Use of Microtiter Plates for Latex Agglutination Immunoassay of Serum Alpha2-Macroglobulin

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Abstract. A simple, sensitive turbidimetric immunoassay is described for analysis of alpha2-macroglobulin (AMG) in serum, using microtiter plates and a commercial kit that features a sensitive latex reagent. Using microwells as reaction vessels and the latex reagent made it possible to minimize the sample size and the amounts of reagents. The procedure involves two pipetting steps and a brief incubation (10 min) at room temperature; the assay measures AMG concentrations ranging from 0.12 to 5 g/L and is unaffected by icterus, hemolysis, lipemia, or rheumatoid factor, even at high concentrations. Recovery of AMG added to three pooled serum samples averaged 95–105%. CVs for replicate analyses ranged from 1.9 to 4.8% (within-run) and 6.1 to 9.3% (between-run). Based on paired analyses of 70 serum specimens, AMG concentrations obtained by this turbidimetric microtiter immunoassay were in close agreement (correlation coefficient = 0.99) with results obtained by an enzyme-linked immunosorbent assay (ELISA). Serum AMG concentrations in 48 healthy adults (mean ± 2SD) were 1.87 ± 1.00 g/L. (received 4 December 2000, accepted 18 January 2001)

Key words: Alpha2-macroglobulin, latex immunoassay, turbidimetry, microtiter plates

Introduction

Although measurements of alpha2-macroglobulin (AMG) concentrations in serum and urine have only limited clinical significance at present [1], accurate and sensitive methods are needed for research on the biological functions and pathophysiology of AMG. For example, measuring the partition of zinc between AMG and albumin in serum requires sensitive analyses of AMG concentrations in serum samples and zinc-binding protein fractions [2]. Several immunoassay techniques for AMG have been developed, including single radial immunodiffusion (SRID) [3,4], rocket immunoelectrophoresis (RIE) [5,6], radioimmunoassay [7,8], enzyme-linked immunosorbent assay (ELISA) [7,9,10], and turbidimetric immunoassay (TIA) using polyethylene glycol (PEG) [2,11–14]. A commercial kit for urine AMG analysis has been introduced that involves a turbidimetric immunoassay, based on the agglutination of antibody-coated latex particles [15]. This technique has been used with an automated immunochemical analyzer [16]. The present paper describes an adaptation of the latex agglutination immunoassay, in conjunction with polystyrene microtiter plates and a microtiter plate reader, for the analysis of serum AMG concentrations. As shown previously with trace metal assays [17,18], this precise, convenient, and economical analytical approach provides significant reductions of sample volume and minimizes the required amounts of analytical reagents.

Materials and Methods

Instrumentation. The following instruments were employed: (1) a microtiter plate spectrophotometer...
with 670 nm interference filter and 10 nm half-bandwidth (model MPR-A4i, Tosoh Corp., Japan), (2) a micromixer for microtiter plates (model V-5, Thermal Chemical Industry, Japan), with mixing speed at setting 2.2, (3) microtiter plates with wells of 400 µl capacity (flat-bottom, 96-well type, #3881, Iwaki, Japan), (4) semiautomatic pipette of the positive displacement type for sampling (model 100, Microman-Bio, Gilson Instruments, USA), and (5) semiautomatic pipettes of the repeater type for dispensing reagents (model 4780, Eppendorf GmbH, Germany).

Reagents. This serum assay uses a commercial kit for urine AMG analysis (“LX-Eiken-AMG,” Eiken Chemical Corp., 5-26-20 Ouzi, Kita-ku, Tokyo 114-0002, Japan), which comprises three reagents: (1) “LX-buffer,” containing HEPES (N-2-hydroxyethylpiperazine-N’-2-ethansulfonic acid) buffer (0.05 mol/L) plus a surface-active agent; (2) “AMG-I” solution, containing bovine serum albumin (BSA); and (3) “AMG-II” suspension, containing rabbit anti-AMG IgG-coated latex particles. These reagents are stable at 4°C for one yr. Human Serum Protein Calibrator (X0908, Dako Corp., Shijo-dori, Sakyo-ku, Kyoto 600-8493, Japan), which contains AMG, 2.00 g/L, was used as stock standard solution. Working standards were prepared by diluting the stock standard to 100, 200, 400, 600, 1000 and 2000 ng/ml with LX-buffer.

Procedure. Reagents, including water, were kept at room temperature during the AMG assay. Water (0.25 ml) was pipetted into the circumferential wells of the microtiter plate (ie, #1-12 of rows A and H, plus #1 and #12 of rows B to G), and into all unused wells. Serum samples were diluted 5000-fold with LX-buffer solution. Samples (40 µl) of reagent blank (water), working standards, and diluted serum samples were pipetted into respective wells, followed by addition of AMG-I solution (150 µl). The microtiter plate was then mixed for 30 sec. AMG-II solution (50 µl) was added and the plate was mixed for 45 sec. At 1 min (T1) and 10 min (T2) after mixing was begun, the absorbance of each well was measured at 670 nm. The increase in absorbance (ΔA = T2–T1) was directly proportional to the AMG concentration (ng/ml) in the working standards and diluted serums. Finally, the values for the diluted serums were multiplied by 0.005 to express the AMG concentration as g/L of undiluted serum.

**Fig. 1.** The effect of reaction time on the calibration curves for AMG immunoassays performed at 20°C. Absorbance measurements were made at T2 = 5 min (+), 10 min (solid circles), 15 min (x), 20 min (open triangles), 25 min (open squares), or 30 min (solid diamonds).

**Fig. 2.** The effect of reaction temperature on the calibration curves for AMG immunoassays performed with T2 = 10 min. The incubation steps and absorbance measurements were at 17°C (open triangles), 20°C (solid circles), or 23°C (open squares).
Method comparisons. Two comparison assays were used: a fully automated procedure using same reagents with an immunochemical analyzer (model LX-2200, Aloka, Japan) [15,16] and the ELISA procedure of Voller et al [9], with modifications [7].

Results

Reaction time. Fig. 1 shows calibration curves for AMG immunoassays with reaction times that ended (T2) at 5, 10, 15, 20, 25, or 30 min after mixing. The reaction period that ended 10 min after mixing was chosen for routine use, since this calibration curve provided adequate sensitivity and was linear throughout the concentration range from 0 to 1000 ng AMG/ml.

Reaction temperature. Fig. 2 shows calibration curves for AMG immunoassays that were performed at 17°C, 20°C, and 23°C. The slopes of the calibration curve were slightly different at the three reaction temperatures, but no significant differences were found in the AMG concentrations of 10 serum samples from patients, assayed at the three temperatures and calculated from the respective calibration curves. The observed AMG concentrations (mean ± SD) were: 17°C, 1.90 ± 0.87 g/L; 20°C, 1.88 ± 0.85 g/L; and 23°C, 1.90 ± 0.86 g/L.

Detection limit. The analytical detection limit (corresponding to the reagent blank + 2SD) was 24.5 ng/ml (ie, 0.12 g AMG/L of serum). The linear range of the AMG assay was 25 to 1000 ng/ml (from 0.125 to 5 g/L of serum).

Interference effects. Potential sources of interference were investigated by analyzing pooled serum samples that had been spiked with bilirubin, hemoglobin, triglycerides, or rheumatoid factor. Hemolysis (≤5 g/L hemoglobin), icterus (≤0.5 g/L bilirubin as bilirubin diturate disodium salt, Porphyrin Products, USA) or lipemia (≤20 mmol/L triglycerides, as “Intralipos,”...
Green Cross, Japan) did not affect AMG determinations. The serum AMG assay was unaffected by spiking with rheumatoid factor (≤500 IU/ml, as “High Level Check·RF,” International Reagents, Japan).

Analytical recovery. The analytical recovery of AMG was measured by adding AMG standard solutions (0.1 to 0.3 g/L) to three pooled serum samples that had been diluted 1:100 with LX-buffer. One volume of AMG standard solution was mixed with 9 volumes of each diluted serum. The recovery of added AMG averaged 95–105%. When 3 serum samples with initial AMG concentrations from 2 to 5 g/L were serially diluted, the results of AMG assays were linearly related to the calculated diluted concentrations.

Precision. Data for within-run and day-to-day precision of serum AMG assays are listed in Table 1, based upon replicate analyses of two pooled serum samples with AMG concentrations of approximately 1.0 and 2.7 g/L. The respective coefficients of variation were 4.8 and 1.9% (within-run) and 9.3 and 6.1% (day-to-day).

Reference intervals. Assays of AMG concentrations in serum samples from 48 healthy adults (both sexes, ages 20–54 yr) gave the following reference interval (mean ± 2SD): 1.87 ± 1.00 g/L.

Method comparison studies. Analytical results for AMG concentrations in patients’ sera by the present method (Y-axis) are compared with those obtained with a fully automated immunoanalyzer, LX-2200 (n = 80, X-axis, Fig. 3), or by an ELISA assay (n = 70, X-axis, Fig. 4). The correlation coefficients (r) were 0.975 and 0.998, respectively (p <0.01).

Discussion

The various immunoassay techniques for AMG determination have their respective advantages and disadvantages. The SRID and RIE techniques give relatively poor precision and SRID requires a long reaction time. The ELISA method is sensitive and accurate, but is considerably more complex than other immunoassays. The kinetic TIA with PEG is simple, rapid, and precise, but shows nonspecific agglutination with IgM and interference from lipemic sera [19]. The TIA using latex particles is 10–100 times more sensitive than the TIA using PEG. However, the TIA using latex particles usually requires an expensive automated apparatus. Using latex agglutination reagents and microtiter plates, Collet-Cassart et al [20,21] assayed serum concentrations of placental lactogen and β2-microglobulin. The present paper describes a TIA assay for serum AMG that uses a commercial kit for urine AMG in conjunction with microtiter equipment.

Collet-Cassart et al [20,21] employed end-point assays that require a longer running time, whereas the present method uses a fixed-time kinetic assay. This system is rapid and has a wide analytical range, but the time and the temperature during the latex agglutination reaction must be maintained accurately. Although differences in reaction temperature slightly changed the slope of the calibration curve, they did not affect the observed concentrations of serum AMG.

In TIA assays that use PEG, human serum albumin in the standards binds nonspecifically to polystyrene and glass tubes [22]. In TIA that involve latex agglutination, the antibody-coated latex particles are also adsorbed to the reaction vessels [23,24]. Moreover, in a previous study [25], serum proteins were adsorbed onto the surface of the polystyrene microwells, causing analytical interference. In the present method AMG-I solution, which contains BSA, is added to microwells.
before the addition of antibody-coated latex particles. This prevents the adsorption of latex particles to the microwell.

Icteric, hemolyzed, or lipemic serum samples did not affect the present assay for AMG, because the serum samples were prediluted by 1:5000 (v/v). The accuracy of the present serum AMG assay, evaluated by analytical recovery and dilution tests, appears satisfactory. The accuracy of AMG measurements by the present method is supported by the correlation coefficient of 0.99 with results by an ELISA assay.

The within-run precision (CV) of the present method (1.9–4.8%), is similar to that (1.9–2.9%) obtained by a kinetic TIA using PEG [2], and that (3.9–4.9%) obtained by laser nephelometry [14].

The author has recently employed the present technique, together with a similar assay for serum albumin [26], for measurements of serum AMG-bound zinc and albumin-bound zinc by affinity chromatography (paper submitted for publication).

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