject to physical examination and stability analysis based on linearity, sensitivity, and precision investigation. A total of 82 serum samples were measured by both the established LiCA and Roche ECL methods. The detection values from the two methods were compared and a correlation curve was plotted.

Serum PCT concentrations from 82 patients were measured by the LICA and Roche electrochemiluminescence assays. The values detected by the two different methods showed comparable results for most samples (**Fig. 1A**). The correlation equation was $y = 0.9452x + 0.6712$, and the correlation coefficient R^2 is 0.9615, indicating that the two methods for detection of PCT do not show any significant difference (**Fig. 1B**). The blood samples from these patients were divided into a low infection group and a high infection group. The low infection group showed a low level of PCT in the blood, as low as 0.05 ng/ml (**Fig. 1C**), while the high infection group had a high level of PCT in the blood, up to 60 ng/ml (**Fig. 1D**). Thus, LiCA is a sensitive method to measure the significant differences in PCT concentration in systemic inflammation. It also demonstrated that the PCT concentration is proportional to the degree of infection.

The sensitivity of LiCA for human serum PCT is 0.025 ng/ml (**Table 1**). The reagent was also stored at 37°C for 7 days, and subject to physical examination including reagent linearity, sensitivity, and precision, which showed good stability at 37°C with the sensitivity of 0.036 ng/ml. Within-run precision for LiCA of PCT, QC L, and QC H are 1.12% and 29.87%, respectively. After 7 days, the QC L and QC H remain stable with 1.01% and 31.01%, respectively. The reagents also retained good particle size distribution without precipitation.

The sample mixed with an equal volume of QC L is named as sample 1, and the sample mixed with equal volume of QC H named as sample 2. The results showed that both samples 1 and 2 had a good recovery rate (**Table 2**). When test sample (9.41 μIU/ml) mixed with QC L (1.06 μIU/ml), the concentration of sample 1 is 5.01 μIU/ml with a recovery rate of
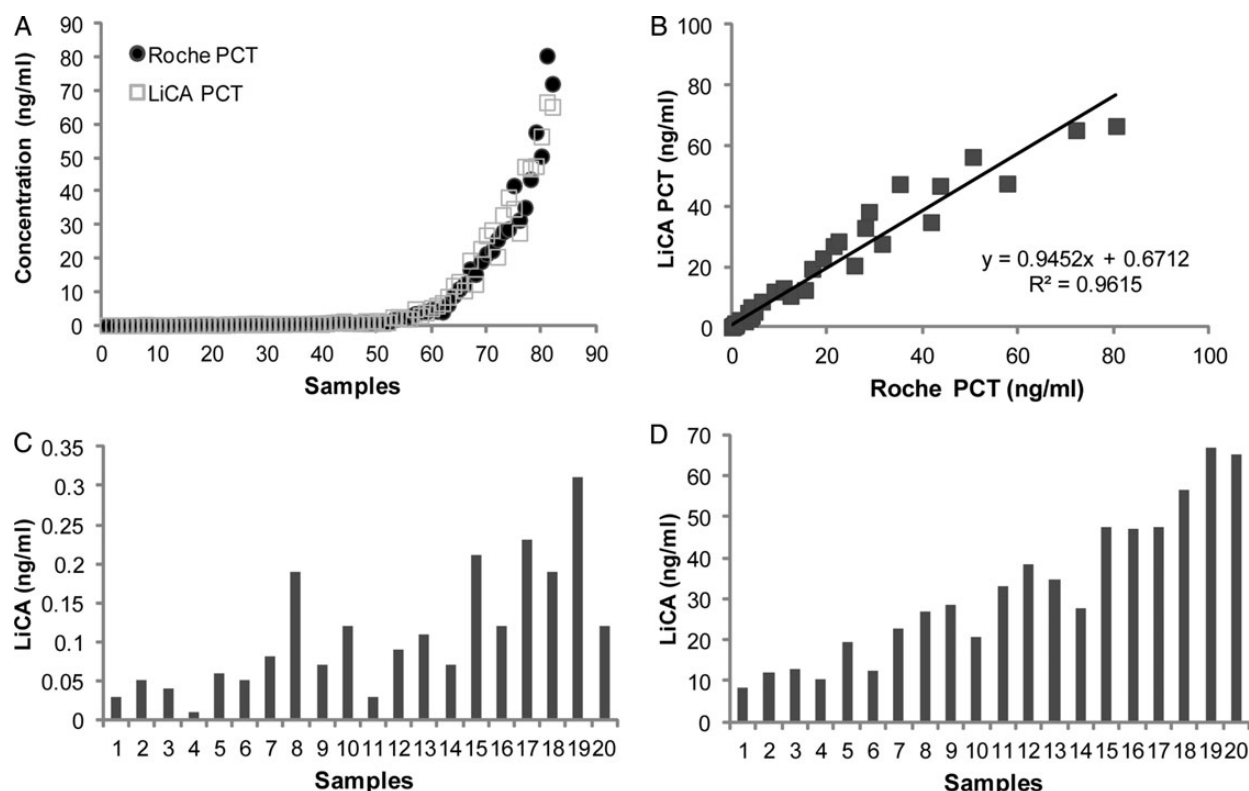
Figure 1. The determination of PCT concentration in blood samples using the LiCA and Roche methods (A) The PCT concentration showed comparable results using either the LiCA or Roche methods. (B) There was a significant correlation of the LiCA and Roche methods in detecting PCT concentration in different samples. (C) LiCA has a high sensitivity of detection for PCT in samples with a low level of infection. (D) LiCA has a wide range of sensitivity and detects PCT in samples with a high level of infection.

Table 1. The stability of PCT by LICA

Item	PCT	
	0 day	7 days
Physical examination	Good particle size distribution, no precipitation	Good particle size distribution, no precipitation
R value for linear correlation	0.9994	0.9989
Sensitivity	0.025 ng/ml	0.036 ng/ml
QC L	1.12	1.01
QC H	29.87	31.01
Testing result	Qualified	Qualified

Table 2. The recovery rate of PCT by LICA

PCT	Value
Sample concentration (μIU/ml)	9.41
QC L concentration (μIU/ml)	1.06
QC H concentration (μIU/ml)	28.62
Mixed sample 1 concentration (μIU/ml)/recovery rate of sample 1 (%)	5.01/95.7
Mixed sample 2 concentration (μIU/ml)/recovery rate of sample 2 (%)	20.45/107.55

95.7%. And when test sample (9.41 μIU/ml) mixed with QC H (28.62 μIU/ml), the concentration of sample 2 is 20.45 μIU/ml with a recovery rate of 107.55%. These results demonstrate the good recovery rate of the LiCA method.

PCT is not only used in the clinically differential diagnosis of inflammation [8], but is also widely used in the clinical diagnosis of sepsis [9] and evaluation of systemic inflammatory response syndrome. PCT is considered as an indicator for disease severity and prognosis [10]. In 2001, PCT was confirmed as a diagnostic indicator of sepsis [11] at the International Sepsis Conference. Recent studies have further shown that PCT is an indicator with high specificity and sensitivity [12,13] in the supplementary diagnosis of sepsis.

LiCA is the second homogeneous immunoassay technology used clinically after luminescent oxygen channeling immunoassay [14]. LiCA Common Reagent with internal dye and internal luminescence emission compounds coated with reactive molecules were mixed with test samples in a homogeneous solution. Particles are coated with active molecules that can capture the target molecule with the advantages of being quicker and more efficient in forming an immune sandwich complex. The reaction is a homogeneous reaction, which can not only accelerate the reaction rate, but also avoid repeated separations and washes. In addition, it can improve the detection sensitivity due to the increase in particle surface area. While the cost of LICA is still a little higher, it is much lower than the current Roche method.

In summary, we established a light initiated chemiluminescent immunoassay for PCT measurement and evaluated its performance. The results shown here demonstrate a low background, high sensitivity, specificity, accuracy, and reproducibility, and short assay time (only 35 min) with LiCA for PCT. At 37°C, 7 days of accelerated stability experiments, no precipitation was observed with uniform particle size distribution. As no significant difference was found in linearity, sensitivity, or precision between storage at 4 and 37°C, the reagent stability is good. Thus, LICA measurement of serum PCT could be suitable for clinical use in the future.

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