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Study of Immunoassay Methods for Recombinant Human Erythropoietin (rhEPO) Using Competitive ELISA

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Abstracts: Two different immunoassay methods, competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) and amplificative competitive indirect ELISA (ACI-ELISA) using biotin-avidin complex system were studied to detect rhEPO. The linear ranges were 50-20000 ng/mL and 10–50000 ng/mL for CI-ELISA and ACI-ELISA, respectively. The low detection limits of CI-ELISA and ACI-ELISA were 62.8 ng/mL and 8.5 ng/mL, respectively.

Keywords: Recombinant human erythropoietin (rhEPO), polyclonal antibody (pAb), competitive indirect ELISA (CI-ELISA), amplificative competitive indirect ELISA (ACI-ELISA).

EPO is a sialoglycoprotein with 165 amino acids, which has a molecular weight of 36,000 daltons. EPO plays a key role in stimulating the proliferation and differentiation of erythroid precursor cells. It has been widely used as a medicament for patients with chronic renal disease, since the DNA-rhEPO was obtained in 1985[1]. Due to its function in promoting the erythropoiesis and difficulty to be detected, rhEPO gradually became a prevalent doping within certain sports, especially in endurance sports in the past ten years; thus the International Olympic Committee Medical Commission (IOC-MC) introduced rhEPO as a banned substance of peptide hormones and analogues in 1990. Haematocrit testing[2], which is subject to some bias, such as sex, age, race, metabolism, and training conditions is the only official detection method of rhEPO accepted by sports organizations. In clinical diagnosis, the EPO-RIA kit is a standard method to detect endogenous EPO, but the use of radioactive element is unfavorable.

In this paper, we studied CI-ELISA and ACI-ELISA method, based upon non-radioactive immunoassay. ACI-ELISA utilized the biotin-avidin amplificative system, which could make improvement on CI-ELISA. The immunoassay conditions of CI-ELISA and ACI-ELISA were explored. The linear ranges and limits of detection (LOD) were determined. Recoveries of both methods were studied by simulant samples of mouse serum.

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Experimental

The anti-rhEPO pAb was obtained from sera of immunized rabbit with rhEPO for five times. The injections of rhEPO were 0.75 mg, 0.5 mg, 0.5 mg, 0.5 mg and 0.2 mg in day 0, 10, 20, 30, and 40, respectively. The anti-rhEPO sera of rabbit were obtained 7 days after the final injection. The pAb used for further ELISA detection was purified according to a modified caprylic acid-saturated ammonium sulfate method [3].

According to anti-rhEPO pAb dilution curves at different coated antigen concentrations, 0.5 μg/mL and 0.015 mg/mL were the optimal concentration of coated antigen and purified pAb used in ELISA detection.

Determinations of the calibration curve and recoveries of CI-ELISA were performed by following procedures. Aliquots of 100 μL coating solutions with rhEPO in concentration of 0.5 μg/mL were added to the wells and incubated for 24 h at 4°C. Blocking step was performed at 37°C for 2 h with 0.8% gelatin-PBS. Then, aliquots of 50 μl of 0.03 mg/mL anti-rhEPO pAb solutions and standard solutions of 50 μl of 40, 16, 4, 1, 0.2, 0.1, 0 μg/mL rhEPO, respectively, were pipetted together into the coated wells. After incubating for 1 h at 37°C, 100 μL of 1:1000 diluted goat anti-rabbit IgG-HRP solution was added and incubated again for one hour at 37 ^{o}C. Subsequently, 100 μl of substrate solution (containing 30% H_2O_2: 6 mg/mL TMB: PB=1.5:10:1000) was added into the wells and the enzyme reaction was developed in the darkness for 15-30 min at room temperature. The reaction was terminated by adding 50 μL of 2 mol/L H_2SO_4 to each wells. The measurement was performed with absorbance and reference wavelength at 450 nm and 492 nm, respectively.

The recoveries of the assay were determined by spiking the mouse serum samples with known amount of rhEPO (15, 5, 0.2 μg/mL). The steps of the assay were similar to the above-described procedure, but were different in adding mouse serum samples instead of standard solutions.

In the ACI-ELISA assay, the addition of goat anti-rabbit IgG-HRP solution in CI-ELISA procedure was substituted by 1:1000 diluted goat anti-rabbit IgG-biotin solution. 100 μL of 1:500 diluted avidin-HRP was added to the wells to form the biotin-avidin complex by incubating at 37°C for 0.5 h. The recoveries of the assay were determined by spiking the mouse serum samples with fixed amount of rhEPO (20, 8, 0.2 μg/mL) using the same steps as the determination of ACI-ELISA calibration curve.

Result and Discussion

Calibration curve of CI-ELISA and ACI-ELISA are shown in **Figure 1** and **Figure 2,** respectively. The linear ranges of rhEPO were 0.05-20 μg/mL and 0.01-50 μg/mL for CI-ELISA and ACI-ELISA.

The linear formulas of these two methods were:

$$Y(Abs.)=0.44003-0.09326\times\lg(C_{rhEPO}) \quad (R=0.997) \text{ for CI-ELISA}$$

$$Y(Abs.)=0.45388-0.07798\times\lg(C_{rhEPO}) \quad (R=0.998) \text{ for ACI-ELISA}$$

The LODs of rhEPO (thrice the standard deviation at zero dose) were 67.8 ng/mL and 8.5 ng/mL for CI-ELISA and ACI-ELISA, respectively.

The recoveries of both methods were summarized in **Table 1** and **Table 2**.

Figure 1 Calibration curve of CI-ELISA

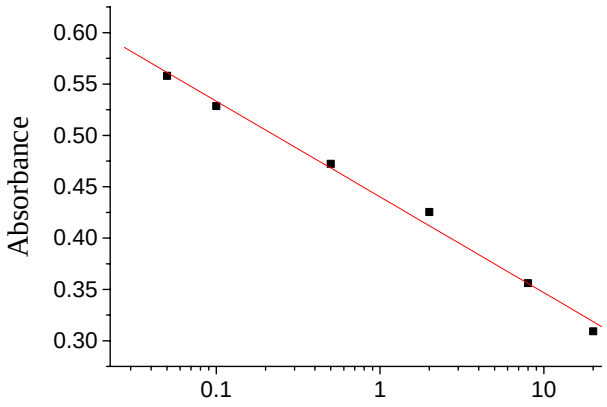

Figure 2 Calibration curve of ACI-ELISA

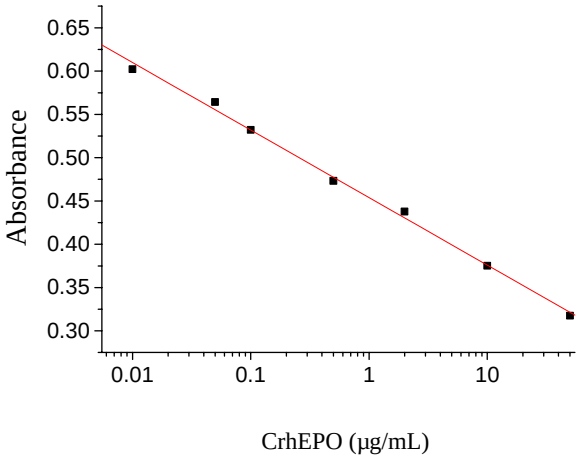

Table 1 The recovery results of rhEPO in mouse serum by CI-ELISA (n=8)

	rhEPO Measured (μg/mL)	Recovery (%)	RSD (%)
15	14.5	96.9	5.7
5.0	5.05	101.0	8.9
0.2	0.29	143.5	10.0

Table 2 The recovery results of rhEPO in mouse serum by ACI-ELISA (n=8)

rhEPO Added (μg/mL)	rhEPO Measured (μg/mL)	Recovery (%)	RSD (%)
20	23.2	116.0	18.7
8.0	9.0	112.5	25.6
0.2	0.16	81.3	22.2

Compared with CI-ELISA, ACI-ELISA was better in the linear range and the detection limits. Theoretically, each avidin molecule can combine four biotin molecules, which can amplify the detection signal severalfold. The use of biotin-avidin complex made obvious improvement on CI-ELISA, but ACI-ELISA had some inherent disadvantages. Avidin is a glycoprotein, which is prone to combine with substance containing carboxyl groups. Consequently, non-specific adsorption of avidin influences its specific combination with biotin. 10% bovine serum-PBS was used as the diluted buffer of avidin-HRP to avoid this problem.

The recoveries of CI-ELISA were preferable in high concentration than in low concentration, and the RSDs were less than 10%. As for the ACI-ELISA, the recoveries in different concentrations were less in variety and the RSDs were relatively distinct, due to the non-specific adsorption mentioned above.

In this study, we successfully established ELISA detection methods for rhEPO using CI-ELISA and ACI-ELISA.

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