Hyaluronic Acid Assays: Turbidimetric or Enzyme-Based Immune Assay? A Method Comparison Study

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Background: Hyaluronic acid (HA) is proposed as a marker of functional liver capacity. The aim of the present study was to compare a new turbidimetric assay for measuring HA with the current standard method. **Methods:** HA was measured by a particle-enhanced turbidimetric immunoassay (PETIA) and enzyme-linked immunosorbent assay (ELISA) in a 40-sample dilution series and 39 intensive care unit (ICU) patients. Agreement was assessed with Bland–Altman's method. **Results:** In the ICU patients, the median HA concentration was 159.0 ng/ml (interquartile range (IQR) 117.5–362.5 ng/ml) with ELISA and 157.5 ng/ml (IQR 92.5–359.6 ng/ml) with PETIA.

The mean difference was 12.88 ng/ml (95% CI, –4.3 to 30.1 ng/ml, $P = 0.14$) and the 95% limits of agreement were –91.17 to 116.9 ng/ml. In the dilution series, the mean difference was –59.26 ng/ml (95% CI, –74.68 to 43.84 ng/ml, $P < 0.0001$) and the 95% limits of agreement were 35.23 to –153.8 ng/ml. **Conclusion:** We found random variation between the PETIA and ELISA test that could affect performance in a clinical context, but only to a lesser extent in a research context. The new clinical biochemistry assay for HA determination will allow for large studies of the clinical utility of HA. *J. Clin. Lab. Anal.* 30:524–528, 2016. © 2015 Wiley Periodicals, Inc.

Key words: ELISA; PETIA; biomarker; validation; liver failure; critical illness

INTRODUCTION

Hyaluronic acid (HA) is a glycosaminoglycan, consisting of repeated pairs of glucuronic acid and glucosamine, and a key component of the extracellular matrix (1). Most human cells, at some point during development, have the capacity to secrete HA, but the circulating HA in human adults is primarily produced in the peripheral soft connective tissue and transported to the systemic circulation via lymph drainage (2). The majority of HA is removed from circulation by hepatic elimination (3). On the basis of this, HA has been proposed as a marker of functional liver capacity across a range of chronic liver conditions (4). The rate of elimination has been shown to be highly

influenced by hepatic blood flow and hepatocyte function during sepsis (5). Consequently, it has been hypothesized that HA could be a marker of liver dysfunction in sepsis and septic shock in intensive care unit (ICU) patients (5).

HA is a challenging molecule to quantitate, because the length of a molecule can extend from hundreds to

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tens of thousands of disaccharides (1). Furthermore, HA is essentially nonimmunogenic, which has excluded direct immunochemical methods of measurement (1). To accommodate this, several advanced methods of measurements have been used including enzymatic degradation; hyaluronic-binding protein (HABP) linked enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography among others. None of these methods are, however, well suited for large-scale testing in large cohorts or in daily clinical practice. The assay evaluated in the present study is a particle-enhanced turbidimetric immunoassay (PETIA). The assay quantitates HA by binding HA, present in the sample, to HABP-bound latex particles. The complex between HA and the HABP-coated latex particles increase the turbidity of the sample, and thus allows for quantification. This is principally the same method used in the reference method that is an HABP-linked ELISA. The purpose of this study was to compare the performance of a new turbidimetric assay for determination of serum HA levels to the current gold standard.

METHODS AND MATERIALS

ICU Samples

We measured HA concentrations in 39 samples of serum from 39 randomly selected ICU patients included in the Procalcitonin and Survival Study (PASS) (6). The trial included, in brief, a broad selection of ICU patients with expected stay in the ICU >24 h and age >18 years. Patient samples were obtained in standard serum separation tubes and placed on ice immediately after sampling. The isolated serum was frozen to -80°C within 5 h. The samples underwent one freeze-thaw cycle prior to analysis. Details of the PASS study and its results have been published elsewhere (6). The PASS study and use of the biobank were approved by the local ethical committee (ref. no. H-KF-272-753) and the Danish Data Protection Agency (ref. no. 2005-54-1779). Written informed consent was obtained from all patients or their legal representative before enrollment. All data and samples were collected and processed in accordance with Good Clinical Practice standards and European Union legislation (EMA CPMP/ICH/135/95).

Dilution Series of HA

A panel of 40 samples was prepared with HA concentrations covering the range of the ELISA and PETIA (target range 25–825 ng/ml). The panel was made by adding increasing amounts of a 20 $\mu\text{g}/\text{ml}$ stock concentrate of HA extracted from rooster combs (H5388, Sigma-Aldrich, St. Louis, MO; used for preparing the calibrators for the

assays) to a pool of serum from healthy human donors. After a brief mix, the samples were frozen to -70°C and did not undergo freeze-thaw cycles prior to analysis.

Measurements

HA was measured with PETIA (Corgenix, Broomfield, CO) in ICU samples on Hitachi 917 (Roche Diagnostics, GmbH, Mannheim, Germany) and in the dilution series on Modular P (Roche Diagnostics GmbH) analyzers (test assay), and by ELISA (HA ELISA, Corgenix) using double determination (reference method). All assays were calibrated against a standardized calibration set (Corgenix, reference 13375) and quality control with a standardized set for this purpose (Corgenix, reference 13376). Both calibration and quality control were done in accordance with the manufacturers' instructions.

Statistical Analysis

Agreement between ELISA and PETIA measurements of HA concentration was analyzed by Bland and Altman's method (7). The distribution of continuous variables was assessed by graphical inspection of histograms and controlled by Kolmogorov–Smirnov's (KS) test. Normally distributed paired continuous data were analyzed by Student's *t*-test and nonnormal data by Wilcoxon's signed-rank test. Data are presented as mean (standard deviation (SD)) or medians (25th–75th interquartile range (IQR)). Linear regression analysis was conducted using the Deming method, assuming a ratio of measurement error of 1. If the fitted model had residuals with a normal distribution, standard parametric methods were used for calculating 95% confidence intervals (CIs) for the parameters and regression line. Correlation was estimated by means of Spearman's correlation coefficient. $P < 0.05$ was considered significant. Analyses were performed with R 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria, <http://r-project.org>).

RESULTS

ICU Patients

In total, 39 samples were measured by ELISA and PETIA. The differences between PETIA and ELISA values convincingly fit a normal distribution curve (KS test, $P = 0.50$), and parametric analysis was performed on the differences. The median HA concentration measured was 159.0 ng/ml (IQR 117.5–362.5 ng/ml) with ELISA versus 157.5 ng/ml (IQR 92.5–359.6 ng/ml) with PETIA. The mean difference between ELISA and PETIA concentrations was 12.9 ng/ml (95% CI, -4.3 to 30.1 ng/ml, $P = 0.14$ (paired *t*-test ELISA vs. PETIA)).

Fig. 1. Bland–Altman plot ELISA versus PETIA in ICU patients. Absolute difference (A) and relative difference (B). Solid line: no difference. Dashed line: mean difference (bias); dotted lines: 95% limits of agreement ($1.96 \times$ standard deviation). HA, hyaluronic acid; ELISA, enzyme-linked immunosorbent assay; PETIA, particle-enhanced turbidimetric immune assay.

The limits of agreement were -91.2 to 116.9 ng/ml (Fig. 1A). There was a notable trend toward high variation in differences with higher concentrations of HA. To evaluate this impact, we repeated the analysis with relative differences (difference between measurements as percent-

Fig. 2. Linear regression (Deming) of PETIA versus ELISA in ICU patients. Thick line: regression line; shade: 95% confidence interval of regression line. Dashed line: line of agreement. HA, hyaluronic acid; ELISA, enzyme-linked immune sorbent assay; PETIA, particle-enhanced turbidimetric immune assay.

age of the reference (ELISA)). The relative differences were convincingly normally distributed (KS test = 0.96) and significantly different from 0 with mean of 7.5 % (95% CI, 0.32–14.6%; $P = 0.04$ vs. no difference). The 95% limits of agreement were -35.7% to 50.7% (Fig. 1B). The Deming regression line (independent variable: ELISA; dependent variable: PETIA) had a slope of 0.98 (95% CI, 0.88–1.07 ng/ml)—which was not significantly different from unity—and an intercept of -7.26 ng/ml (95% CI, -36.8 to 22.28 ng/ml), which was not significantly different from 0 (Fig. 2). Passing–Bablok regression did not alter the signal of the Deming regression analysis excluding significant influence of outliers. The residuals were approximated to normality (KS test, $P = 0.36$). The Spearman correlation coefficient was 0.94 ($P < 0.0001$).

Dilution Series

A total of 40 samples were measured by ELISA and PETIA. The differences between ELISA and PETIA values adequately fitted a normal distribution curve (KS test, $P = 0.27$). Parametric analysis was performed on the differences; however, the results were confirmed in a nonparametric analysis. The median HA concentration measured was 337.50 ng/ml (IQR 180.5–483.0 ng/ml) with ELISA versus 411.5 ng/ml (IQR 213.4 to 617.7 ng/ml) with PETIA. The mean difference between

Fig. 3. Bland–Altman plot ELISA versus PETIA in a dilution series. Absolute difference (A) and relative difference (B). Solid line: no difference. Dashed line: mean difference (bias); dotted lines: 95% limits of agreement ($1.96 \times$ standard deviation). HA, hyaluronic acid; ELISA, enzyme-linked immune sorbent assay; PETIA, particle-enhanced turbidimetric immune assay.

measured concentrations (ELISA–PETIA) was -59.26 ng/ml (95% CI, -74.68 to 43.84 ng/ml; $P < 0.0001$ (paired t -test ELISA vs. PETIA)) and the limits of agreement were 35.23 to -153.8 ng/ml (Fig. 3A). This was confirmed by Wilcoxon's signed-rank test ($P < 0.0001$).

From these results and the graphical inspection, there was a substantial systematic bias toward high HA concentrations measured with PETIA compared to ELISA. To explore this bias, we investigated the relative differences between ELISA and PETIA. The relative differences were convincingly normally distributed (KS test = 0.87) and significantly different from 0 with mean of -21.1% (95% CI, -17.2% to -24.9% ; $P < 0.0001$ vs. no difference). The 95% limits of agreement were 2.6 to -44.7% (Fig. 3B). The mean intra-assay coefficient of variation (%CV) in the PETIA was 1.9% (range 0.13 – 7.62).

DISCUSSION

We set out to compare the performance of a new turbidimetric assay for determination of serum HA levels with the current gold standard. We found that in ICU patients, there was no systematic bias between ELISA and PETIA. However, we observed random variation that seemed to increase with higher concentrations. The relative difference from the reference method (ELISA) was a moderate 7.6% . In a dilution series, we found a systematic and substantial bias toward lower values measured with ELISA compared to PETIA. The bias increased with higher concentrations, and relative difference from the reference method was substantially higher (-21.1%).

The current study evaluated PETIA for measurement of HA in ICU patients. The results should therefore be interpreted in the context of the normal variation of HA in ICU patients. In a recent study, the levels of HA in 164 ICU patients has been investigated. The median concentration of HA was 270.5 ng/L (IQR approximately 100 – 750 ng/ml, range 0 – $2,842$ ng/ml) (8). The limits of agreement in our study were -91.2 to 116.9 ng/ml and we did not find any systematic bias, but only random variation. On the basis of these two observations we conclude that the bias could, although to a lesser extent, influence the clinical interpretation of test results. Since there was no systematic bias, the PETIA assay can be used without limitation in a research context comparing populations, for example, septic versus nonseptic patients. The PETIA assay will substantially reduce the resources and time needed for large-scale measurement of HA. In the light of the variation found, it would be relevant to investigate if patient-related characteristics influence the performance of either assays.

In the dilution samples, we found a substantial systematic bias toward higher values in PETIA assays compared to the ELISA. The reason for this bias is unclear, but could potentially be attributed to either difference between the human HA in the sample and rooster HA in the calibrators used in the dilution series or measurement error.

The clinical potential for HA is substantial, but presently there is no known use. Determining this is the

goal of ongoing investigations. The new assay will make it feasible to do these investigations in populations of a size that is suited for inferring clinical utility. However, the variation found in the current study should be taken into account.

CONCLUSION

In the current study, we have compared a turbidimetric HA assay to the current gold standard and found random variation between the ELISA and PETIA test that could affect performance in a clinical context, but only to a lesser extent in a research context. In a dilution series, we found substantial systematic bias. The new clinical biochemistry assay for HA determination will allow for large studies of the clinical utility of HA.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

REFERENCES

1. Fraser JR, Laurent TC, Laurent UB. Hyaluronan: Its nature, distribution, functions and turnover. *J Intern Med* 1997;242:27–33.
2. Laurant T, Fraser JR. Hyaluronan. *FASEB J* 1992;6:2397–2404.
3. Henriksen JH, Bentsen KD, Laurent TC. Splanchnic and renal extraction of circulating hyaluronan in patients with alcoholic liver disease. *J Hepatol* 1988;6:158–166.
4. Rostami S, Parsian H. Hyaluronic Acid: From biochemical characteristics to its clinical translation in assessment of liver fibrosis. *Hepat Mon* 2013;13:e13787.
5. Berg S. Hyaluronan turnover in relation to infection and sepsis. *J Intern Med* 1997;242:73–77.
6. Jensen JU, Hein L, Lundgren B, Bestle MH, Mohr TT, Andersen MH, et al. Procalcitonin-guided interventions against infections to increase early appropriate antibiotics and improve survival in the intensive care unit: A randomized trial. *Crit Care Med* 2011;39:2048–2058.
7. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–310.
8. Yagmur E, Koch A, Haumann M, Kramann R, Trautwein C, Tacke F. Hyaluronan serum concentrations are elevated in critically ill patients and associated with disease severity. *Clin Biochem* 2012;45:82–87.