Evaluation of the first automatized hyaluronic acid assay

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INTRODUCTION

Hyaluronic acid (HA, hyaluronan), ubiquitously distributed in the extracellular spaces, is a linear polysaccharide built from repeating disaccharide units ([D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]$_n$) of molecular weight 10^4-10^7 daltons.

In the liver, HA is mostly synthesized by the hepatic stellate cells and degraded by the sinusoidal endothelial cells. In chronic liver diseases, increases in serum HA levels occur together with the development of liver fibrosis. First, enhancement of HA production by the activated stellate cells may contribute to the increase in serum HA levels observed in patients with chronic liver disease. Later, when cirrhosis is constituted, reduced degradation by sinusoidal endothelial cells may cause greater HA increases. In patients with cirrhosis, high serum HA levels were associated with the occurrence of complications or death and correlated with clinical severity .

HA serum determination is used alone or in association with other tests for non invasive assessment of liver fibrosis in patients with chronic liver disease. Currently, only an enzyme-linked binding protein assay proposed as a self contained kit in the 96well ELISA format (HA Test Kit, Corgenix, Westminster, USA) is commercially available. This assay needs duplicate determinations (standards and samples), must be proceeded in batches and, unless the laboratory possesses an automated ELISA plate processor, is time-consuming.

We evaluated a new reagent for the quantitative determination of HA using the latex agglutination method that can be applied to clinical chemistry analyzers (hyaluronic acid detection reagent, Latex method, Wako, Osaka, Japan).

MATERIALS AND METHODS

Principle : A sample is mixed with recombinant hyaluronic acid binding protein (rHABP), and HA in the sample combines specifically with rHABP.
In order to make an insoluble aggregate, latex particles coated with anti-HABP antibody are added, and the latex binds to the above complex. As a result, the insoluble aggregate increases turbidity in the solution. The degree of turbidity of the solution can be measured optically and is proportional to the HA concentration in the serum.

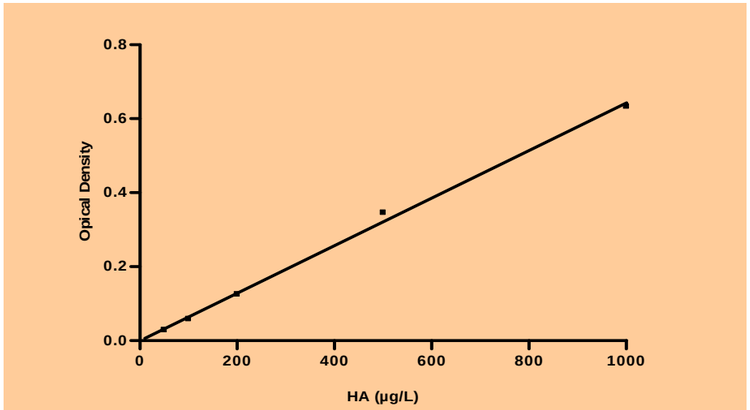
Reagents : The reagents are designed to be used in a commercially available automated analyzer. Reagent 1: Recombinant hyaluronic acid binding protein (rHABP): 32 mL, ready to use and stored at 2-10°C. After opening the bottle, it is stable for 30 days at 2-10°C. Reagent 2: Latex sensitized with anti-HABP antibody (mouse, monoclonal): 12 mL, ready to use and stored at 2-10°C. After opening the bottle, it is stable for 30 days at 2-10°C.
Calibrators: Solutions containing 50, 100, 200, 500 and 1000 µg/L HA, ready to use and stored at 2-10°C.

Test procedure : An OLYMPUS AU640 (Tokyo, Japan) analyzer was used.
Temperature: 37°C; At T_0 : Sample/Calibrator (3.0 µL) and Reagent 1 (180µL) are mixed; After 3.5 min: addition of Reagent 2 (60 µL); OD measurement at 800 nm from T=4.2min to T=8.4min.
One calibration per week was made with double measurements of saline blank (0 µg/L) and calibrators. Autodilution of the sample (1/5) was programmed for specimens with HA concentration above 1000 µg/L.

Study protocol An analytical study, including evaluation of the imprecision, the inaccuracy and the limit of detection, was conducted. HA concentrations measured with the Wako reagents were compared with those obtained routinely (HA Test Kit, Corgenix, Westminster, USA). This test uses HABP-coated on the microwell surface to capture HA. An enzyme (horseradish peroxydase) conjugated with HAPB is used to detect the bound HA with a chromogenic reaction (tetramethylbenzidine and hydrogen peroxide).
For a comparison of serum vs. heparinized plasma**,** plasma samples (n=37) used came from blood samples for other tests requested at the same time as HA determination.
Three pools of serums at three levels of concentration were prepared for within-run and between-run assays. They were fractioned and kept at -20°C until assays.
For dilution tests, serial two-fold dilutions of serum samples with high levels HA were performed in saline solution up to 1/16 or 1/64 according to the initial concentration. For recovery tests, low level serum samples were added with HA (25, 50, 100, 200, 500, µg/L).
Serum samples (n=140) used for the correlation study came from blood samples referred to the laboratory in order to determine HA levels. Using the Corgenix HA test all the determinations were performed in duplicate.
For the determination of the detection limit, 10 measurements of standard 0 (saline solution) were made in the same assay. The concentration corresponding to a signal 3 SD above the mean was calculated.
Statistical analysis : Graphpad Prism® software was used for data analysis.

RESULTS

Standard curve



Dilution test

Dilution	Measured (µg/L)	Expected (µg/L)	Recovery
1/2	1224	1256	97,5%
1/4	648	628	103,2%
1/8	318	314	101,3%
1/16	158	157	100,6%
1/32	78	78	100,0%
1/64	38	39	97,4%

Recovery test

Added (µg/L)	Measured (µg/L)	Expected (µg/L)	Recovery
0	84		
25	113	109	103,7%
50	133	134	99,3%
100	184	184	100,0%
250	339	334	101,5%
500	587	584	100,5%

Imprecision

Within-run imprecision (20 determinations in the same run)

	Pool A (µg/L)	Pool B (µg/L)	Pool C (µg/L)
Mean	48.7	174.4	901.8
SD	0.92	3.69	8.48
CV	1.90%	2.12%	0.94%

Between-run imprecision (1 determination per day for 20 days)

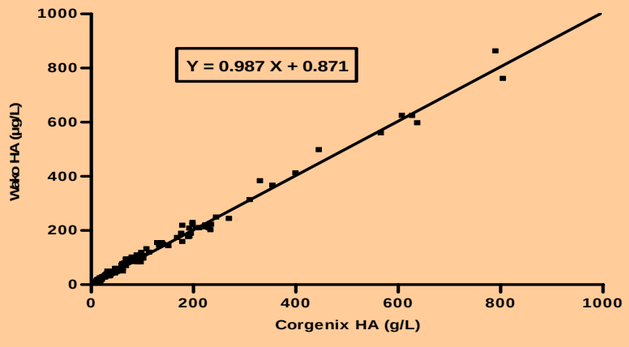
	Pool A (µg/L)	Pool B (µg/L)	Pool C (µg/L)
Mean	49.9	171.7	900.7
SD	2.03	4.15	24.66
CV	4.08%	2.42%	2.74%

Detection limit

1.5 µg/L with 99% confidence.

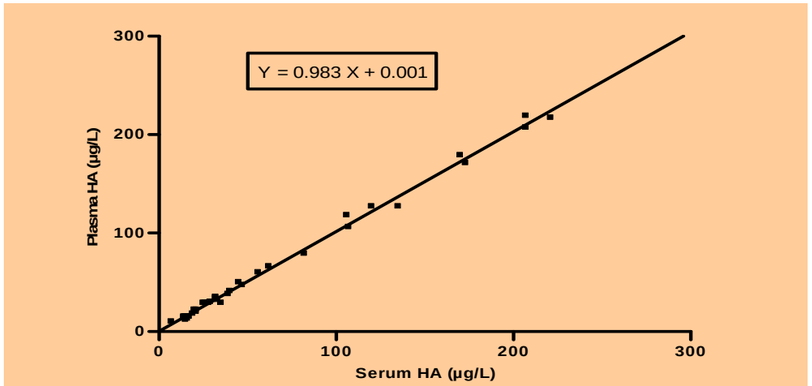
Inaccuracy 1

Comparison between LT Auto Wako Hyaluronic acid (using OLYMPUS AU640) and Corgenix HA-Test® in 138 samples: T-test showed no significant differences between the results (p=0.449).



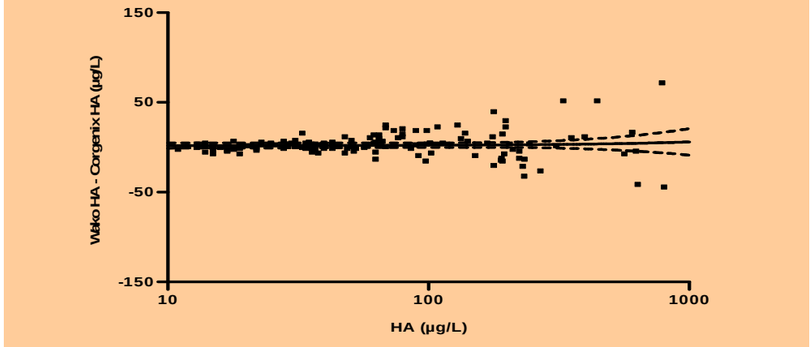
Sampling

Comparison between serum and heparinized plasma determinations (n=37)



Inaccuracy 2

Two samples had to be diluted: the results were 1546 µg/L and 2512 µg/L with Wako reagents and 1700 µg/L and 2500µg/L with the Corgenix assay.



DISCUSSION

This study shows that the reagents for the automatized determination of HA in serum or heparinized plasma developed by Wako method (hyaluronic acid detection reagent, Latex method) showed analytical characteristics that make it appropriate for clinical practice. Sensitivity and range of measured concentrations are adapted to clinical analysis, particularly in chronic liver diseases. Imprecision is improved when compared to methods previously commercialized. No inaccuracy problem was detected. According to the data presented by the Wako company, no effect of common interferents like lipids, haemoglobin or bilirubin was observed. Correlation with the most used commercialized product is excellent. Consequently, 1/ the reference values previously determined could be used and 2/ the indexes for predicting liver fibrosis using combined serum markers including HA levels could be calculated using the described algorithms.

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