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CURRENT TECHNIQUES

Automated assay of hyaluronic acid in serum
Dosage sérique automatisé de l'acide hyaluronique

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Summary Measured alone or combined with other serum markers, hyaluronic acid (HA) has been reported as being relevant for predicting liver fibrosis in patients with chronic liver disease and is proposed as a non-invasive alternative to liver biopsy. However, until now, only manual assays have been available. The latex agglutination method (HA detection reagent, Latex method, Wako, Osaka, Japan) using recombinant hyaluronic acid binding protein (rHABP) and latex sensitized with anti-HABP monoclonal antibody was evaluated using an Olympus AU640 analyzer (Tokyo, Japan). Within-run imprecision was between 1 and 2.2%. Between-run imprecision was between 2 and 4%. Correlation with Corgenix HA-Test®was $y = 0.987 \times -0.871$ ($r = 0.995$). No accuracy problem was detected. Serum or heparinized plasma can be used equally. The detection limit was estimated as 1.5 μg/L. A totally automated serum HA assay is now available for clinical chemists so that its determination in assessing liver fibrosis, particularly in composite indices can be largely proposed.
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MOTS CLÉS
Acide hyaluronic ;
Dosage ;
Fibrose hépatique

Résumé Mesuré seul ou avec d'autres marqueurs sériques pour le calcul de scores, la concentration circulante d'acide hyaluronique (HA) est un marqueur du stade de développement de la fibrose au cours des maladies chroniques du foie qui a été proposé comme alternative à la ponction biopsique hépatique. Alors qu'à ce jour il n'existe que des techniques de dosage manuelles, une méthode turbidimétrique automatisable (*HA detection reagent, Latex method*, Wako, Osaka, Japon) utilisant une protéine recombinante (hyaluronic acid binding protein, rHABP) et des particules de latex sensibilisées avec un anticorps monoclonal anti-HABP est mise sur le marché. Cette nouvelle méthode a été évaluée sur un automate Olympus AU640 (Tokyo, Japon). L'imprécision intra-essai est comprise entre 1 et 2,2 % et l'imprécision intra-essai comprise entre 2 et 4 %. La corrélation avec la méthode la plus utilisée (Corgenix HA-Test®) est caractérisée par une régression $y = 0{,}987 \times -0{,}871$ ($r = 0{,}995$). Aucune difficulté liée à un problème de spécificité n'a été observée. Des échantillons de sérum ou de plasma hépariné peuvent être utilisés indifféremment. La limite de détection à 99 % est estimée à 1,5 μg/L. La

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disponibilité d'un dosage sérique, totalement automatisé, de l'acide hyaluronique doit permettre à un grand nombre de laboratoires de proposer ce marqueur et/ou les scores composites qui l'utilisent dans le cadre de l'évaluation de la fibrose hépatique chez les patients atteints d'une maladie chronique du foie.
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Introduction

Hyaluronic acid (HA, hyaluronate, 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 [1].

In patients with chronic liver diseases of various etiologies, increases in serum HA levels occur together with the development of liver fibrosis [2–5]. 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 without cirrhosis. Later, when cirrhosis is constituted, reduced degradation by sinusoidal endothelial cells may cause greater HA increases. The development of hepatic sinusoids capillarization may explain the reduced HA degradation. Clinical data have shown that in patients with cirrhosis, high serum HA levels were associated with the occurrence of complications or death and correlated with clinical severity [6,7].

HA serum determination is used alone [3–5] or in association with other tests [8–11] for non-invasive assessment of liver fibrosis in patients with chronic liver disease [12].

Currently, only a few laboratories offer serum HA measurement since it is available commercially in most countries as an enzyme-linked binding protein assay proposed as a self-contained kit in the 96 well ELISA format (HA Test Kit, Corgenix, Westminster, USA). Efficient use requires at least a plate washer to perform the multiple rinses and washes as well as a plate reader to read the final absorbance. This assay which needs duplicate determinations (standards and samples), must be proceeded in batches and, unless the laboratory possesses an automated ELISA plate processor, is time-consuming.

Increasing demands explain the need for reagents allowing automated measurement serum HA on the largely used commercial analyzers.

This study aimed to evaluate a new reagent for the quantitative determination of serum HA developed by Wako using the latex agglutination method that can be applied to general clinical chemistry analyzers (hyaluronic acid detection reagent, Latex method, Wako, Osaka, Japan).

Materials and methods

Principle of the method

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 for the study.

- Temperature: 37 °C.
- At T_0: Sample/Calibrator (3.0 μL) and Reagent 1 (180 μL) are mixed.
- After 3.5 min: addition of Reagent 2 (60 μL).
- Absorbance measurement: at 800 nm from $T = 4.2$ min to $T = 8.4$ min.

Every day, Reagent 2 was homogenized by slowly turning the bottle upside down.

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.

Specimen collection and preparation

HA determinations were made in serum samples in parallel with routine determinations in patients with chronic liver diseases. Serum was separated as soon as possible and kept at −20 °C until assays.

Table 1 Imprecision.

	Pool A (μg/L)	Pool B (μg/L)	Pool C (μg/L)
Within-run imprecision (20 determinations in the same run)			
Mean	48.7	174.4	901.8
S.D.	0.92	3.69	8.48
CV	1.90%	2.12%	0.94%
Between-run imprecision (1 determination per day for 20 days)			
Mean	49.9	171.7	900.7
S.D.	2.03	4.15	24.66
CV	4.08%	2.42%	2.74%

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 versus 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 three S.D. above the mean was calculated.

Statistical analysis

Graphpad Prism® software was used for data analysis.

Results

Standard curve

The standard curve is reported in Fig. 1. For four calibrations, the duplicate measured absorbance were 0.0001 ± 0.0006; 0.0287 ± 0.0009; 0.0581 ± 0.0003; 0.1259 ± 0.0020; 0.3468 ± 0.0019 and 0.6260 ± 0.0049 for 0, 50, 100, 200, 500 and 1000 μg/L HA standards, respectively.

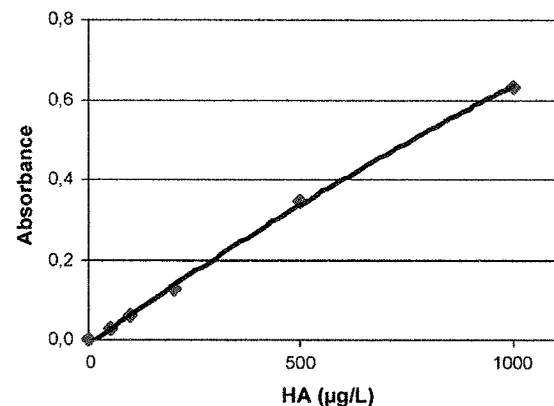

Figure 1 Standard curve.

Sampling

Comparison of the results obtained with serum and heparinized plasma are given in Fig. 2. There were no significant differences between the results (T-test, $p = 0.471$).

Imprecision

Estimates of within-run and between-day standard deviations are shown in Table 1.

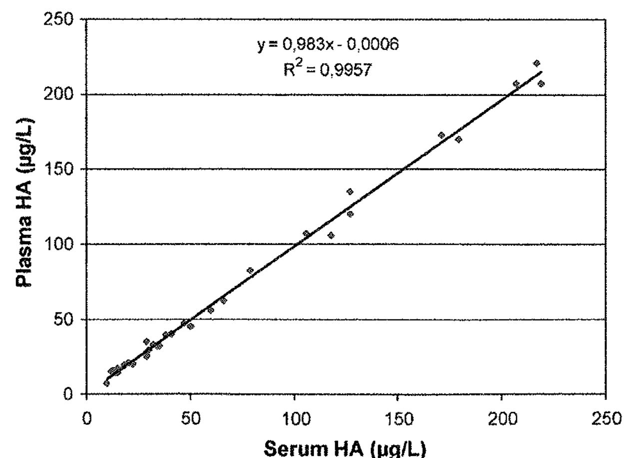

Figure 2 Comparison between serum and heparinized plasma determinations ($n = 37$).

Table 2 Dilution tests.

Dilution	Measured (μg/L)	Expected (μg/L)	Recovery (%)
Assay 1			
1	559		
1/2	274	279	98.2
1/4	140	140	100.0
1/8	72	70	102.9
1/16	34	35	97.1
Assay 2			
1	2512		
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

Dilution and recovery tests

The results of dilution tests and recovery tests are shown in Tables 2 and 3 respectively.

Inaccuracy

Fig. 3 gives the results of the comparison between LT Auto Wako Hyaluronic acid (using Olympus AU640) and HA Test® for 138 samples with HA levels in the measure range of the two assays. *T*-test showed no significant differences between the results ($p = 0.449$). The coefficient of correlation was 0.995, the equation of the linear regression was $y = 0.987 \times -0.871$ and the equation of the Deming regression was $y = 0.991 \times -1.384$). Two samples had to be diluted: the results were 1546 μg/L and 2512 μg/L with Wako reagents and 1700 μg/L and 2500 μg/L with the Corgenix assay.

Table 3 Recovery tests.

Added (μg/L)	Measured (μg/L)	Expected (μg/L)	Recovery (%)
Assay 1			
0	24		
25	48	49	98.0
50	76	74	102.7
100	123	124	99.2
250	274	274	100.0
500	527	524	100.6
Assay 2			
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

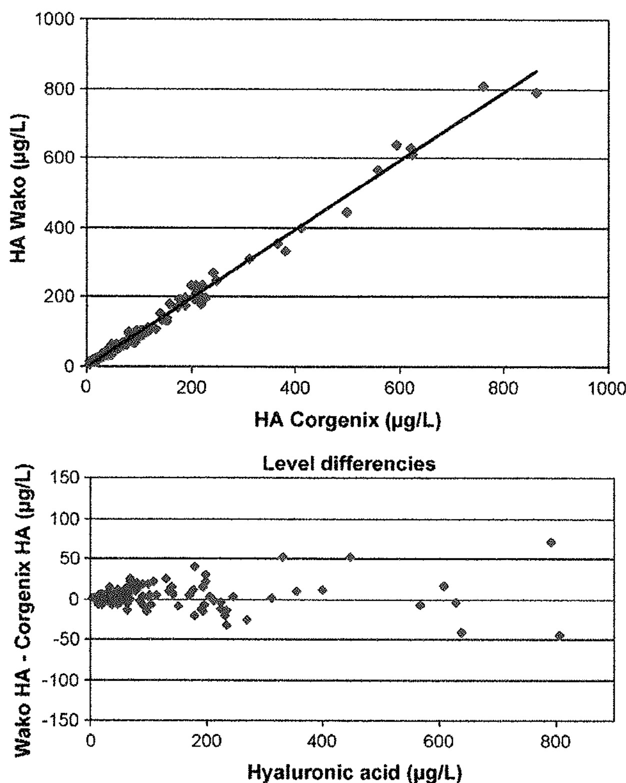

Figure 3 Comparison between LT Auto Wako Hyaluronic acid (using Olympus AU640) and Corgenix HA-Test® ($n = 138$).

Detection limit

The lowest concentration that can reliably be distinguished from a zero concentration is 1.5 μg/L with 99% confidence.

Discussion

This study shows that the reagents for the quantitative determination of HA in serum developed by Wako which use the latex agglutination method (hyaluronic acid detection reagent, Latex method, Wako) showed analytical characteristics that make it appropriate for clinical practice.

Total automation is possible. Serum and heparinized plasma concentrations are equivalent. Sensitivity and range of measured concentrations are adapted to human serum analysis, particularly in chronic liver diseases.

Imprecision is largely improved when compared to other methods previously commercialized. For instance, imprecision of the HA Test as evaluated from the within-run CV for 10 assays of samples with HA concentrations of 25, 53 and 502 mg/L was 10.4, 4.7 and 6.9%, respectively [13].

No inaccuracy problem was detected. According to the data presented by the Wako company [14], no effect of common interferents like lipids, haemoglobin or bilirubin was observed. Correlation with the most used commercialized product is excellent. Consequently, firstly the reference values previously determined [15] could be used and secondly, the indexes for predicting liver fibrosis using

combined serum markers including HA levels could be calculated using the described algorithms.

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