Immunoassay Using a Metal-complex Compound as a Chemiluminescent Catalyst. III. Flow-through Analysis of a Labeled Antigen Bound by Immune Reaction

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(Received May 26, 1983)

Synopsis. By measuring the chemiluminescence intensity by the flow-through method rather than the conventional batchwise method, the sensitivity and precision of immunoassay and the time required for the measurement of the chemiluminescence intensity were improved as follows: 20 ng—50 μg of human albumin per tube, 5.8—7.2% (n=9) coefficient of variation, and 4 min measuring time.

Studies on automatic and continuous immunoassay have been made for non-labeled immunoassays such as laser nephelometry[1-3] and latex-photometric immunoassay,[4] but few such attempts have been made for a labeled immunoassay like enzyme immunoassay (EIA), because the enzyme as a labeling reagent is more unstable than an ordinary chemical reagent and requires some reaction time.

Chemiluminescence complex catalyst immunoassay(CLCCIA), in which a synthesized metal-complex compound acting as a labeling reagent, was reported by the authors for the determination of human serum albumin(HSA) as a model compound for the protein.[5] CLCCIA was also shown to provide the sensitivity necessary for practical immunoassay.[6] Moreover, CLCCIA has the following advantages in comparison with EIA: 1) The labeling reagent, that is, the metal-complex compound is more stable. 2) The analysis utilizing chemiluminescence reaction is more rapid. Judging from these advantages, CLCCIA is suitable for automatic and continuous immunoassay. As the first step for determining such a procedure, a flow-through apparatus by which the chemiluminescence intensity(CL intensity) could be continuously measured was set up and its performance was investigated.

The present method was superior to the previous batchwise method in the following points: 1) The time necessary for the measurement of the CL intensity was reduced to about one-tenth. 2) The limit of detection was also reduced to about one-half. And 3) the coefficient of variation for the measurement was smaller.

Experimental

The following procedures were done as explained in the previous paper:[6] synthesis of iron(III) 4,11,18,25-tetrakis-(chlorocarbonyl)phthalocyanine (TCCP-Fe(III)) as a labeling reagent, labeling of TCCP-Fe(III) to the HSA chosen as an analyte model, preparation of glass beads immobilized with antibodies(Ab-bead), and the competitive immune reaction between labeled HSA and HSA.

A schematic flow diagram of the apparatus used for the measurement of the CL intensity is shown in Fig. 1. Each bottle was filled with (a) a 1.0×10^{-3} mol/dm^3 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) solution (pH 11.4, a 0.014 mol/dm^3 disodium tetraborate–0.03 mol/dm^3 sodium hydroxide buffer solution (BS-I)), (b) BS-I as a liberating solution, (c) a washing solution (pH 7.2, a 0.0072 mol/dm^3 disodium hydrogenphosphate buffer solution (BS-II)), and (d) a 7.5×10^{-3} mol/dm^3 hydrogen peroxide (H_2O_2) solution.

Each solution was fed at the flow rate of 2.5 cm^3/min by means of pump (e) (ATTO, Perista Mini-pump SJ1211). The CL intensity at the flow-cell (i), which was obtained by winding spirally a 40 cm × 0.8 mm diam. transparent poly(vinyl chloride) tube, was measured by means of a photon counter(k)(high voltage=−900 V, gate time=0.1 s, discriminator=330 mV) (Hamamatsu TV, C1230) equipped with a photomultiplier(j) (Hamamatsu TV, R464). The output was recorded with a recorder(l) (Matsushita Communication Industrial, VP6521A).

The CL intensity was measured as follows: 1) The Ab-bead (g) which had been washed with BS-II after the immune reaction was put into a sampler (f) and washed with a washing solution (c) for 2 min. 2) A liberating solution was sent onto an Ab-bead in a sampler from (b) by operating a six-way cock (h) and some labeled antigen was liberated from an immune complex. The liberated HSA and labeled HSA were both introduced into a flow-cell (i), and the chemiluminescence of luminol, which was based on the catalytic action of TCCP-Fe(III) on a labeled HSA, was measured. The maximum CL intensity was obtained in about 30 s after operating a six-way cock, and a standard curve of HSA was obtained from the maximum CL intensity. 3) The inside of the sampler was washed for 1 min with a washing solution by operating again a six-way cock 1 min after its first operation. The Ab-bead in the sampler was replaced by a new Ab-bead and the same procedure was repeated.

Results and Discussion

An immune complex dissociates in a strongly acidic or basic solution as well as a high concentration of salt solution. In order to examine the liberation of antigen from an immune complex, each Ab-bead after immune reaction was washed for 3 min with each pH of solution and the CL intensity due to the labeled HSA on an

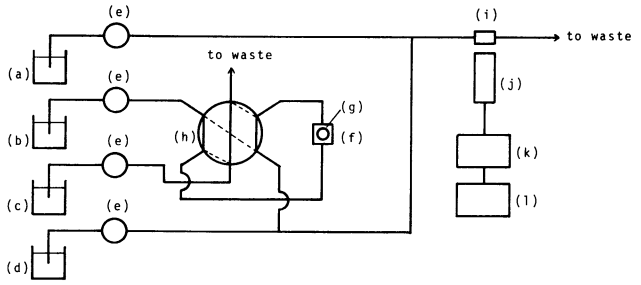

Fig. 1. Schematic flow diagram.
a: Luminol solution (pH 11.4), b: liberating solution (pH 11.4), c: washing solution (pH 7.2), d: H_2O_2 solution, e: pump, f: sampler, g: Ab-bead, h: six-way cock, i: flow-cell, j: photomultiplier, k: photon counter, and l: recorder.

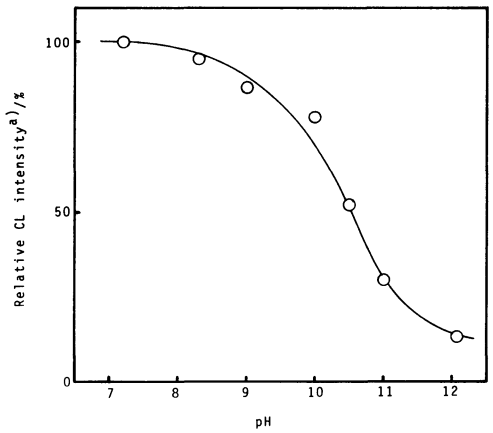

Fig. 2. Effect of pH of a washing solution on the CL intensity.
a) CL intensity at pH 7.2 of a washing solution was normalized to 100%.

Ab-bead was measured by the previous batchwise method (Fig. 2).

As shown in Fig. 2, the CL intensity decreased with an increasing pH value and this decrease corresponded to the amount of labeled HSA which was lost during washing. Most of the immune complex was found to dissociated only by putting an Ab-bead into a washing solution having pH value over 11. On the basis of this result, we achieved a rapid and accurate immunoassay, in which an antigen was liberated from an Ab-bead after the immune reaction and in which the catalytic effect of TCCP-Fe(III) in a liberated antigen solution on the chemiluminescence between luminol and H_2O_2 was measured.

A flow-through apparatus was set up to measure the CL intensity (Fig. 1). Both a luminol solution(a) and a liberating solution(b), shown in Fig. 1, were held at the same pH value and their pH values were changed simultaneously. This change was followed by measuring the CL intensity (Fig. 3). As can be seen from Fig. 3, the CL intensity increased with an increasing pH value of a luminol solution and a liberating solution, and gave a constant value in the vicinity of pH 12. By taking the large background of chemiluminescence at high pH into account, pH 11.4 was chosen as both the luminol solution and the liberating solution in the following experiments.

The relationship between the CL intensity and washing time of an Ab-bead with washing solution(c) shown in Fig. 1 was examined. No change in the CL intensity was observed in 1 to 10 min of washing time; so 2 min washing was recommended.

A standard curve of HSA was obtained under the above-mentioned optimum conditions (Fig. 4). According to the present method, up to 50 μg/tube HSA can be estimated with a limit of detection of 20 ng/tube

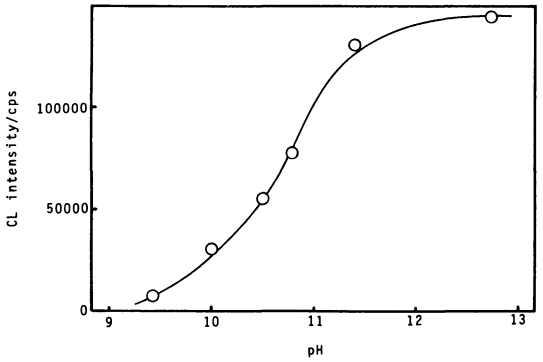

Fig. 3. Effect of pH of a luminol solution and a liberating solution on the CL intensity.

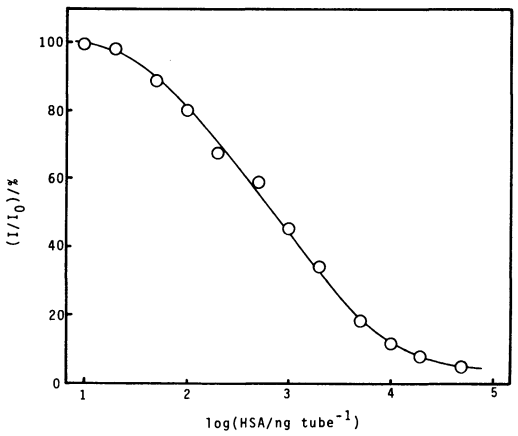

Fig. 4. Standard curve for HSA.
I, I_0: The CL intensity of the sample in the presence or absence of HSA.

and with coefficient of variation 5.8—7.2% for 9 analyses. The present method was superior to the previous batchwise method (limit of detection 50 ng/tube HSA and coefficient of variation 6—16%). The time necessary for analysis of a sample after immune reaction could be reduced to 4 min from 35 min in the previous batchwise method.

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