Estimation of Serum Hyaluronidase Activity Overcoming the Turbidity Interference

SHIVAIAH NAGARAJU, K SUBBAIAH GIRISH, YI PAN, KIRK A EASELY, KEMPAIAH KEMPARAJU

ABSTRACT: The assay of mammalian hyaluronidases (HAases) is important in understanding the role of the hyaluronan-hyaluronidase (HA-HAase) system in various pathophysiological processes. Despite several quantitative assay method options, the Morgan-Elson colorimetric method modified by Reissig et al [1] is considered the best for determining the activity in clinical samples. However, the sensitivity of the method was greatly limited by presence of protein above 400 µg due to turbidity interference that led to chromogen quenching. Therefore, an effort has been made to reinvestigate the Reissig et al method. In the re-investigated method, a standardized optimal 0.32 M potassium tetraborate (PTB) was used against 0.13 M (native) to overcome the turbidity interference. The estimated mean OD at 585 nm of serum for native method was 0.043 (95% CI: 0.040 to 0.045), while that for the re-investigated method was 0.138 (95% CI: 0.133 to 0.143, $p<0.0001$). The mean OD at 585 nm of serum of native method was significantly lower than that of re-investigated method ($p<0.05$) at all protein levels. This was also true for estimated mean OD at 585 nm of plasma. The mean intrasample CVs for native and re-investigated methods were 0.9% and 0.5%, respectively, for normal serum. Furthermore, the repeatability coefficient of normal serum for native was 0.003 IU, while re-investigated method experienced that of 0.002 IU.

INDEX TERMS: Hyaluronidase; Hyaluronan; Human serum; Colorimetric method; Turbidity interference

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INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight non-sulfated glycosaminoglycan composed of repeating disaccharide unit of D-glucuronic acid (Glc UA) and *N*-acetyl-D-glucosamine (NAG). The HA is recognized as a major participant in important biological processes as cell motility, proliferation, differentiation and migration. The HA degrading enzymes, the HAases are found in various human organs and body fluids and in the external secretions of various other organisms.[2-5] There are three groups of HAases according to their HA

degradation mechanisms namely,[1] hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase: EC 3.2.1.35),[2] hyaluronate 3-glycanohydrolase (hyaluronoglucuronidase: EC 3.2.1.36), and (3) hyaluronate lyase (EC 4.2.2.1).[6] The HAases present in various mammalian tissues belong to the first group of enzymes and are of particular clinical interest as they have been demonstrated to be involved in the pathophysiology of many human disorders.[2-11] Due to their physiological importance, a rapid and sensitive method to measure HAase activity has become increasingly necessary. A variety of assay methods have been used to measure HAase activity, i.e., turbidometric, viscometric, and colorimetric methods, and newer methods such as spectrophotometric, fluorogenic, radiometric, agarose plate based, ELISA-like, HPLC, zymography, capillary electrophoresis, FACE- based and ECL-assisted assays.[2,12-14]

Despite several quantitative assay methods available, the Morgan-Elson colorimetric method modified by Reissig et al[1] is considered to be the best and widely used method to determine the HAase activity in clinical samples including serum, plasma, saliva, urine and other body fluids. In the Morgan-Elson reaction the NAG in the reducing end is successfully transformed into a chromogen under alkaline condition at 100° C and subsequently by the action of *p*-dimethyl amino benzaldehyde (*p*-DMAB) in glacial acetic acid and hydrochloric acid mixture (9:1; v/v) to give a reddish purple colored product that can be detected at 585 nm. However, this method is limited by the interference of variation in the pH of the sample; presence of other sugars, amino acids and Mg^{+2} and also the final color development often require 90 minutes. Reissig et al[1] improved this method by substituting a concentrated PTB reagent (0.8 M, pH 9.1; 50 µl/ assay volume of 300 µl, to a final concentration of 0.13 M PTB in the reaction mixture) for the original carbonate buffer. As a result of modification, the final color development required just 20 minutes and about 2 folds increase in the color yield. In spite of the significant improvement, the estimation of HAase activity in clinical samples such as serum, plasma and other body fluids was not satisfactory as there was a massive interference by turbidity upon increasing the protein concentration above 400 µg in the reaction mixture. While, less than 400 µg of protein in the reaction mixture did not cause any turbidity. Hence, removal of turbidity in cases when excess of protein used was only through centrifugation at a high centrifugal force of about 18,000x g. Although, the adoption of the curvilinear interpolation by Asteriou et al.[15] overcomes the turbidity interference, this approach was not suitable especially for the assays of HAases performed at acidic pH, since the resulting turbidity colored reaction mixture was often unstably suspended. Hence, balancing the protein content of the assay in order to get the measurable activity and at the same time preventing the turbidity formation is a critical practical difficulty. Therefore, it is highly unlikely to pick up the HAase activity can be picked up in cases of lesser protein levels (< 400 µg protein) or results in turbidity in cases of higher protein levels (> 400 µg protein) in specimens turbidity would cause anomalous results. However, use of larger concentrations of the clinical sample (protein content > 400 µg) is quite essential to get the measurable activity. Therefore, considering these limitations, we attempted to re-investigate the Reissig et al.[1] method by using an optimal concentration of PTB reagent (2 M, pH 9.1; 50 µl/ assay volume of 300 µl, to a final concentration of 0.33 M PTB in the reaction mixture) to drastically reduce turbidity.

MATERIALS AND METHODS

Healthy donors (20-25 yrs age group), osteoarthritic and diabetic patients were recruited with informed consent according to the Declaration of Helsinki and as per University of Mysore institutional review board protocols to donate up to 5 ml of whole blood. Hyaluronic acid (HA) and *N*-acetyl glucosamine were purchased from Sigma Chemicals Co. St Louis, USA. All other chemicals used were of analytical grade.

Determination of hyaluronidase activity

Hyaluronidase activity was determined according to the native method of Reissig et al (1955) and reinvestigated Reissig et al method.

Reissig et al method: Enzyme was incubated with HA (50 µg) in a final reaction volume of 300 µl of 0.1 M sodium formate buffer (pH 3.8) containing 300 mM NaCl and incubated for 150 min at 37° C. The reaction was stopped by adding potassium tetraborate, (0.8 M,

pH 9.1, 50 µl/assay volume of 300 µl to a final concentration of 0.13M). The reaction mixture was kept in boiling water bath for exactly 3 min. The coloring reagent *p*-dimethyl amino benzaldehyde (*p*-DMAB) in glacial acetic acid and hydrochloric acid (1.5 ml, 9:1, v/v) was added to give a reddish purple colored product that can be detected at 585 nm. The change in absorbance was monitored at 585 nm. Activity was expressed as µ moles of N-acetyl glucosamine released/min/mg protein.

In reinvestigated method: To stop the reaction, 2 M potassium tetraborate (pH 9.1; 50 µl/ assay volume of 300 µl, to a final concentration of 0.33 M PTB in the reaction mixture) was used instead of 0.8 M.

The primary endpoint (OD at 585 nm; NAG estimation), was measured using both native and re-investigated methods at different protein levels 0, 500, 1000, 1500 and 2000 µg. Repeated-measures analyses for OD was done with a means model with SAS Proc Mixed (version 9.2, mixed linear models). The repeated-measures analyses were done separately for the plasma and serum samples. A heterogeneous compound symmetry variance-covariance form among the repeated measurements was assumed for each outcome, and robust estimates of the standard errors of parameters were used to do statistical tests and construct 95% confidence intervals. T tests were used to compare the pairwise differences between the model-based means (least squares means) at each protein level, providing separates of the means by method (native or re-investigated) and protein level.

Statistical analysis:

Native and re-investigated methods were used to evaluate the enzyme activity of normal serum on the same 25 subjects. Each sample was measured three times by each method. Similar studies were performed for 25 diabetic samples and osteoarthritis samples (data not shown). Intra-sample coefficient of variation (CV) was used to assess the repeatability of replications within each method. Inter-sample CV was also calculated.

Furthermore, the repeatability coefficient was calculated to quantify the repeatability of each method from replicated measurements obtained by the same method. The repeatability coefficient[16] is defined as 1.96 $\sqrt{2^2 W}$ where σ^2_w is the within subject variance for enzyme activity. Estimation of σ^2_w is provided using the mean square error from an ANOVA model with 3 replicates from each sample (sample was the blocking factor for the ANOVA model).

Additionally a plot of the mean difference (i.e., 1st minus 2nd enzyme activity measurement) versus the mean of the two replicate enzyme activity levels was included to graphically summarize the repeatability of each method. The repeatability limits (± $2.77S_w$) were added to the graph (as horizontal lines on the Y axis of Figure 2b). The mean difference (i.e., mean bias) is not plotted on the graph since the mean difference is statistically zero (as expected). Since the mean bias for the other 2 plots (1st minus the 3rd measurement and 2nd minus the 3rd measurement) were very similar to the first plot these 2 plots were not included in the manuscript.

RESULTS AND DISCUSSION

Figure 1a represents the estimation of standard NAG (Sigma-Aldrich, St. Louis, USA) by native Reissig et al method[1] and re-investigated Reissig et al method using 0.8 M and 2 M PTB reagent respectively. The results show no difference in optical activity. The final colored

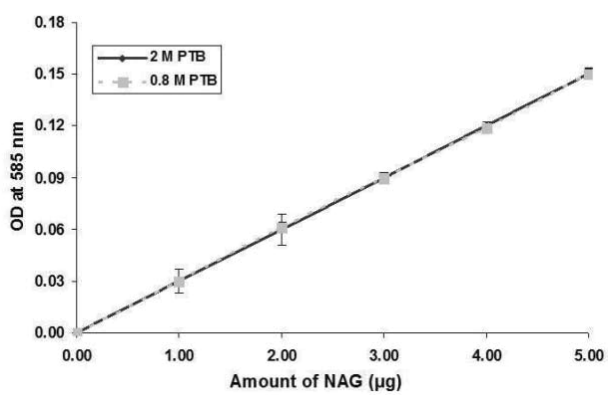

Figure. 1a. Estimation of standard N-acetyl glucoseamine (NAG). The reaction mixture contained different amounts NAG (0 - 5 µg) in 300 µl of 0.1 M sodium formate buffer pH 3.8 containing 300 mM NaCl. The PTB reagent was then added. The sample tubes were heated vigorously in boiling water bath for exactly 3 min and cooled in tap water, followed by the addition of 1.5 ml of *p*-DMAB reagent. The tubes were maintained at 37° C for 20 min for color development and measured at 585 nm. The PTB reagent, 0.8 M (native) and 2 M (re-investigated) were used and processed according to Reissig et al method [1]. The results were presented as mean ± SEM of five independent determinations.

solution was clear with no signs of turbidity and no visible precipitate was observed when centrifuged at 18000x *g* for 10 min in both the cases. Thus, use of either 0.8 M or 2 M PTB reagent did not affect the intensity of the chromogen generated.

Further, when standard NAG was estimated in presence of serum and plasma samples of varied amounts of proteins (0 to 2000 µg) using native Reissig et al[1] method (0.8 M PTB), we observed the formation of turbidity which required centrifugation prior to optical density measurement. When comparing the re-investigated and native methods using serum protein, at protein 0 µg, the estimated mean OD at 585 nm using re-investigated method was 0.150 nm (95% CI: 0.147 to 0.153) and at 2000 µg protein this value was 0.138 nm (95% CI: 0.133 to 0.143). In contrast, the estimated mean OD at 585 nm using native method at protein 0 µg and 2000 µg were 0.140 nm (95% CI: 0.137 to 0.144) and 0.043 nm (95% CI: 0.040 to 0.045) respectively. The mean OD at 585 nm of serum of native method was significantly lower than that of re-investigated method ($p<0.05$) at all protein levels (Figure 1b). Analyses of OD at 585 nm of plasma showed similar results (Figure 1c).

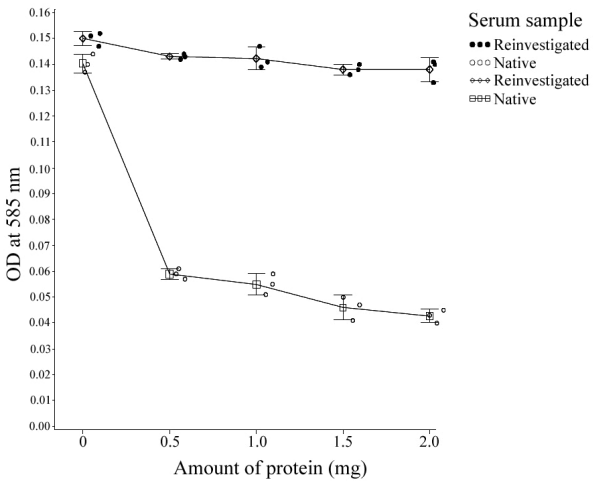

Figure 1b. Estimation of *N*-acetyl glucosamine in the presence of serum proteins. The reaction mixture, 300 µl of sodium formate buffer pH 3.8 (0.1 M with 0.05 M NaCl) containing 5 µg each of NAG were added independently with the increasing amounts of total serum protein (0 -2000 µg). The PTB reagent, 0.8 M (native) and 2 M (re-investigated) were used and processed according to Reissig et al method [1]. The results were presented as mean ± 95% Confidence Interval as well as raw data points.

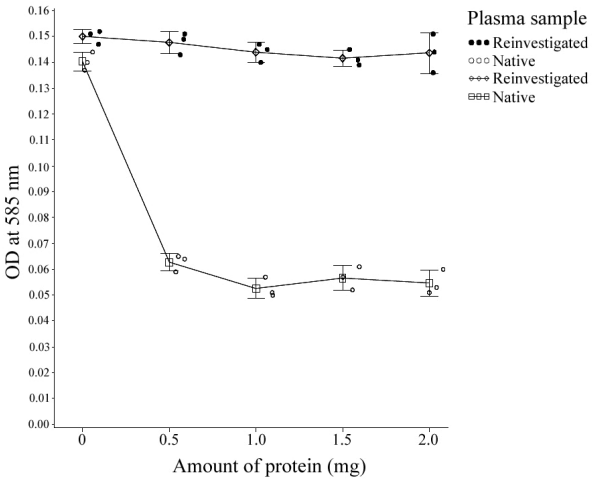

Figure 1c. Estimation of *N*-acetyl glucosamine in the presence of plasma proteins. The reaction mixture, 300 µl of sodium formate buffer pH 3.8 (0.1 M with 0.05 M NaCl) containing 5 µg each of NAG were added independently with the increasing amounts of total plasma protein (0 -2000 µg). The PTB reagent, 0.8 M (native) and 2 M (re-investigated) were used and processed according to Reissig et al method [1]. The results were presented as mean ± 95% Confidence Interval as well as raw data points.

However, when similar estimation was done using 2 M PTB reagent (re-investigated Reissig et al method); no visible turbidity formation was seen. This modification was not only independent of centrifugation but also did not reduce the color intensity (Fig. 1b, 1c). In addition the estimation of NAG in the presence of total serum and plasma proteins and in presence of other protein preparations such as bovine serum albumin, casein and gelatin, the results obtained showed similar trend. The protein content was determined by Biuret method[17] using bovine serum albumin as standard.

In order to verify this finding, the normal serum HAase activity was estimated. Figure 2a indicate the independent determination of HAase activity in terms of the end product NAG estimation using 0.8 M (native) and 2 M (re-investigated) PTB reagent. In presence of 0.8 M PTB, we observed the formation of turbidity as well as reduction in the color intensity. In addition, the color intensity was further reduced when we attempted to obtain clearer supernatant by centrifugation. We observed a drastic reduction in optical density. This poses a serious set back in assessing the actual level of HAase activity in clinical samples. In contrast, when we used 2 M PTB, the samples not only recorded an increased optical density, the samples were

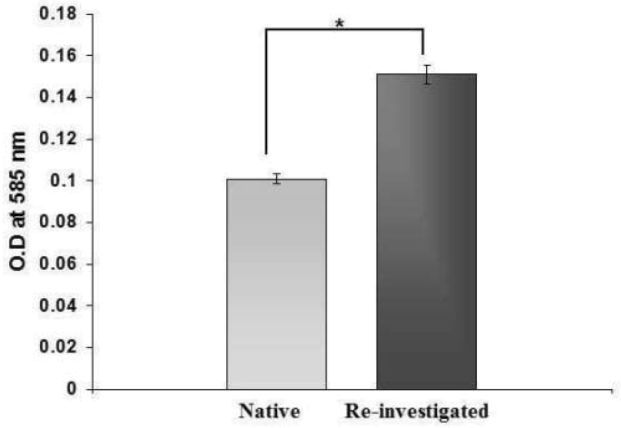

Figure. 2a. Estimation of normal serum HAase activity by the native and the re-investigated Reissig et al methods. The assay mixtures contained buffered substrate solution (50 µg of HA in 300 µl of 0.1 M sodium formate buffer pH 3.8 containing 0.05 M NaCl) and normal serum sample (containing 1500 µg protein) in a final reaction volume of 300 µl and incubated for 150 min at 37° C. The reaction was initiated independently by adding the serum samples. The reducing terminal NAG was estimated using native and re-investigated Reissig et al method [1] as described in legend of fig.1a. A difference in normal serum HAase activity between the native and the re-investigated Reissig et al methods was considered statistically significant when $P < 0.001$ (*).

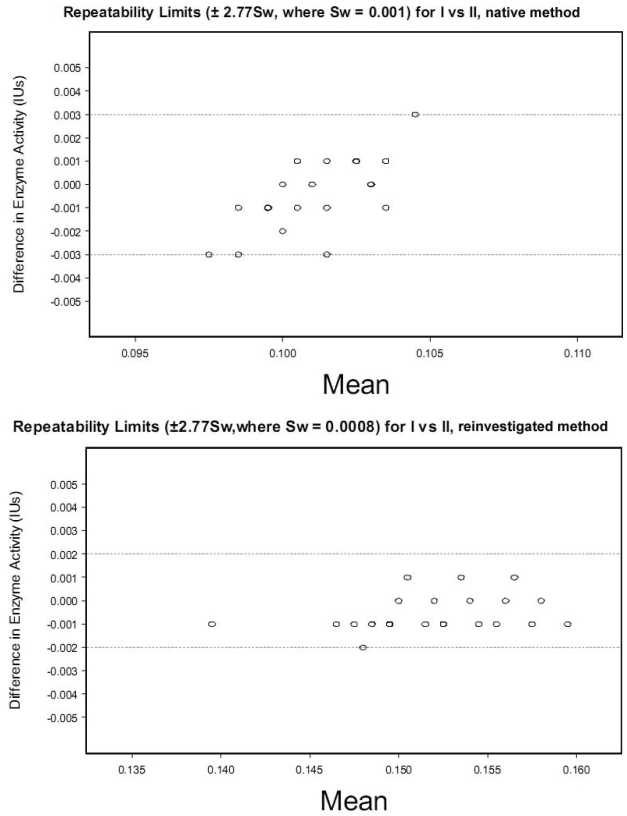

Figure. 2b. Analysis of normal serum HAase activity by the native and the re-investigated Reissig et al methods. Limits of Repeatability: The mean difference (i.e., 1st minus 2nd enzyme activity measurement) was plotted versus the mean of the two replicate enzyme activity levels. The repeatability limits (± 2.77S_w) were added to the graph.

clear with no traces of turbidity and no prior centrifugation was required before measuring the optical density. Thus, use of 2 M PTB instead of 0.8 M, ensures clearer solutions even at higher protein levels of clinical samples with the protein content as high as of about 2000 µg, that is about 5 folds more than that used (about 400 µg) by the native Reissig et al method.[1] However, further increase in the concentration of >2 M PTB, did not have any influence on the turbidity nor the intensity of the color formation (data not shown). All the serum samples used for the study were procured by Government Ayurvedic Medical College, Mysore, India with the prior consent from donors.

To access repeatability on the same sample, descriptive statistics of intrasample CV (%) of normal serum was carried out. The mean intrasample CVs for native and re-investigated methods were 0.9% and 0.5%, respectively. On the other hand, the intersample CV for the native method was 1.7% while the number was 2.8% for the re-investigated method. Repeatability coefficient of the native method was estimated as 1.96 $\sqrt{2S^2W} = 1.96\sqrt{2(0.000001139)} = 0.003$ IU. In other words, 0.003 IU was the difference that would be exceeded by only 5% of pairs of measurements made by the native method on the same subject. Re-investigated method's repeatability coefficient was estimated as 1.96 $\sqrt{2S^2W} = 1.96\sqrt{2(0.000000627)} = 0.002$ IU. In other words, 0.002 IU was the difference that would be exceeded by only 5% of pairs of measurements made by the native method on the same subject. Figure 2b shows the repeatability limits of re-investigated (± 0.003 IU) and native methods (± 0.002 IU) for normal serum. No 1st minus 2nd enzyme activity measurements exceeded the repeatability limits for both methods.

In conclusion, the estimation of HAase activity in clinical samples appears to be tedious and results in anomaly. This is due to turbidity interference when higher protein concentrations (>400 µg) are used. Also

there is, the fact that underscores is the difficulty in obtaining the measurable activity with lower protein concentrations in samples. The re-investigated method we have tested and are proposing a satisfactory and effective means of determining the HAase activity even when high proteins are present in clinical samples. The reaction mixtures were not turbid but clear, permitting a more accurate estimation of the activity of the enzyme in clinical samples.

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