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Measurement of hyaluronidase activity in normal human serum

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Abstract

A detailed evaluation of the assay for serum hyaluronidase (HAE) activity originally developed by Bonner and Cantey [W.M. Bonner, Jr. and E.Y Cantey, Clin. Chim. Acta, 13 (1966) 746–752] is described, together with studies of its precision. The method is based on the liberation of saccharides with *N*-acetylglucosamine (NAG) end-groups from hyaluronic acid. The NAG is quantitated by heating with alkaline tetraborate to form an intermediate which reacts with *p*-dimethylaminobenzaldehyde in acidic medium to form a coloured product.

The optimised assay, which requires less that 50 μl of serum, was used to study the HAE activity of 70 normal sera. The mean HAE activity was 17.1 μmol NAG min^{-1} l^{-1} (range 11.5–27.0 μmol NAG min^{-1} l^{-1}); there was no significant difference with age ($t = 1.65$, $0.5 > P > 0.1$) or sex ($t = 0.33$, $P > 0.5$).

Keywords: Hyalurondiase; Human serum; Colorimetric assay; Endoglycosidase; Hyaluronic acid

1. Introduction

Hyaluronidase is an endoglycosidase which cleaves internal β-*N*-acetyl-D-glucosaminidic linkages of hyaluronic acid (HA) to liberate saccharides containing equimolar glucuronic acid and *N*-acetylglucosamine (NAG). The enzyme has been demonstrated in a wide variety of mammalian tissues, synovial fluid and serum [2] but the origin of the serum hyaluronidase (HAE) is not known.

Clinical interest in serum HAE activity has arisen from the observation that patients with disseminated neoplasm have decreased activity of this enzyme [3–5]. In 1991 Stern et al. [6] described elevated levels of HAE in urine of children with Wilm's tumour and suggested that HAE may be used as a tumour marker. Fiszer-Szafarz [7] postulated that HAE may play a role in cancer invasiveness.

A variety of methods have been described for the determination of serum HAE activity including assays based on measuring changes in viscosity [8] and turbidity [9] of solutions of HA due to the activity of the HAE. All have been complex and time-consuming and have lacked detailed evaluations. This paper describes a thorough evaluation of the colorimetric method of Bonner and Cantey [1] in which serum HAE digests HA substrate to liberate NAG end-groups. The NAG was quantitated using the colorimetric method of Reissig et al. [10] in which NAG is heated with

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alkaline tetraborate to form an intermediate, possibly a glucoxaline, which then reacts with *p*-dimethylaminobenzaldehyde (DMAB) in acidic medium to form a coloured product.

This is the first detailed study of HAE activity in normal sera.

2. Materials and method

2.1. Materials

Fresh normal sera from five males and five females in each decade from 20–89 years were obtained from healthy blood donors and well person clinics. The serum glucose, urea and liver function tests were normal. The samples were stored at −20°C prior to assay.

Potassium tetraborate, DMAB and NAG were obtained from Sigma Chemical Co., Poole, UK. HA (from human umbilical cord) was obtained from ICN Biomedicals, Oxfordshire, UK. Pooled human serum was used as a control.

2.2. Method

Patient serum samples and the pooled human serum control were diluted 1 in 5 with 0.15 mol l^{-1} NaCl before assay. Aliquots (0.1 ml) of NAG standards (50, 100 and 200 mg l^{-1} in water), diluted serum, diluted control or water (reagent blank) were added to 0.1 ml acetate buffer (0.3 mol l^{-1} containing 0.45 mol l^{-1} NaCl, pH 3.8). HA substrate (0.1 ml, 4 mg ml^{-1} in water) was added and the samples were incubated for 24 h at 37°C in a thermostatically controlled room. Serum blanks were prepared by adding the HA substrate after the 24 h incubation. The reaction was terminated by heating at 100°C in an aluminium heating block for 5 min. After cooling, potassium tetraborate (60 μl, 0.8 mol l^{-1} in water, pH 10.0) was added with mixing before heating for 5 min at 100°C. The reaction mixtures were cooled in an ice-water bath before adding 2 ml of DMAB reagent (stock DMAB reagent—10% w/v in 12.5% v/v concentrated hydrochloric acid in glacial acetic acid; stock reagent diluted 1 in 10 with glacial acetic acid before use) and were then incubated for 20 min at 37°C in a water bath. The reaction mixtures were centrifuged immediately (1500 $g \times 10$ min) and the absorbance of the supernatant read at 582 nm within 30 min. HAE activity was expressed as the mean μmol NAG min^{-1} l^{-1} serum.

3. Results

3.1. Basic assay

The within-assay relative standard deviation (RSD) for normal pooled sera was 4% ($n = 10$) and the between-assay RSD was 14% ($n = 16$) with a mean of 18.4 μmol NAG min^{-1} l^{-1}.

3.2. Investigation of the serum HAE assay

3.2.1. Concentration and pH optimum of potassium tetraborate

Studies using a solution of NAG (120 mg l^{-1}) showed that the optical density (OD) obtained increased with the concentration of the potassium tetraborate solution (pH 10.0). A concentration of 0.8 mol l^{-1} was used routinely, higher concentrations being insoluble. The OD also increased rapidly as the pH of the potassium tetraborate (0.8 mol l^{-1}) was increased from 8.9 to 9.8, reaching a plateau between pH 9.8 and 10.5. A pH of 10.0 was used routinely.

3.2.2. Time of heating NAG with potassium tetraborate

Comparison of the ODs produced by heating the NAG standards with potassium tetraborate (0.8 mol l^{-1}, pH 10.0) at 100°C for 5, 10, 15 and 20 min, showed that the final OD produced in the assay increased proportionally with the time of heating. A time of 5 min was chosen for the routine assay because it produced suitable ODs.

3.2.3. Wavelength optimum of the NAG assay and linearity of the standard curve

The most intense λ_{max} value for a NAG standard (100 mg l^{-1}) and for the NAG produced by assay of a normal serum diluted 1 in 5 with 0.15 mol l^{-1} NaCl was 582 nm (apparent ε ($\pm$ SE) =

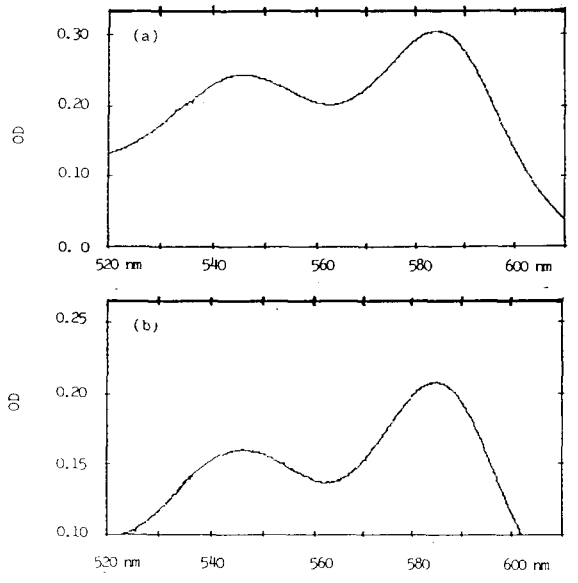

Fig. 1. Scans of the absorption spectra obtained by assaying: (a) normal serum; (b) NAG standard (100 mg l^{-1}).

1.57×10^4 ($\pm 2.91 \times 10^2$) 1 mol^{-1} cm^{-1}, [$n = 20$]) (Fig. 1). Fig. 2 shows that the ODs at 582 nm were proportional to the concentration of NAG

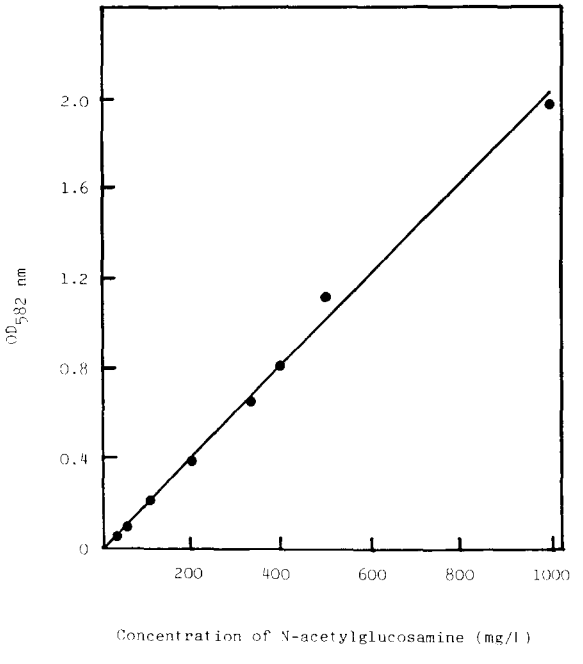

Fig. 2. The NAG standard curve.

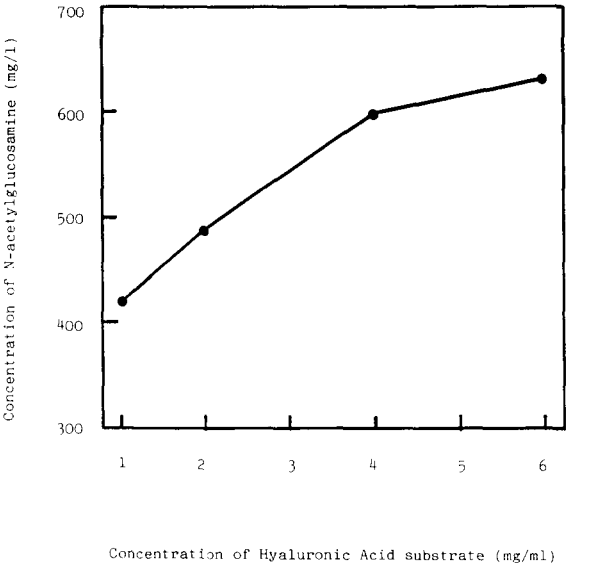

Fig. 3. The concentration of NAG produced by assaying a normal serum at varying concentrations of HA substrate.

up to at least 1000 mg NAG l^{-1}.

3.2.4. *Time course and stability of Reissig colour development*

The OD produced by assaying the NAG standards (50, 100 and 200 mg l^{-1}) increased with the time of incubation with DMAB up to 20 min. Longer incubation times resulted in a gradual decrease in OD. After centrifugation the final colour was stable for up to 30 min.

3.2.5. *Optimum buffer pH for serum HAE activity*

Two normal sera (diluted 1 in 5 with 0.15 mol l^{-1} NaCl) were assayed after incubating in acetate buffers pH 3.2–4.8. The optimum pH for serum HAE activity was 3.6–4.4. A pH of 3.8 was chosen for routine use.

3.2.6. *Optimum concentration of HA substrate*

The concentration of NAG produced by incubating normal sera (diluted 1 in 5 with 0.15 mol l^{-1} NaCl) with HA substrate increased proportionally with the concentration of the substrate (Fig. 3). A concentration of 4 mg ml^{-1} was chosen for routine use since higher concentrations were too viscous for accurate pipetting.

3.2.7. Optimum time of incubating serum with HA substrate

The concentration of NAG produced after incubating two normal sera (diluted 1 in 5 in 0.15 mol l^{-1} NaCl) at 37°C for 0.5–48 h is shown in Fig. 4. A 24 h incubation was chosen for routine use.

3.2.8. Optimum dilution of test sera

Normal sera ($n = 7$) were assayed neat and diluted 1 in 2, 1 in 5, 1 in 10 and 1 in 20 with 0.15 mol l^{-1} NaCl prior to assay. Maximum HAE activity was obtained with sera that had been diluted 1 in 5 (Fig. 5).

3.2.9. Stability of HAE in sera

There was no decrease in the HAE activity in blood samples left unseparated for 4 h or in serum samples left at room temperature for 2 h, at 4°C for 48 h or at −20°C for 22 days.

3.3. Serum HAE activity in normal human sera

The distribution of HAE activities of normal sera is shown in Fig. 6. Statistical analysis of non-normality (χ^2, [11]) showed that the activities did not conform to a Gaussian distribution ($\chi^2 = 30.59$, $P < 0.001$). The mean activity ($\pm$SD) was 17.1 ($\pm$3.6) μmol NAG min^{-1} l^{-1}. The mean activity for females (17.3 ($\pm$3.7) μmol NAG min^{-1} l^{-1}) and for males (17.0 ($\pm$3.6) μmol NAG min^{-1} l^{-1}) were compared using Student's t-test [12]. There was no significant difference

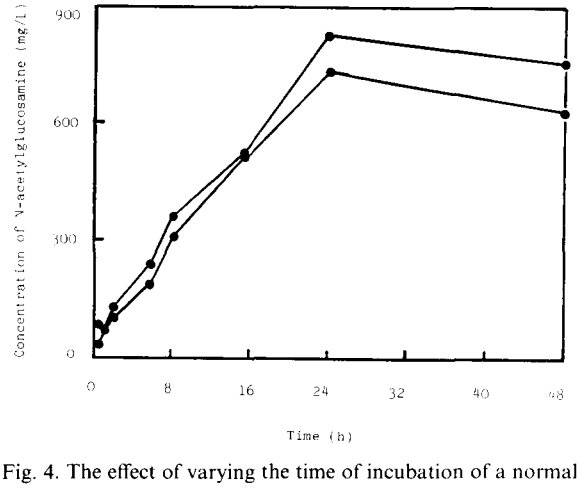

Fig. 4. The effect of varying the time of incubation of a normal serum with HA substrate.

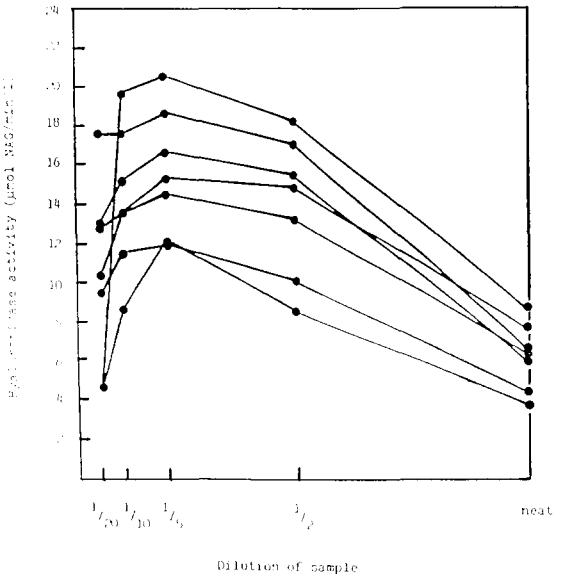

Fig. 5. The HAE activity of different concentrations of normal sera.

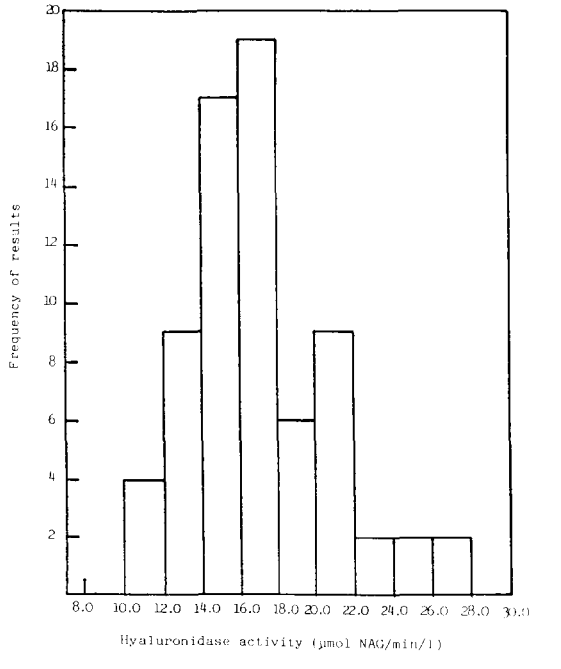

Fig. 6. The distribution of HAE activities of normal sera.

between activities in females and males ($t = 0.33$, $P > 0.5$). The mean HAE activities for subjects less than 55 years of age (17.8 ($\pm$ 3.8) μmol NAG min^{-1} l^{-1}) and for subjects 55 years and over (16.4 ($\pm$ 3.3) μmol NAG min^{-1} l^{-1}) were compared using Student's t-test. There was no significant different in HAE activities with age ($t = 1.65$, $0.5 > P > 0.1$).

4. Discussion

The colorimetric method of Bonner and Cantey [1] was chosen for further evaluation since a number of workers [4,13–15] have used variations of this method. This study confirmed the reports of Aminoff et al. [16] and Reissig et al. [10] that the concentration and pH of borate markedly influenced the colour production. A variety of wavelengths (544–585 nm) [10,13,15] have been used by different workers for measuring the NAG product. This study showed that 582 nm was the optimum wavelength for maximum sensitivity and other workers have reported that there were minimal effects from interfering substances at this wavelength [10].

The pH optimum of serum HAE was suprisingly low but is similar to that of lysosomal HAE (pH 3.5) and significantly lower than that of testicular HAE (pH 4.5–6.0) [2], suggesting that serum HAE is unlikely to originate from the latter. The low pH optimum also suggests that this enzyme is not normally involved in the degradation of HA in serum.

The time course for serum HAE activity using optimum conditions was unusually slow compared to most enzymes in that a prolonged incubation was required for maximum HA depolymerisation. This agreed with the work of Bonner and Cantey [1] and Bollet et al. [17]. The latter showed that this was not due to microbial contamination since cultures failed to reveal organisms and, as shown in this study, the highest rate of product formation occurred during the first few hours of incubation. The slow reaction kinetics of serum HAE and the fact that the maximum HAE activity was obtained using diluted serum suggests the possibility of HAE inhibitors or the presence of HAE complexes in serum. Mathews and Dorfman [18] reported the presence of a magnesium-dependent physiological inhibitor of HAE in serum. Northup et al. [5] were unable to confirm the presence of this inhibitor and, on the contrary, reported a thermolabile activator of HAE. Kolarova and Brada [13] showed that serum contained a thermostable activator of HAE but also reported that HAE was present as a complex with an unspecified inhibitor that dissociated on dilution. Further work is required to establish the presence and nature of activators and inhibitors of HAE in normal serum.

There are few reports of HAE activity in normal sera. A comparison of these studies is difficult because of the use of different assay conditions and units of HAE activity [1,8,15]. Bonner and Cantey [1] assayed the HAE activity of 35 patients with rheumatoid arthritis and compared these to the HAE activity of 18 normal adults, mainly medical students and hospital employees. They made no comment on any differences in HAE activity with age or sex. Northup et al. [5] assayed the serum HAE activity of 50 patients with neoplastic disease and compared this to 50 age-matched controls. They commented that females in the control group had a slightly higher mean HAE activity than males but found no variation with age. The lack of any significant differences in the serum HAE activity with age or sex in this study suggests that serum HAE activity is unlikely to originate from testicular tissue.

The fully evaluated assay presented in this paper is suitable for routine use and offers the possibility of further studies to establish the presence and nature of inhibitors and activators of HAE in normal serum and to establish the HAE activity in individual patients with a variety of pathological disorders.

Acknowledgements

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