Polyclonal Antibody-Based Enzyme-Linked Immunosorbert Assay of \(\alpha_1\)-Acid Glycoprotein

Béatrice Tisot, Nathalie Seta, Geneviève Durand, and Joanne Feger

This is a noncompetitive enzyme-linked immunosorbent assay for measuring low concentrations (2 to 100 \(\mu\)g/L) of human \(\alpha_1\)-acid glycoprotein (AGP; orosomucoid). The method is based on a simple "sandwich" technique involving polyclonal rabbit antiserum against AGP. Mean within-run and total (between-run) CVs were 6.2% and 9.7%, respectively. Analytical recovery, tested in various biological fluids, averaged 101%. The technique has been successfully applied to diluted biological fluids such as bronchoalveolar lavage, cerebrospinal and amniotic fluids, and hepatocyte-culture supernates. Because of its analytical validity and the commercial availability of the reagents, this assay is suitable for large-scale determinations of AGP concentrations in those biological fluids in which its concentration is relatively low.

Additional Keyphrases: orosomucoid · acute-phase proteins · radial immunodiffusion compared

\(\alpha_1\)-Acid glycoprotein (AGP, orosomucoid) is one of the most extensively studied acute-phase proteins in serum. Increased concentrations of AGP are known to be associated with various inflammatory diseases (1, 2), trauma (3), malignancies (4, 5), myocardial infarction (6), and even chronic pain (7). For these numerous investigations, several immunological methods have been described, including single radial immunodiffusion (8), electroimmunoassay (9), laser-based immunonephelometry (10, 11), and immunoturbidimetry (12, 13). Although these methods are well adapted for assays of AGP in plasma or other fluids in which it is present in high concentration, assay of AGP in biological fluids where it is less concentrated has received little attention. A more sensitive radioimmunoassay (14) and an enzyme-linked immunosorbent assay involving monoclonal antibodies (15) have been reported for evaluation of AGP in such fluids, but these methods are not convenient for routine use in a clinical laboratory.

For large-scale clinical investigations, a simple, accurate, extremely sensitive, and inexpensive test is still needed for quantifying low concentrations of AGP in body fluids other than serum. The assay presented here appears to meet these requirements.

Materials and Methods

Reagents

We used the following reagents: calf intestinal alkaline phosphatase (EC 3.1.3.1), grade I, with a specific activity >2500 U/L (Boehringer Mannheim, Mannheim, F.R.G.); bovine serum albumin (Sigma Chemical Co., St. Louis, MO); Tween 20 surfactant and diethanolamine (Prolabo, France); p-nitrophenyl phosphate and glutaraldehyde, 250 g/L (Merck, Darmstadt, F.R.G.); and Ultrogel ACA 22 (I.B.F., Villeneuve-La-Garenne, France). The AGP we used was either kindly provided by Behring (Marburg, F.R.G.) or purified by affinity chromatography in our laboratory from pooled serum from adults. Antiserum to human AGP was either purchased from Behring or raised in rabbits (blanc du Bouscat strain).

Specific anti-AGP immunoglobulins: These were obtained by using affinity chromatography on Ultrogel ACA 22 bound to human AGP, according to the manufacturer's instructions (I.B.F.). After purification and concentration, immunoglobulins kept at \(-20^{\circ}C\) could be used for as long as six months.

Conjugate: Purified anti-human AGP immunoglobulins were labeled with alkaline phosphatase according to the one-step glutaraldehyde-coupling procedure described by Avrameas et al. (16). Stored at \(-20^{\circ}C\), these could be used for as long as six months. Before use, the immunoglobulins were diluted 1000-fold in a solution containing 10 g of bovine serum albumin and 0.15 mol of NaCl per liter.

Buffers: Coating solution, 50 mol/L solution of anti-AGP immunoglobulins, was dissolved in sodium bicarbonate buffer (100 mmol/L, pH 9). The assay diluent was 10 g of bovine serum albumin per liter of 0.15 mol/L NaCl. The washing solution was 50 g of Tween 20 surfactant per liter of 0.15 mol/L NaCl and the substrate solution was a 1 g/L solution of p-nitrophenyl phosphate in 1 mol/L diethanolamine buffer containing, per liter, 1 mmol of MgCl₂·6 H₂O, adjusted to pH 9.8 with 12 mol/L HCl. This solution is to be prepared just before use.

Standards: We diluted in assay diluent a solution (1 g/L; \(e^{1%}_{278} = 8.93\)) of purified human AGP in 0.15 mol/L NaCl to yield concentrations of 2, 10, 25, 50, 75, and 100 \(\mu\)g/L.

Apparatus

We used 96-well flat-bottomed microtiter plates (Biokema, Nimes, France). Absorbance was measured in an EL-308 EIA Reader (Biotek Instrument Inc., OSI, Paris, France).

Samples

Serum was obtained from healthy volunteers. Biological fluids such as bronchoalveolar lavage and cerebrospinal and amniotic fluids were collected from patients who were undergoing specialized tests. Culture supernates of Hep G2 hepatocytes were obtained after two and five days of culture under standard conditions without hormones in the medium (17). All samples were stored at \(-20^{\circ}C\) until use.

Procedure

Optimal reaction conditions (incubation times, temperature, and antibody concentrations for the different enzyme-linked immunosorbert assay steps) were determined in preliminary experiments and were chosen to be practical in routine use. All reagents and samples were added to the wells in 250-\(\mu\)L volumes, and each incubation step was followed by a washing step (three washes with the washing solution, the last one lasting 10 min).

Microtiter plates were coated with the coating solution by incubating the plates for 4 h at 37 \(^\circ\)C and then washed.
They could be used after being stored for one week at 4 °C with the buffer in the wells or, dry, for one month at −20 °C.

Samples, prediluted or not, and standards were incubated in duplicate for 2 h at 37 °C. Then, we added alkaline phosphatase-labeled immunoglobulins and incubated for 2.5 h at 4 °C. Freshly prepared substrate solution was applied to all wells and the absorbance at 405 nm was measured after 10 min at 37 °C.

Results

Standardization: Figure 1 shows a typical standard curve. The relation between 2 and 50 μg/L was linear. The standard curve obtained with purified AGP paralleled that obtained with a serially diluted serum specimen (Figure 1), indicating the specificity of the assay for AGP in human serum. Sensitivity, defined as the lowest concentration that could be differentiated from the zero standard with 95% confidence, was 1.5 μg/L. The detection limit, defined as the lowest concentration that could be differentiated from the results for the dilution buffer with 95% confidence (18), was <1 μg/L.

Precision: Table 1 summarizes within- and between-run coefficients of variation (CV). The former was estimated with 12 successive assays of three control samples (33, 64, 93 μg/L), in a single run (overall mean CV 6.2%), the latter by assaying the same control samples for five consecutive days (mean CV 9.7%).

Specificity and accuracy: Analytical recoveries, measured after adding increasing amounts of purified AGP to the different biological fluids, averaged 101% (range 96% to 108%). Recovery was identical for all fluids (Table 2). Human serum albumin did not affect the assay at concentrations of 5 mg/L or less in the diluted sample (results not shown).

Comparison with radial immunodiffusion: Results obtained with the present assay (y) for serum samples with AGP concentrations ranging up to 1.5 g/L were compared with those obtained by single radial immunodiffusion (x). Linear least-squares regression analysis gave the following equation: y = 1.03x − 0.06 g/L (n = 53, r = 0.916).

Concentrations in AGP in different fluids: Using the present assay, we measured concentrations of AGP in samples of cerebrospinal fluid, bronchoalveolar lavage fluid, and amniotic fluid. In cerebrospinal fluids (n = 12), AGP ranged from 4 to 16 mg/L; bronchoalveolar lavage fluids (n = 19) contained between 0.4 and 1.2 mg/L; and amniotic fluids (n = 5) contained between 25 and 45 mg/L.

AGP was determined in the culture supernates of Hep G2 cells after only a two- to 10-fold dilution. On the second day of culture, 240 μg of AGP had been secreted per liter, corresponding to 1.2 μg/10⁶ cells, and on the fifth day, we measured 500 μg of AGP per liter, corresponding to 1.56 μg/10⁶ cells.

Discussion

Quantification of AGP in biological fluids is of great interest, not only in exploring various inflammatory states but also in pharmacology, because AGP is the major binding protein for many basic drugs (19, 20). Different types of assay have been developed and are well adapted to determinations of AGP in serum or fluids with similar AGP concentration, but are not sensitive enough for directly measuring AGP in most other biological fluids. We present here a simple “sandwich” immunoassay for human AGP, which has the advantages of being sensitive and economical. Its working range is better than that in other techniques for AGP determinations (15, 21). The procedure we have chosen takes into account optimal working conditions but is also adapted to routine laboratory situations. Polyclonal antisera against AGP are commercially available and more economical than monoclonal antibodies in immunoassays (15).

The analytical performances of the proposed method were confirmed in various biological fluids that contained previously established concentrations of AGP. Our results are similar to those reported for bronchoalveolar lavage fluid (22) and cerebrospinal fluid (23). Formation of the antigen–antibody complex depends on a minimum protein concentration in the assay medium. Because of the high sensitivity of the assay, samples and standards are diluted...
in the same protein-containing assay diluent. This offers easy standardization of the protein concentration in our assay, something that is difficult with other immunochromatographic methods such as laser nephelometry. Moreover, because of its high sensitivity, this procedure can be used with any body fluids and culture media without a preliminary concentration step, which might alter the structure of the protein and (or) lead to some loss.

Dr. M. Petit (INSERM U131, Clamart, France) provided us with Hep G2 culture media. We thank Drs. D. Biou and D. Porquet for their advice. This work was supported by a grant from INSERM (contrat libre externe n. 857-007) and CNRS URA 622.

References

Dichlorobenzoquinone Chloroimine Colorimetry of Uric Acid in Urine

Peter Miller and Victor Oberhofer

In this nonenzymatic colorimetric method, dichlorobenzoquinone chloroimine is used as a color reagent to measure uric acid in urine. This method is less subject to the interferences that make enzymatic methods unreliable and is inexpensive, simple, and fast.

Most methods currently used to measure uric acid are based on the use of uricase (urate oxidase, EC 1.7.3.3) to convert urate to allantoin, followed by measuring the hydrogen peroxide formed by coupling the reaction to the formation of NADPH or to the oxidation of a colored dye. These methods are expensive, the reagents are labile, and use of the correct temperature is critical. Although satisfactory for serum, these methods are subject to many interferences (e.g., by bilirubin, xanthine, and ascorbate) when used for urine (1, 2).

The phosphotungstic acid method previously widely used is also subject to many interferences, e.g., ascorbate, thiols, caffeine, theobromine, gentisic acid (a metabolite of salicylates), paracetamol (acetaminophen), and glucose. Of the color formed by this method when it is used for analyzing urine, only 80-90% is ascribable to urate (3).

Here we report a simple, specific method for measuring uric acid in urine. It requires only inexpensive stable reagents and a bare minimum of equipment, and could be easily automated. Dichlorobenzoquinone chloroimine has been used previously to make uric acid visible on thin-layer chromatography plates (4). To our knowledge, its use for quantifying uric acid has not been reported previously.