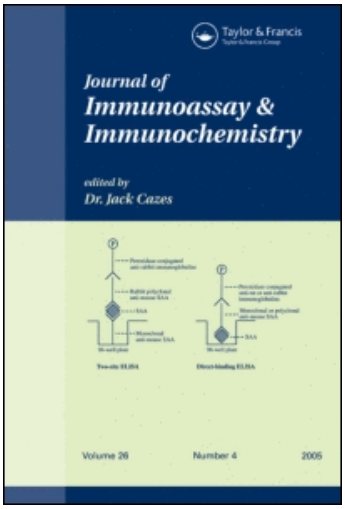

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Yoshiyuki G. Takei[a]; Tamotsu Honma[a]; Akio Ito[a]
[a] Research and Development Center, Yuka Medias Corporation, Inashiki, Ibaraki, Japan

Online publication date: 04 March 2002

To cite this Article Takei, Yoshiyuki G. , Honma, Tamotsu and Ito, Akio(2002) 'QUANTITATION OF HYALURONIC ACID IN SERUM WITH AUTOMATED MICROPARTICLE PHOTOMETRIC AGGLUTINATION ASSAY', Journal of Immunoassay and Immunochemistry, 23: 1, 85 — 94
To link to this Article: DOI: 10.1081/IAS-120002276
URL: http://dx.doi.org/10.1081/IAS-120002276

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 23(1), 85–94 (2002)

QUANTITATION OF HYALURONIC ACID IN SERUM WITH AUTOMATED MICROPARTICLE PHOTOMETRIC AGGLUTINATION ASSAY

Yoshiyuki G. Takei,* Tamotsu Honma, and Akio Ito

Research and Development Center,
Yuka Medias Corporation, 8-5-1 Chuo,
Ami, Inashiki, Ibaraki 300-0033, Japan

ABSTRACT

A microparticle photometric agglutination assay to quantitate hyaluronic acid (HA) in serum has been developed. The principle of the method is that hyaluronic acid binding protein covalently sensitized on microparticle surface initiates the particles to agglutinate in the presence of HA. By measuring the time-course of transmittance changes at 800 nm, due to the particles' agglutination, HA concentration is estimated as a function of the magnitude of agglutination in a fully automated immunochemistry analyzer. The analytical range for HA was found to be between 10 and 1200 ng/mL. The precision (CV) was between 3.0 and 8.4% in the intra-assay ($n = 10$), and 4.8 and 8.9% in the inter-assay ($n = 3$). The lower limit of detection was 10 ng/mL. The deviation of the linearity study indicated within 8% of expected values, and

*Corresponding author. E-mail: med0033@cc.m-kagaku.co.jp

the analytical recovery was between 96.5 and 106.3%. The correlation coefficient against HA plate ELISA was 0.989 with a slope of 1.01 ($n = 31$).

INTRODUCTION

Hyaluronic acid (HA) has been recognized and focused as a marker to indicate the changes in the basic structure of liver.(1–5) The increase in HA concentration in sera was observed for some liver diseases, such as liver cirrhosis with hepatic sinusoidal capillarization or active fibrosis.(6,7) Detection and quantitation of HA is of interest in many fields of biology, including that of clinical chemistry.

Hyaluronic acid binding protein (HABP), with a molecular weight of 6.7 kDa, is well known to exhibit high affinity with HA, and to avoid cross-reacting with other polysaccharides and glucossamines.(8) The specific interaction of HABP with HA has been applied to quantitate HA levels in biological fluids. Tengblad et al. described a radioimmunoassay using purified ^{125}I-HABP and HA-immobilized sepharose beads for the determination of HA in human sera and other biological fluids.(9,10) While this method has enabled to quantitate low levels of HA in biological samples, it necessitates the frequent preparation of ^{125}I-HABP, due to a relatively short half-life. An enzyme linked immunosorbent assay (ELISA) has been developed by Delpech et al., using the brain-derived hyaluronectin.(11) However, as HA has low affinity for the microplates, the method requires immobilization of hyaluronectin to the wells and this has presented some difficulties in the routine application of this approach. Other modified ELISA techniques have been developed using monoclonal antibodies against the HA-binding region of proteoglycan monomer or keratan sulfate.(12,13) These methods demonstrated sufficient sensitivity and specificity for HA, but the complicated handling, such as preparation of monoclonal antibodies and the procedures of measurement were indispensable.

We have developed a microparticle photometric agglutination (MPA) assay for HA measurement utilizing the affinity of HABP to HA. The principle of the reaction is that HABP covalently sensitized on a microparticle surface initiates the particles' agglutination and alters the transmittance at 800 nm. Since the time-course of transmittance changes at 800 nm, due to the particles' agglutination, HA concentration was automatically monitored in the analyzer and is estimated as a function of the magnitude of agglutination. We have applied MPA assay for the quantitative measurement of HA in sera on the LPIA-S500, a fully automated immunochemistry analyzer

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(Mitsubishi Chemical Co., Tokyo, Japan). Here, we describe the development and analytical performance of this assay.

EXPERIMENTAL

Automated MPA Assay for HA

The LPIA-S500, a fully automated immunochemistry analyzer, was used for HA assay. The principle of this assay is based on the ability of HABP to bind HA. A schematic representation of the assay is shown in Figure 1. The agglutination reaction is initiated by recognizing the binding region on HA with the specific HABP sensitized on microparticle surface.

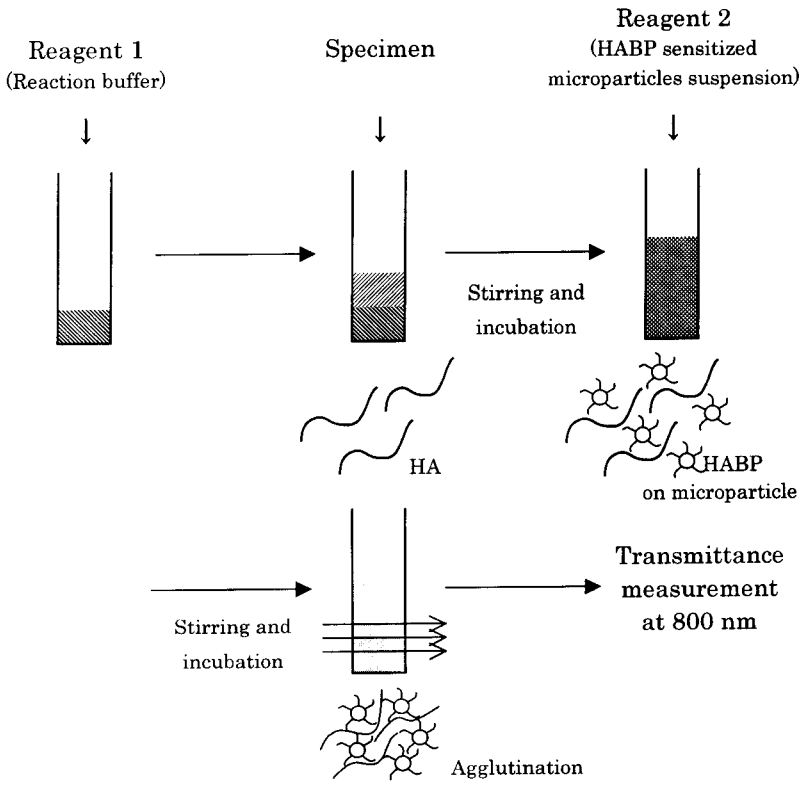

Figure 1. Schematic outline of the microparticle photometric agglutination (MPA) assay procedure.

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This reaction proceeds to make the agglutination clumps, which are optically detected in the analyzer. The transmittance at 800 nm is monitored for 10 min at 37°C, and the agglutination reaction rate was defined by the formula as follows (14):

$$\text{Agglutination reaction rate } (V) = ([\text{dAbs}]/[10\,\text{min}]) \times 10^4$$

where dAbs is the integration of transmittance changes during 10 min. The analyzer automatically converts the transmittance changes into HA concentration according to a pre-established calibration curve.

Reagents

HA was purified from bovine nasal cartilage by the method of Laurent et al..(9) Carboxylated polystyrene microparticles (average diameter $0.400 \pm 0.04\,\mu m$) were purchased from Japan Synthetic Rubber Corporation, Japan, as a 10 w/v% aqueous suspension. 2-(*N*-Morpholino)-ethanesulfonic acid (MES) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, HCl salt (EDC) were obtained from Dojin Chemical, Japan. Unless otherwise noted, all other chemicals were reagent grade and were purchased from Wako Pure Chemicals, Japan.

HABP was coupled covalently with the microparticle surface in aqueous media. These coupling procedures were performed at room temperature. First, 3.2 mL of 50 mmol/L MES (pH 5.5) and 0.4 mL of 10 w/v% microparticle suspension were added to a reaction tube with stirring. Then, 100 μL of EDC aqueous solution (10 mg/mL) was added into the reaction mixture with stirring. After 15 min stirring, 100 μL of HABP (2 mg/mL in 50 mmol/L MES, pH 5.5) was added into the reaction mixture. After 90 min of HABP addition with stirring, 2 mL of bovine serum albumin (BSA, 1 wt% in 50 mmol/L MES, pH 5.5) was added into the reaction mixture, and then the mixture was stirred continuously for 40 min. After centrifugation, the supernatant was aspirated, and then the pellet of HABP-sensitized microparticles was resuspended in 4 mL of 0.03 wt% NaN_3 aqueous solution to wash with stirring for 30 min. After washing twice with the procedure described above, HABP-sensitized microparticles were centrifuged and resuspended in 0.03 wt% NaN_3 aqueous solution. Finally, the HABP-sensitized microparticle suspension was adjusted its concentration to 0.20 wt% and was used for the assay.

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Assay

Reagent 1 as a reaction buffer contains 0.2 M *tris*, 0.15 M NaCl, 0.5 wt% BSA, 0.8 wt% dextran sulfate, and 0.03% NaN_3. Reagent 2 is 0.20 wt% of HABP-sensitized microparticles suspension in 0.03 wt% NaN_3 aqueous solution. First, reagent 1 (210 μL) and sample (16 μL) to be analyzed were pipetted into a reaction cuvette and incubated at 37°C in the LPIA-S500 for 5 min. Second, 80 mL of reagent 2 (HABP-sensitized microparticles suspension) was pipetted into the reaction mixture. After vigorous stirring for 2 s, measurement the transmittance at 800 nm was started. From the results of 10 min measurement, the LPIA-S500 software program calculated the agglutination reaction rate. During these procedures, the reaction cuvette was kept at 37 ± 1.3°C. All the procedures described above were performed automatically.

The calibration curve for HA was constructed using linear coordinates of the HA concentration against the agglutination reaction rate. The HA concentration in tested samples was determined by comparing the agglutination reaction rate relative to this calibration curve. As the calibrators, hyaluronic acid was dissolved in saline and diluted to the concentration of 20, 60, 200, 600, and 1200 ng/mL.

Precision of HA assay was evaluated by the analysis of three levels of samples and the calibrators. The lower limit of detection was evaluated by determination of the minimum detectable concentration of HA that could be statically distinguished from the concentration of the zero calibrator.

To validate linearity over the dynamic range of the assay, two serum samples (197.80 and 311.70 ng/mL) were diluted with saline to generate the series of dilutions over the entire calibration range. The dilution samples containing 100, 80, 60, 40, 20, and 0% of initial serum samples were assayed. Linear regression analysis for the results of the diluted samples, compared to the expected values, was used to assess linearity. For the recovery of HA added to serum, calibrators of 600, 1200, and 2000 ng/mL were used as the additives. Samples to be analyzed were prepared as the mixture of calibrator and serum, in which the volume ratio was 1 : 9.

The HA microtiter plate ELISA (Chugai Pharmaceuticals, Japan) was used according to the manufacturer's instructions. For the method comparison study, 31 serum samples were tested in duplicate, using both the MPA assay and the ELISA. Assay values generated from the methods were compared by least-squares regression statistics.

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RESULTS AND DISCUSSION

As shown in Figure 2, the calibration curve was analyzed by the least-squared linear regression analysis and was linear over the range studied ($y = 0.344\,x + 2.084$, $r = 0.999$, $n = 5$). Linearity was confirmed in the range of 10 to 1200 ng/mL. No high-dose hook effect was observed when HA was added to samples up to 10 000 ng/mL. The reproducibility of this method was ascertained by measuring the lower calibrator, 4.9% (CV) at 20 ng/mL, $n = 10$. Kondo et al. reported that the level of HA in serum of healthy subjects was 30 ng/mL, and the level of HA was approximately 200 ng/mL in typical rheumatoid arthritis patients.(15) From these results, automated MPA assay for HA exhibited sufficient analytical range and reproducibility.

For precision data of HA measurement, the results of three levels of samples are represented in Table 1. The intra-assay CV was determined by analyzing three samples with low, intermediate, and high concentration of HA; the means $\pm$ SD (CV) were 310.95 ± 0.37 ng/mL (2.0%), 198.39 ± 0.99 ng/mL (1.6%) and 108.14 ± 2.33 ng/mL (4.2%), respectively.

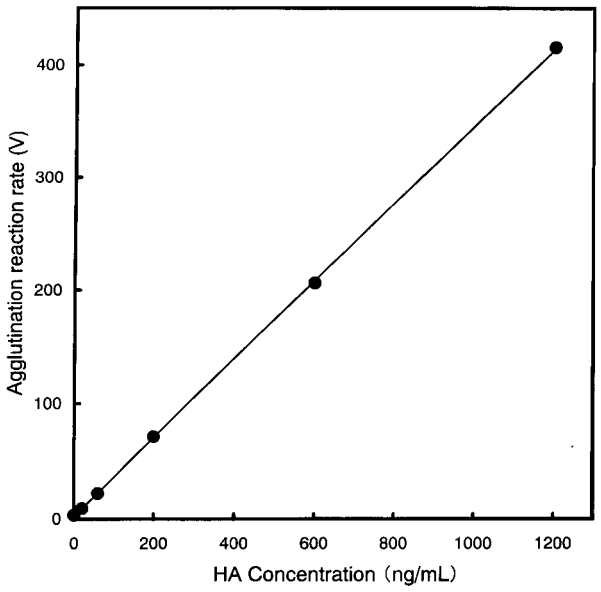

Figure 2. Calibration curve of the MPA assay for hyaluronic acid. The hyaluronic acid concentrations of calibrators were 0, 20, 60, 200, 600, and 1200 ng/mL. The calibration curve was constructed by fitting the concentration (x) vs agglutination reaction rate (y) with least-squared linear regression ($y = 0.344x + 2.084$, $r = 0.999$).

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Table 1. Intra- and Inter-Assay Precision

	Intra-Assay			Inter-Assay		
	Mean (ng/mL)	SD (ng/mL)	CV (%)	Mean (ng/mL)	SD (ng/mL)	CV (%)
Sample 1	310.95	0.37	2.0	311.17	2.33	0.7
Sample 2	198.39	0.99	1.6	198.41	3.22	0.6
Sample 3	108.14	2.33	4.2	107.56	0.23	1.7

The inter-assay variation (three determinations performed on three days with a new calibrator for each day) gave the means ± SD (CV); 311.17 ± 2.33 ng/mL (0.7%), 198.41 ± 3.22 ng/mL (0.6%), and 107.56 ± 0.23 ng/mL (1.7%), respectively. The lower limit of detection, defined as the concentration of 2.5 SD above the value for the level zero calibrator, was 10 ng/mL. Since some authors reported that HA concentration in normal human sera indicated approximately 20 ng/mL (7,8), the results of this assay exhibited sufficient accuracy for clinical use.

Linearity was examined by measuring HA in six serial dilutions of two high HA concentration serum samples diluted with saline. Table 2 shows the results for analysis of dilutions of serum samples with HA concentrations of 197.80, and 311.70 ng/mL. Linear regression of the observed HA (y) vs the calculated HA (x) gave the following equation: $y = 1.0041x - 0.864$ ($r = 0.9993$) for 311.70 ng/mL, and $y = 0.9562x + 1.492$ ($r = 0.9986$) for 197.80 ng/mL. These results indicated a deviation between 92.7 and 104.1%, respectively. As shown in Table 3, the HA recovery was determined by adding known amount of HA to samples. It varied between 96.5 and 106.3% of the theoretical values. These results demonstrated that there were no inhibitory or interfering substances in serum.

Further validation of the method was done by comparing the HA values of the MPA assay to the ELISA. A total of 31 serum samples were tested in duplicate in both the MPA assay and HA plate ELISA, as shown in Figure 3. The concentration range for the MPA assay was 60 to 600 ng/mL. A comparison of the results obtained with the method presented here and those of the ELISA indicated good agreement between the methods. The correlation coefficient between the values obtained by these two methods was 0.989 with a slope of 1.01.

The purpose of the present study was to establish a simple and sensitive MPA assay for HA measurements. Most methods for determining HA in biological samples are time-consuming, or need sample pretreatment. The LPIA-S500 system, a fully automated immunochemistry analyzer, is

Table 2. Dilution Linearity

Dilution Factor	Hyaluronic Acid (ng/ml)		Recovery (%)
	Calculated	Observed	
Sample 1			
1.0		311.70	
0.8	249.36	252.13	101.1
0.6	187.02	182.35	97.5
0.4	124.68	127.58	102.3
0.2	62.34	58.57	94.0
0.0	0.00	1.03	
Sample 2			
1.0		197.80	
0.8	158.24	150.85	95.3
0.6	118.68	115.00	96.9
0.4	79.12	82.35	104.1
0.2	39.56	36.66	92.7
0.0	0.00	0.88	

The samples containing 311.70 and 197.80 ng/mL hyaluronic acid were diluted with saline to the concentration indicated.

Table 3. Recovery of Hyaluronic Acid Added to Serum

Hyaluronic Acid (ng/mL)			Recovery (%)
Added	Calculated	Observed	
Sample 1			
0		42.55	
50	88.30	85.82	97.2
100	138.30	144.66	104.6
200	238.30	253.31	106.3
Sample 2			
0		216.21	
50	244.59	236.03	96.5
100	294.59	300.48	102.0
200	394.59	408.01	103.4

For the additives, the calibrator of 600, 1200, and 2000 ng/mL were used. Samples to be analyzed were prepared as the mixture of calibrator and serum, in which the volume ratio was 1 : 9.

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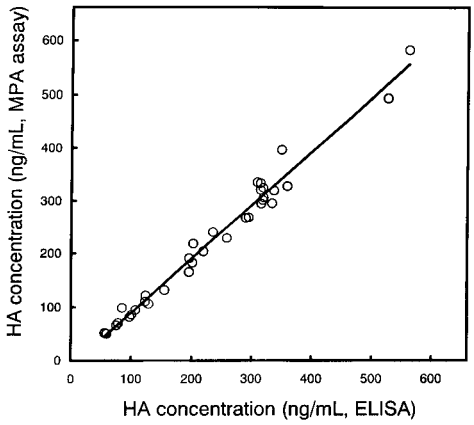

Figure 3. Method comparison of the results from the MPA assay (y) and the ELISA (x) for hyaluronic acid. The equation for the line is: $y = 1.01\,x - 11.70$ ($r = 0.989$, $n = 31$).

extremely simple to handle and does not require any complicated procedures. Considering the performance shown here, we conclude that MPA assay enables us to observe HA changes in sera more extensively with high reliability. This is the first description of a fully automated assay for HA in serum and the first development of reagents for the MPA assay utilizing the specificity of binding proteins. Finally, because the turnaround time of the method is as short as 15 min per sample, it is well suited for the clinical field, where large numbers of samples are analyzed.

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Received May 14, 2001
Accepted November 10, 2001
Manuscript 3013

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