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An efficient and reliable determination of hyaluronidase supports revision of the United States Pharmacopeia USP46-NF41 monograph – Hyaluronidase for injection

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ABSTRACT

Industrial scale manufacture of pharmaceutical grade hyaluronic acid increasingly uses a recombinant source of hyaluronidase to achieve size-specific polymers, necessary for both cosmetic and medical purposes. A turbidimetric assay is currently recommended by the United States Pharmacopeia (USP) to measure specific hyaluronidase enzyme activity. However, this monograph lacks critical detail of experimental methodology, uses difficult to source reagents and is technically challenging to perform. Herein, a simplified assay to measure the specific activity of hyaluronidase with improved reliability, consistency and flexibility is presented. Results demonstrated that the source and concentration of substrate significantly influenced turbidity formation, which occurred only under acidic conditions and when measured by absorbance at 400 nm rather than 640 nm. Replacement of horse serum as the binding protein agent with bovine serum albumin enhanced accuracy and precision, consistently meeting acceptance criteria. The improved protocol also introduced flexible timing, enabling reliable measurements within a 30 mins to 1 h window, compared to a fixed 30-minute time point. Inference by different compounds at concentrations normally used in standard buffers and fermentation media was not significant. However, Tris-HCl and Na_2HPO_4 significantly reduced the specific activity of hyaluronidase and are, therefore, not recommended in growth media for yeast cultivation, buffers used for enzyme purification, and in reagents necessary for monitoring production of size-specific hyaluronic acid polymers. A robust assay that can be validated is an important part of any enzyme dependent manufacturing process and based on our empirical experimentation, hereby propose revision of the existing USP monograph.

1. Introduction

Hyaluronidases ([HAse] EC3.2.1.35) are a class of enzymes belonging to the endoglucanases that hydrolyse β-1,4-glycosidic bonds between N-acetylglucosamine and glucuronic acid residues in the hyaluronic acid (HA) polymer into smaller oligosaccharides and disaccharides. HA chains containing between 0.4 and 1 MDa are termed low molecular weight, chains containing between 1 and 1.8 MDa are termed medium molecular weight, and chains above 1.8 MDa are termed high mass. This classification is based on the ability of HA to penetrate human dermal cells, and this is important as the size of the HA chain impacts its use. High molecular weight chains are used in medical applications via injection (ampoules) and low molecular weight chains are used in various cosmetics (moisturizers, lotions, lipsticks) for topical application to the skin [1,2].

There are various methods used to determine HAse enzyme activity, which can be classified into three main types: chemical, physicochemical, and biological methods. A summary of the advantages and disadvantages of each method is presented in Supplementary File 1, with extensive references. However, following an extensive search of worldwide pharmacopeias, only viscometry and turbidimetric assays are described in monographs for measuring HAse enzyme activity. The viscoelastic method for determining HAse activity is based on the ability of HAse to degrade HA, leading to changes in the viscoelastic properties

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of the HA solution [3]. The activity of HAse is quantified in International Units (IU) by comparing the rate of reduction in HA viscosity induced by the HAse being tested, against the rate obtained using the Standard for HAse [4]. The turbidimetric method involves observing a change in turbidity of a HA complex with protein in an acidic environment, after incubation with HAse. The principle is that a high-molecular mass of HA is able to form precipitates with diluted acidified protein serum, whereas depolymerised HA forms this complex to a lesser extent and the solution is, therefore, less turbid [5,6]. In the turbidimetric assay, horse serum, rabbit serum, human serum or human plasma, as well as purified protein fractions including horse serum albumin or bovine plasma albumin are used as precipitating reagents [6–8]. Moreover, quaternary ammonium salts such as, cetyltrimethylammonium bromide or cetylpyridinium chloride have been used as a precipitating agent in some studies [9,10].

The viscometry assay is provided as a monograph in the British Pharmacopoeia 2024 and the European Pharmacopoeia 11.0, whilst the turbidimetric assay is described in the United States Pharmacopoeia (USP) USP46-NF41 and the Indian Pharmacopoeia 2022 [11–14]. Both methods show no significant difference in the measurement of HAse enzyme activity [4,15–17]. However, the viscometry assay has some limitations as it requires multiple determinations, making it time-consuming. On the other hand, the turbidimetric assay is more efficient as it requires only a single turbidity measurement for each sample, making it ideal for serial testing [7,8,18].

Consequently, the turbidimetric method following the detailed protocol described in the USP was selected for our research, as it offers more comprehensive guidance compared to the Indian Pharmacopoeia. Unfortunately, the USP HAse standard enzyme appears to be no longer available, rendering the invalid since 2023. Additionally, we noted that the USP monograph does not specify the protein concentration of the horse serum reagent, a critical factor for turbidity formation. A study by Alburn and Whitley [19] highlighted key factors influencing results obtained using the turbidimetric assay including serum concentration, salt concentration, substrate concentration, pH, and storage time for turbidity formation. These possible inferences have also not been included in the current methodology of the USP monograph, neither has any advisory for interpretation of results. Rigorous quality control measures to ensure validation of HAse purity and activity are absolutely essential to ensure consistency and reliability in enzyme performance for quality assurance of resulting healthcare products. Like any biopharmaceutical, there is a risk of adverse reactions when using HAse, for example, potential allergic reactions. The aim of this study was to herein revisit the USP46-NF41 monograph and further investigated key factors influencing the usefulness of this protocol.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA) sodium salt from rooster comb (MW 757 kDa), bovine serum albumin (BSA), hydrolyzed gelatin, horse serum (HS), 1 M NaOH, European Pharmacopoeia (EP) HAse reference standard enzyme (577 IU/mg), $MgCl_2$, and $(NH_4)_2SO_4$ were purchased from Sigma (UK). The Pierce™ BCA Protein Assay Kit (Catalog number 23227, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States), glacial CH_3COOH, 5 M HCl, and Tris-base were sourced from Thermo Fisher Scientific Inc. NaCl and $Na_2HPO_4{\cdot}7\ H_2O$ were obtained from Acros Organics (Thermo Fisher Inc.), while NaH_2PO_4 (anhydrous) was acquired from Fluka Chemicals Ltd (Gillingham, Dorset, UK). $C_2H_3NaO_2$ anhydrous was supplied by VWR International Ltd (Lutterworth, Leicestershire, UK) and high molecular weight HA (MW 2 MDa) was purchased from WPA Chemical (Pudong, Shanghai, China). Absorbance was measured using a Jenway 7315 UV spectrophotometer (Fisher Scientific, Loughborough, Leicestershire, UK). Adjustments of pH were carried out using an inoLab pH meter level 2 equipped with a Sentix 42 pH electrode (Fisher Scientific). The incubation temperature for enzyme reactions was controlled by a Grant Instruments SUB 28 water bath (Royston, Hertfordshire, UK).

2.2. Methodology

2.2.1. Preparation of stock solutions

The stock solution of HA (MW 757 kDa) was prepared using phosphate buffer saline (PBS) to achieve a concentration of 500 μg/mL. The PBS (pH 6.3) was prepared by dissolving 2.5 g of NaH_2PO_4, 1.89 g of $Na_2HPO_4{\cdot}7\ H_2O$, and 8.2 g of NaCl in water and adjusting to 1 L. The HA stock solution was stored at temperatures not exceeding 5 °C and used within 30 days. The PBS solution can be stored at 15–25 °C in tightly sealed containers for several months. Before being used in the assay, the HA stock solution was diluted with an equal volume of PBS to obtain a concentration of 250 μg/mL. The HS stock solution was prepared by diluting HS in acetate buffer (pH 4.3) at a 1:9 ratio. The acetate buffer was made by dissolving 14 g of CH_3CO_2K and 20.5 mL of glacial CH_3COOH in deionized water, adjusting the volume to 1 L. After preparation, the pH of the HS stock solution was adjusted to 3.1 using 4 M HCl, and was left to stand at room temperature (22 °C) for 18–24 hs. The HS stock solution was stored at 0–4 °C and used within a few days. The acetate buffer could be stored at 15–25 °C in tightly sealed containers for several months. Before use in the assay, the stock solution was further diluted with acetate buffer at a 1:3 ratio, resulting in a final solution with a pH of 4.2. The HAse reference standard was dissolved in a cold diluent for the enzyme solution to produce a fresh standard solution with a concentration of 1.5 IU/mL. To prepare the diluent for the enzyme solution, mix 250 mL of PBS with 250 mL of water. Dissolve 330 mg of hydrolyzed gelatin in this mixture within 2 hs before use.

2.2.2. Scrutinizing the USP46-NF41 HAse assay protocol

Five different concentrations of standard HAse (0, 0.6, 0.9, 1.2, and 1.5 IU/mL) were prepared in duplicate to establish a standard enzyme activity calibration curve. First, 0.5 mL of HA solution (MW 757 kDa, 250 μg/mL) was added to each standard test tube, while 0.5 mL of PBS was added to a blank test tube. The enzyme diluent was added in the following volumes to the standard test tubes: 0.5 mL, 0.3 mL, 0.2 mL, 0.1 mL, and 0 mL. Additionally, 0.5 mL of the enzyme diluent was added to the blank test tube. Next, the specific volume of the standard enzyme was then added to each standard test tube with a 30-second gap between additions to reach a final volume of 1 mL. The mixture was gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was removed from the water bath sequentially at 30-secs intervals and immediately supplemented with 4 mL of HS solution. The final mixture was shaken well and allowed to stand at room temperature (22 °C) for 30 mins. Absorbance was measured at two wavelengths: 640 nm and 400 nm. Notably, the sample needed to be shaken for 5 s before each measurement. An overview of this method is provided as an easy-view graphical format in Supplementary File 2.

2.2.3. The Bicinchoninic Acid (BCA) protein assay

There are various methodologies for determining total protein concentration, such as the Bradford, Lowry and BCA assays. The BCA protein assay was selected because it offers better tolerance to interference compounds and can be carried out as a one-step process, unlike the two-step Lowry assay [20]. Additionally, the BCA assay is more precise than the Bradford assay, making it a preferred choice for accurately determining protein concentrations in various samples [21]. A concentration-response curve for the protein standard was established, ranging from 0 to 1000 μg/mL. This curve was created by diluting a 2000 μg/mL BSA standard solution obtained from the Pierce™ BCA Protein Assay Kit with Milli-Q water in a 2-fold dilution series. In this assay, the protein content of 1 mL of HS and 1 mg/mL BSA was precisely determined.

To determine the total protein content, 0.1 mL of samples or standard solutions was thoroughly mixed with 2.0 mL of BCA working reagent.

The BCA working reagent, prepared by mixing BCA Reagent A with BCA Reagent B at a ratio of 50:1, was also obtained from the Pierce™ BCA Protein Assay Kit. The mixture was then incubated in a water bath maintained at 37 °C for 30 mins. After incubation, each tube was cooled to room temperature (22 °C) for approximately 5 mins. Finally, the absorbance of each mixture was measured at 562 nm, with Milli-Q water serving as the blank. All samples and standard solutions were prepared in triplicate. An overview of this method is provided as an easy-view graphical format in Supplementary File 3.

2.2.4. Optimizing factors affecting assay performance

2.2.4.1. Influence of type of protein and concentration on turbidity formation. A 2-fold dilution series of HS and BSA was prepared, with concentrations of 2, 4, 8, and 16 mg/mL of protein content. The pH was adjusted to 3.1 by adding 4 M HCl, and the solutions were left to stand at room temperature (22 °C) for at least 18–24 hs. Before being used in the optimized USP assay, the HS and BSA stock solutions were diluted with acetate buffer solution at a ratio of 1:3. The results were reported as the final protein concentration in the mixture.

Samples were prepared by adding 500 μL of HA solution (MW 757 kDa, 250 μg/mL) to each test tube, while 500 μL of PBS was added to a blank test tube. Each concentration of HS and BSA sample required its own blank. Next, 500 μL of enzyme diluent was added sequentially to the test tubes at 30 s intervals. The mixtures were gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was removed from the water bath in sequence, and 4 mL of HS or BSA solution was immediately added. The final mixture was shaken well and allowed to stand at room temperature (22 °C) for 30 mins. Finally, absorbance was measured at 640 and 400 nm after shaking for 5 s.

2.2.4.2. Influence of pH on turbidity formation. A pH series of HS solutions was prepared by first mixing HS with acetate buffer (pH 4.3) at a 1:9 ratio, followed by further dilution at a 1:3 ratio. The pH was then adjusted to 2, 3, 4, 5, 7, and 11 by adding either 4 M HCl or 1 M NaOH. Additionally, the HS solution prepared according to the USP monograph was also evaluated. Samples were prepared by using 500 μL of HA solution (MW 757 kDa, 250 μg/mL), while 500 μL of PBS was added into a blank test tube. Each pH of HS needed to have its own blank. Then, 500 μL of diluent of enzyme was sequentially added into each test tubes at 30 s intervals. The mixture was gently mixed well and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was sequentially removed from the water bath, and 4 mL of HS solution at the desired pH level was immediately added. The final mixture was shaken well and left at room temperature (22 °C) for 30 mins. Finally, the absorbance of the mixture was measured at wavelengths 400 and 640 nm. The results were reported as pH of the final mixtures.

2.2.4.3. Influence of storage time on turbidity formation. Samples were prepared by using 500 μL of HA solution (MW 757 kDa, 250 μg/mL), while 500 μL of PBS was added into a blank test tube. Then, 500 μL of diluent of enzyme was also added into both test tubes with a gap time of 30 s. The mixture was gently mixed well and incubated in a water bath at 37 °C for 30 mins. After the incubation, each tube was taken out from the water bath sequentially at 30 s intervals, and immediately 4 mL of HS solution was added. The final mixture was shaken well, and the absorbance of the mixture was measured at wavelengths 400 and 640 nm at different time points: 5, 10, 15, 30, 45, and 60 mins.

2.2.4.4. Influence of ionic strength on turbidity formation. A 1.6 M NaCl stock solution was prepared in Milli-Q water and diluted to achieve concentrations of 0.4, 0.8, 1.2, and 1.6 M (stock solution). Four different concentrations of ionic strength samples were prepared by using 500 μL of HA solution (MW 757 kDa, 250 μg/mL), while 500 μL of PBS was added to a blank test tube. Then, 250 μL of enzyme diluent was added to each test tube. Next, 250 μL of each different NaCl solution was added to the respective tubes to achieve a final volume of 1 mL, with a gap time of 30 s. The mixture was gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was taken out of the water bath sequentially at 30 s intervals, and immediately 4 mL of HS solution was added and left at room temperature (22 °C) for 30 mins. The absorbance of the mixture was measured at wavelengths of 400 and 640 nm.

2.2.5. Method validation of the assay

2.2.5.1. Method validation assumptions. The general chapter USP46-NF41 < 1225 > [22] and The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2(R2) [23] describes method validation as the process to demonstrate that the characteristics of a procedure meet the requirements for the intended purpose. The purpose of analysis in General chapter USP46-NF41 < 1225 > can be categorized into 4 categories, for example, quantification of active ingredients, determination of impurities in drug product, determination of the drug release or dissolution and Identification, respectively. This category is also similar to ICH guideline Q2(R2). Different intended analytical applications require different characteristics for performance as is shown in Supplementary File 4 [22,23]. The HAse enzyme activity assay is classified as category I. Therefore, the parameters that are required to be performed were specificity, range, linearity, accuracy and precision.

2.2.5.2. Terminology to describe validation parameters. Definitions of specificity, range, linearity, accuracy and precision followed the General chapter USP46-NF41 < 1225 > [22], namely: 1) Specificity is described as the capability of accurately identifying and measuring an analyte, demonstrating that its identification and/or quantitation is not impacted by the presence of other expected components. 2) The range refers to the interval between the high and low concentrations of the substance of interest in a sample. This range, typically determined from linearity studies, depends on the intended application of the procedure. Within this concentration range, the method has been proven to possess acceptable precision, accuracy, and linearity. For the assay of a drug substance, the range generally covers 80–120 % of the test concentration. 3/ Linearity refers to its capacity to produce test results that are directly proportional to the concentration of the substance of interest within a specified range. This relationship is typically evaluated by visually inspecting a plot of the analytical response versus the analyte concentrations. If a linear relationship is evident, statistical methods such as the least squares regression analysis are employed to quantify the degree of linearity. Key parameters, including the correlation coefficient, y-intercept, and slope of the regression line should be reported. For accurate linearity assessment, testing should be conducted using a minimum of five different concentrations. 4/ Accuracy refers to the closeness of the measured value to the true or accepted reference value. This characteristic, also known as trueness, must be established across the procedure's entire range. For assays involving drug substances, accuracy can be determined by applying the analytical method to a substance of known purity, such as a reference standard. It is typically calculated as the percentage of recovery of a known quantity of analyte added to the sample. The ICH guidelines suggest that accuracy should be assessed using at least nine determinations over three different concentration levels, covering the specified range of the procedure (i.e., three concentrations with three replicates each). This can be achieved by evaluating the percent recovery of the analyte across the assay range. 5/ Precision refers to how consistently a procedure produces the same results when applied repeatedly to multiple samples of a homogeneous sample. It is typically measured using the standard deviation, relative standard deviation (RSD), or coefficient of variation (CV) of a set of measurements. Precision can be divided into three categories:

repeatability, intermediate precision (ruggedness), and reproducibility. In this study, it is essential to determine both repeatability and intermediate precision. Repeatability involves using the same analytical procedure within a single laboratory over a short period, with the same analyst and equipment. It should be assessed through at least nine determinations covering the procedure's specified range (three concentrations with three replicates each) or at least six determinations at 100% of the test concentration. Intermediate precision refers to determining the variability within a laboratory, considering different days, analysts, or equipment with the same analytical procedure.

2.2.5.3. Assessment of assay specificity. The spectrum of the HA solution (MW 757 kDa, 250 μg/mL), HS solution (pH 4.2), and a sample without HAse enzyme (0 IU/mL), prepared following the USP46 NF41 assay protocol [12], was scanned at wavelengths ranging from 400 to 800 nm to assess specificity, as shown in Supplementary File 5.

2.2.5.4. Assessment of assay range and linearity. The standard concentration-response curve, ranging from 0 to 1.5 IU/mL with five different concentrations following the assay protocol, was performed to evaluate the range and linearity of the assay. The procedure is shown in Supplementary File 6.

2.2.5.5. Assessment of assay accuracy. Before determining accuracy, a calibration curve was established following the USP protocol (Supplementary File 6). Three concentrations of the standard enzyme (0.6, 1.0, and 1.4 IU/mL) were prepared in triplicate. Each sample tube was added 0.5 mL of HA solution (MW 757 kDa, 250 μg/mL) and varying amounts of enzyme diluent (300 μL, 167 μL, and 33 μL), while the blank test tube was added 500 μL of PBS and 500 μL of enzyme diluent. Standard enzyme was added to each sample tube at 30-second intervals to reach a final volume of 1 mL. The mixtures were gently mixed and incubated at 37 °C for 30 mins. After incubation, each tube was sequentially removed at 30-second intervals, and 4 mL of HS solution (pH 4.2) was immediately added. The mixtures were shaken well and left at room temperature (22 °C) for 30 mins. Absorbance was measured at 640 and 400 nm as shown in Supplementary File 7. Results were reported as % recovery given by:

$$\%\text{Recovery} = Y_f/Y_c \times 100$$

Where Y_f is the measured concentration (IU/mL), and Y_c is the actual or true concentration (IU/mL) [31].

2.2.5.6. Assessment of assay precision. Before determining precision, a daily calibration curve was established following the USP protocol shown in Supplementary File 6. A 1.0 IU/mL standard enzyme solution was prepared in six replicates. Firstly, 0.5 mL of HA solution (MW 757 kDa, 250 μg/mL) was added to each sample tube, and 0.5 mL of PBS was added to a blank test tube. Then, 167 μL of enzyme diluent was added to each sample tube, and 500 μL to the blank test tube. Next, 333 μL of standard enzyme was added to each test tube at 30-second intervals to reach a final volume of 1 mL. The mixtures were gently mixed and incubated at 37 °C for 30 mins. After incubation, each tube was sequentially removed at 30 s intervals, and 4 mL of HS solution (pH 4.2) was immediately added. The mixtures were shaken well and left at room temperature (22 °C) for 30 mins. Absorbance was measured at 640 and 400 nm, as shown in Supplementary File 8. Repeatability measurements were conducted within a single day, while intermediate precision measurements were taken over three days using the same protocol. Results were reported as %RSD, given by:

$$\text{SD} = \sqrt{\frac{\sum_{i=1}^{n}(Y_i - \overline{Y})}{n-1}};\ \text{RSD} = \text{CV} = \frac{SD}{Mean} \times 100\%$$

where Yi is the individual value, $\overline{Y}$ is the sample mean, n is the sample size, SD is the standard deviation, and CV is the coefficient of variation [24].

2.2.5.7. Acceptance Criteria for Method Validation of the USP46-NF41 HAse Assay Protocol. To validate the analytical compendial assay for HAse, assessments of specificity, range, linearity, accuracy, and precision must be performed to meet the required acceptance criteria. These criteria, detailed in Supplementary file 9, were modified from the AOAC and FDA method validation guidelines [25,26], as both guidelines consider the concentration of the sample, especially in our assay, which uses samples in the ppm unit.

2.2.6. Optimization of HA concentration for improved HAse determination

Four different concentrations of HA (MW 2 MDa) were prepared: 250 μg/mL, 300 μg/mL, 400 μg/mL, and 500 μg/mL, reacting with 1.63 mg/mL HS or 3.75 mg/mL BSA. These concentrations were evaluated with enzyme concentrations of 0, 1, and 5 IU/mL, each in duplicate. Briefly, 0.5 mL of HA solution at each concentration was added to each standard test tube, while 0.5 mL of PBS was added to a blank test tube. The enzyme diluent was then added to the standard test tubes in the following volumes: 0.5 mL, 0.4 mL, and 0 mL, with an additional 0.5 mL of the enzyme diluent also added to the blank test tube. The specific volume of the standard enzyme was added to each standard test tube with a 30 s gap between additions to achieve a final volume of 1 mL. The mixture was gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was sequentially removed from the water bath at 30 s intervals and immediately supplemented with 4 mL of HS or BSA solution. The final mixture was shaken well and allowed to stand at room temperature (22 °C) for 30 mins. Finally, the absorbance of the mixture was measured at 400 nm after shaking for 5 s

2.2.7. Stock solution preparation for improved for improved HAse determination

Due to the difficulty in accessing the previous HA (757 kDa), high molecular weight HA (2 MDa) was used as a replacement and prepared in PBS at a concentration of 600 μg/mL. Prior to use in the assay, the HA stock solution was diluted with an equal volume of PBS to achieve a final concentration of 300 μg/mL. The HS stock solution was prepared following the optimized original stock preparation protocol. Additionally, the BSA stock solution was prepared at a concentration of 16 mg/mL, with the pH adjusted to 3.1 using 4 M HCl. Both the HS and BSA solutions were allowed to stand at room temperature (22 °C) for 18–24 hs The BSA and HS stock solutions were stored at 0–4 °C and used within a few days. Prior to use in the assay, each stock solution was further diluted with acetate buffer at a 1:3 ratio, yielding a final pH of 4.2. For the HAse standard, the enzyme was dissolved in a cold enzyme diluent solution to produce a fresh standard solution at a concentration of 5 IU/mL.

2.2.8. Revised protocol for improved HAse determination

A standard concentration-response curve was prepared using six different concentrations of standard HAse (0, 1, 2, 3, 4, and 5 IU/mL), each in duplicate to establish a calibration curve. Briefly, 0.5 mL of HA solution (MW 2 MDa, 300 μg/mL) was added to each standard test tube, while 0.5 mL of PBS was added to a blank test tube. The enzyme diluent was added in the following volumes to the standard test tubes: 0.5 mL, 0.4 mL, 0.3 mL, 0.2 mL, 0.1 mL, and 0 mL, with 0.5 mL of the enzyme diluent also added to the blank test tube. The specific volume of the standard enzyme was added to each standard test tube with a 30 s gap between additions to reach a final volume of 1 mL. The mixture was gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was removed from the water bath sequentially at 30 s intervals and immediately supplemented with 4 mL of HS or BSA solution. The final mixture was shaken well and allowed to stand at room temperature (22 °C) for 30 mins. Finally, the absorbance of the

mixture was measured at 400 nm after shaking for 5 s, as shown in Supplementary File 10.

2.2.9. Method validation for improved HAse determination

2.2.9.1. Specificity. The spectrum of the HA solution (MW 2 MDa, 300 μg/mL), 1.63 mg/mL protein content from HA, 3.75 mg/mL of BSA solution, and a sample without HAse (0 IU/mL), was scanned at wavelengths ranging from 400 to 800 nm to assess specificity, as shown in Supplementary File 11.

2.2.9.2. Range and Linearity. The standard concentration-response curve, ranging from 0 to 5 IU/mL with 6 different concentrations following the assay protocol, was performed to evaluate the range and linearity of the assay. The procedure is shown in Supplementary File 10.

2.2.9.3. Accuracy. A calibration curve was established following the improved USP assay protocol (Supplementary File 10). Three concentrations of the standard enzyme (1.5, 2.5, and 3.5 IU/mL) were prepared in triplicate. Each sample tube was added 0.5 mL of HA solution (MW 2 MDa, 300 μg/mL) and varying amounts of enzyme diluent (350 μL, 250 μL, and 150 μL), while the blank test tube was added 500 μL of PBS and 500 μL of enzyme diluent. Standard enzyme was added to each sample tube at 30 s intervals to reach a final volume of 1 mL. The mixtures were gently mixed and incubated at 37 °C for 30 mins. After incubation, each tube was sequentially removed at 30 s intervals, and 4 mL of 1.63 mg/mL protein content from HS or 3.75 mg/mL of BSA solution was immediately added. The mixtures were shaken well and left at room temperature (22 °C) for 30 mins. Absorbance was measured at 400 nm as shown in Supplementary File 12. Results were reported as % recovery.

2.2.9.4. Precision. Before determining precision, a daily calibration curve was established following the USP protocol shown in Supplementary File 10. A 2.5 IU/mL standard enzyme solution was prepared in six replicates. Firstly, 0.5 mL of HA solution (MW 2 MDa, 300 μg/mL) was added to each sample tube, and 0.5 mL of PBS was added to a blank test tube. Then, 250 μL of enzyme diluent was added to each sample tube, and 500 μL to the blank test tube. Next, 250 μL of standard enzyme was added to each test tube at 30 s intervals to reach a final volume of 1 mL. The mixtures were gently mixed and incubated at 37 °C for 30 mins. After incubation, each tube was sequentially removed at 30 s intervals, and 4 mL of 1.63 mg/mL protein content from HS or 3.75 mg/mL of BSA solution was immediately added. The mixtures were shaken well and left at room temperature (22 °C) for 30 mins. Absorbance was measured at 400 nm, as shown in Supplementary File 13. Repeatability measurements were conducted within a single day, while intermediate precision measurements were taken over three days using the same protocol. Results were reported as %RSD. After the method was validated, the factors of reading time and salt interference were evaluated again using the new method.

2.2.9.5. Acceptance Criteria for Method Validation of improved HAse determination. The acceptance criteria for the improved method for HAse determination, detailed in Table 1, are based on modifications to the AOAC and FDA method validation guidelines [25,26]. These guidelines were chosen because they account for the sample concentration factor, which is crucial for establishing acceptance criteria that align with small sample quantities (in the ppm range). These criteria are similar to those for the method validation of the USP46-NF41 HAse Assay Protocol, described in 2.2.5.7. However, the precision acceptance criteria were made stricter due to the increased sample concentrations and the expanded range. Five key parameters, including specificity, range, linearity, accuracy, and precision, must be evaluated and compared against the established acceptance criteria.

Table 1
Acceptance criteria for method validation of improved hyaluronidase determination, modified from AOAC and FDA guidelines [25,26], including specificity, range, linearity, accuracy, repeatability, and intermediate precision.

Parameter	Acceptance criteria
Specificity	Inferences from other substances does effect the absorption spectrum.
Range	At least 80 % - 120 % of the test concentration
Linearity	$R \geq 0.995$
Accuracy	%Recovery: 80 % – 110 %
Repeatability	% RSD < 7.3 %
Intermediate precision	% RSD < 11 %

2.2.10. Determination of factors affecting improved HAse determination

2.2.10.1. Effect of storage time on turbidity formation. The four concentrations of the standard enzyme (0, 1, 2.5, and 5 IU/mL) were prepared in triplicate and measured at various time points. Briefly, 0.5 mL of HA solution (MW 2 MDa, 300 μg/mL) was added to each sample tube, while 500 μL of PBS was added to a blank tube. Varying amounts of enzyme diluent (500 μL, 400 μL, 250 μL, and 0 μL) were added to the sample tubes, with 500 μL added to the blank tube. Standard enzyme was then added to each sample tube at 30 s intervals to reach a final volume of 1 mL. The mixtures were gently mixed and incubated in a 37 °C water bath for 30 mins. Following incubation, each tube was sequentially removed from the water bath at 30 s intervals, and 4 mL of 3.75 mg/mL BSA solution was immediately added. The final mixture was thoroughly shaken, and absorbance was measured at 400 nm at intervals of 5, 10, 15, 30, 45, and 60 mins.

2.2.10.2. Salt inferences on turbidity measurements. The screening experiment was conducted by preparing 0.4 M solutions of potential salt interferences present in the medium and buffer, including NaCl, $MgCl_2$, $(NH_4)_2SO_4$, NaH_2PO_4, Na_2HPO_4, CH_3COONa, and Tris base in enzyme diluent. Samples were prepared by adding 500 μL of HA solution (MW 2 MDa, 300 μg/mL) to each test tube, while 500 μL of PBS was added to a blank tube. Next, 250 μL of each salt solution was added to the respective test tubes, followed by 250 μL of enzyme diluent to achieve a final volume of 1 mL, with a 30 s gap between additions. The mixtures were gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was removed sequentially at 30 s intervals, immediately supplemented with 4 mL of BSA solution at a concentration of 3.75 mg/mL and left to stand at room temperature (22 °C) for 30 mins. The absorbance of each mixture was then measured at 400 nm. Based on the screening results, a 2-fold dilution series of the significant salt interferences was further evaluated at five concentrations: 0, 25, 50, 100, and 200 mM, using the same protocol.

2.2.10.3. Salt inferences on HAse enzyme activity. Similarly, the screening experiment was conducted by preparing 0.4 M solutions of potential salt interferences present in the medium and buffer, including NaCl, $MgCl_2$, $(NH_4)_2SO_4$, NaH_2PO_4, Na_2HPO_4, CH_3COONa, and Tris base in enzyme diluent. Samples were prepared by adding 500 μL of HA solution (MW 2 MDa, 300 μg/mL) to each test tube, while 500 μL of PBS was added to a blank tube. Next, 250 μL of each salt solution was added to the respective test tubes, followed by 250 μL of a 5 IU/mL standard enzyme solution to achieve a final volume of 1 mL, with a 30 s gap between additions. The mixtures were gently mixed and incubated in a water bath at 37 °C for 30 mins. After incubation, each tube was removed sequentially at 30 s intervals, immediately supplemented with 4 mL of BSA solution at a concentration of 3.75 mg/mL and left to stand at room temperature (22 °C) for 30 mins. The absorbance of each mixture was then measured at 400 nm. Based on the screening results, a 2-fold dilution series of the significant salt interferences was further evaluated at five concentrations: 0, 25, 50, 100, and 200 mM, using the

same protocol.

3. Results and Discussions

3.1. Total protein content measurement by Bicinchoninic Acid (BCA) assay

Firstly, the BCA assay was used to quantify the total protein content in the HS for the USP assay. Additionally, BSA was also determined because it is commonly used in turbidimetric enzyme activity assays instead of HS [27,28]. The principle of the BCA assay relies upon the reduction of Cu^{2+} to Cu^{+1} by protein in a basic environment. After that, Cu^{+1} will chelate with 2 molecules of bicinchoninic acid in the reagent, resulting in a purple-colored complex, which can be measured for absorbance at 562 nm [29]

3.2. Calibration curve of BCA assay

Before revalidating the turbidimetric assay, it was important to know the exact protein content in HS to for the method to be reproducible. The USP assay only provided the ratio of HS used, but did not specify the precise protein concentration. HS contains various proteins including albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin, the concentrations of which can vary between different batches and sources, leading to variations in turbidity results. The concentration-response curve for the protein standard is shown in Supplementary File 14 which provided R = 0.9967. This curve demonstrated a linear increase in absorbance, with rising protein concentrations within the range of 0–1000 μg/mL. The analysis of BSA and HS revealed that a concentration of 1 mg/mL of BSA corresponded to a protein content of 1.00 ± 0.01 mg. In contrast, 1 mL of HS contained a total protein content of 69.22 ± 0.58 mg. The difference in protein concentration was due to the form of the raw material. BSA was prepared from pure albumin powder with a known concentration of albumin, while HS is a solution that contains various concentrations of different proteins.

3.3. Optimization of factors affecting the turbidimetric method

3.3.1. The influence of type of protein and concentration on turbidity formation

The turbidity assay is based on the formation of insoluble complexes involving HA and non-catalytic proteins. A study by Dorfman et al. found that both HS and BSA, which are non-catalytic proteins, can form complexes with HA [18]. However, the USP monograph does not provide details of the concentration of HS. Therefore, the effects of different protein types (i.e., HS and BSA) and concentrations on turbidity formation were investigated. The results demonstrate that increasing the concentration of either protein results in a corresponding increase in turbidity. Significant differences in turbidity are observed at each concentration and both wavelengths, except between concentrations of 1.75 mg/mL and 3.47 mg/mL for HS, where no difference in turbidity is evident, as illustrated in Fig. 1. However, HS demonstrated a superior ability to produce turbidity compared to BSA at similar concentrations when measured at the same wavelength. This is because HS contains various proteins, while BSA contains only albumin. Regarding the turbidity complex formed by the interaction between HA and proteins, primarily through electrostatic interactions, the varying properties of the proteins can result in higher or lower positive charges under the conditions measured. Bełdowski et al. (2021) described that HSA and γ-globulin interact differently with HA due to different isoelectric points. This indicated that the pKa values of the ionizable groups of HSA and γ-globulin were different and would, therefore, provide different degrees of turbidity [30]. Based on the results illustrated in Fig. 1, HS was selected for further study as it produced a higher absorbance value compared to the BSA-HA complex.

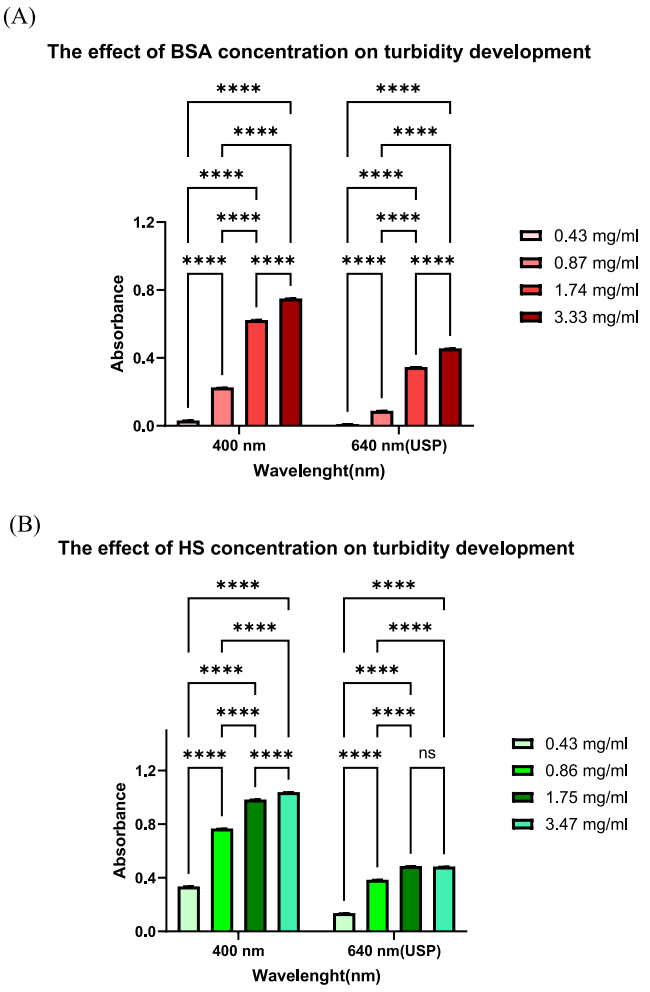


Fig. 1. Comparison of turbidity development between BSA (A) and horse serum (HS) (B) at four different concentrations. The effect of protein on turbidity development was assessed at wavelengths of 400 nm and 640 nm. Each sample was run in triplicate (n = 3), and the absorbance values are reported as Mean ± SD. *Indicates a significant difference in absorbance for each sample compared to a different time point (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001). The results are reported as the final protein concentration in the mixture.

3.3.2. The influence of pH on development of turbidity

Understanding the mechanism of complex formation is essential. The formation of complexes occurs through electrostatic forces between HA which carries a negative charge and proteins, which have a positive charge. HA exhibits a negative charge when the pH exceeds its pKa of 2.9, whereas proteins carry a positive charge when the pH is below their pKa values, as illustrated in Supplementary File 15. The isoelectric pH of HS was not known because of the inconsistent composition of this mixed protein solution. An effect of pH was constructed to find the optimum pH for turbidity development. The results showed that a final pH environment, lower than 5, could influence the overall positive charge on the surface of proteins, leading to complex formation. The pH recommended in the USP protocol is pH 4.1, which provided the greatest turbidity at both wavelengths measured, as shown in Fig. 2. This was comparable to the results from the study of Lenormand et al. where turbidity could be measured between pH 2.3–6.5, with a maximum between pH 3.3 and pH4 [31]. Therefore, pH 4.1 as recommended by the USP monograph was selected for further study.

3.3.3. The influence of storage time on turbidity formation

Understanding the rate of turbidity formation between HA and HS is

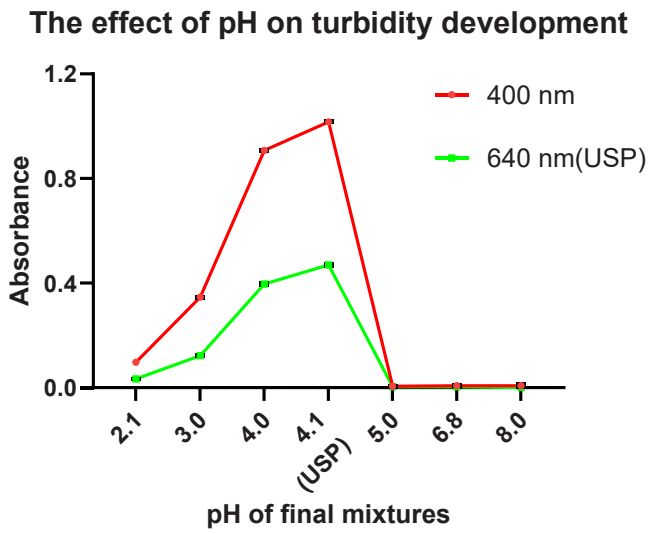


Fig. 2. Effect of pH on turbidity development in the optimized USP hyaluronidase activity assay method. The results are presented for final mixture pH values of 2.1, 3.0, 4.0, 4.1, 5.0, 6.8, and 8.0. Turbidity development was measured at wavelengths of 400 nm and 640 nm. Each sample was performed in triplicates (n = 3), with absorbance values reported as Mean ± SD.

a fundamental parameter to provide accurate turbidity reading. Thus, turbidity formation was monitored by measuring the turbidity of the solution at different time intervals. The results, shown in Fig. 3, demonstrated an increase in turbidity over time, with significant differences observed except between 10 and 45 mins at 400 nm. Similarly, during the same interval, there was notable variation in turbidity at 640 nm. Therefore, the 10–45-minute interval at 400 nm appeared most suitable for measurement without significant variation. Raw data are available in Supplementary File 16.

3.3.4. The Influence of ionic strength on turbidity development

Inferences to assay performance can be caused by salts, such as NaCl, $MgCl_2$ and $(NH_4)_2SO_4$, that are included as ingredients in many standard buffers or media used during the fermentation and purification processes for HAse production. Therefore, it was important to study the effect of salt interference on turbidity development by varying the NaCl concentration in reaction mixtures. The results demonstrated that increasing NaCl concentration interferes with HA-HS complex formation, leading to reduced turbidity. Significant differences were observed, except when comparing absorbance at NaCl concentrations between 0 mmol/L and 20 mmol/L at 640 nm, as depicted in Fig. 4. A study by Dorfman et al. reported a comparable trend, with increasing salt concentration resulting in decreased turbidity development [18]. This phenomenon occurs because HA and proteins primarily interact through electrostatic forces; the ionic charge from the salt can neutralize these

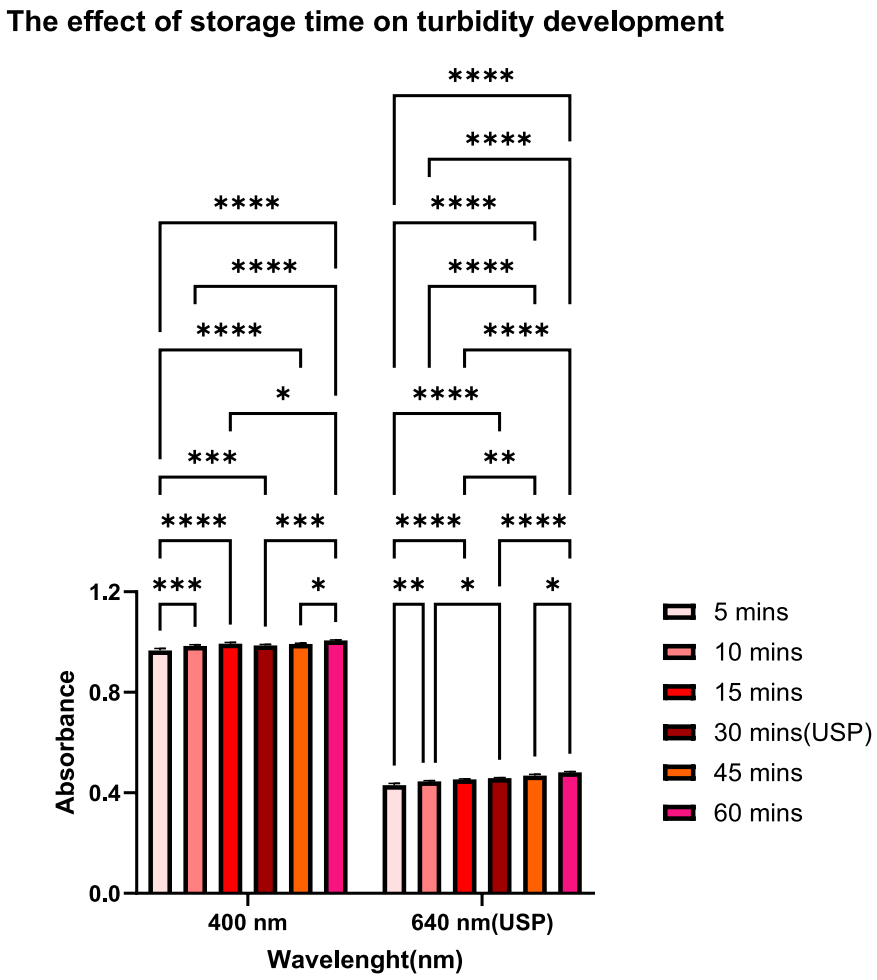


Fig. 3. Effect of storage time on turbidity development in the optimized USP hyaluronidase activity assay at different time points, measured at 400 nm and 640 nm. Each sample was performed in triplicates (n = 3), and absorbance values are reported as Mean ± SD. *Indicates a significant difference in absorbance for each sample compared to a different time point (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001), with raw data to clarify these statistical difference given in Supplementary File 16.

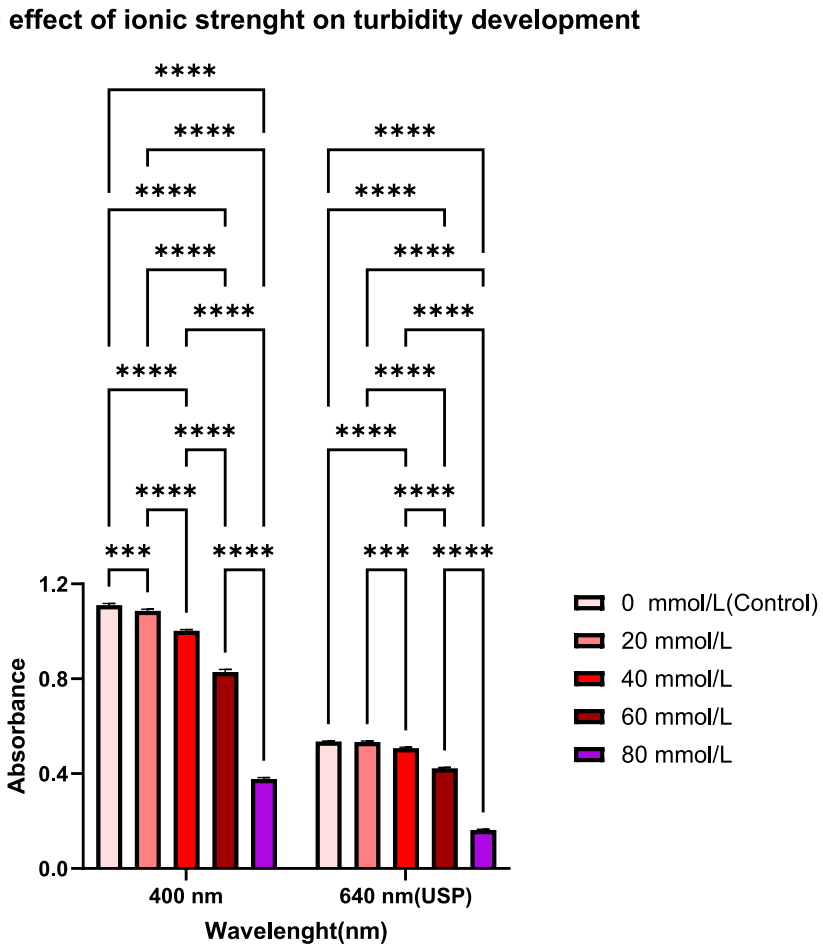


Fig. 4. The effect of NaCl interference on turbidity development in the optimised USP hyaluronidase activity assay at four different concentrations, ranging from 0 to 80 mmol/L. Absorbance of the mixture was measured at wavelengths of 400 and 640 nm. Each sample was run in triplicate (n = 3), and absorbance values are reported as Mean ± SD. *Indicates a significant difference in absorbance for each sample compared to a different time point (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001).

charges on the surfaces of HA and proteins. This neutralization diminishes electrostatic interactions, thereby preventing complex formation [32].

3.4. Method validation

3.4.1. Specificity of the USP46-NF41 HAse Assay Protocol

The spectrum of the HA-HS complex, HA solution (MW 757 kDa, 250 μg/mL) and HS solution, prepared according to the USP protocol, was evaluated by measuring the absorption of the complex between 400 and 800 nm, to illustrate the specificity of the USP assay. The results demonstrated that the spectrum of the HA-HS complex was not compromised by other components of the assay mixture, as shown in Fig. 5. This confirms the specificity of the method and its compliance with the acceptance criteria, as shown in Supplementary File 9. Additionally, the spectrum presented an optimal starting point for the turbidimetric assay at an absorbance of around 1, because when test HAse is added, it will result in a reduction in turbidity (i.e., absorbance). Therefore, a wavelength of 400 nm was selected for further study and compared with the 640 nm wavelength recommended in the USP monograph.

3.4.2. Range and Linearity of the USP46-NF41 HAse Assay Protocol

In the next phase of method validation, the range of concentrations was evaluated to ensure reliability of the assay. In the absence of a USP standard enzyme, the HAse European Pharmacopoeia standard enzyme was assayed at concentrations ranging from 0 to 1.5 IU/mL at pH 4.1 and 22 °C. Then, the absorbance of these solutions was measured, and a calibration curve plotted. The correlation coefficient and linear regression equation were next determined. The response of the enzyme was found to be linear within the investigated concentration range, with a linear regression equation $y = -0.5209x + 0.9181$ and a correlation coefficient of 0.9981 at 400 nm, and $y = -0.2114x + 0.3711$ with a correlation coefficient of 0.9989 at 640 nm as shown in the Supplementary File 17. Both calibration curves passed the acceptance criteria outlined in Supplementary File 9, with correlation coefficients exceeding 0.9995.

3.4.3. Precision of the USP46-NF41 HAse Assay Protocol

Accuracy refers to how closely the observed value aligns with the true or reference value, expressed as percentage recovery. The % recovery results provided in Table 2 range from 98.23 % to 117.67 % at 400 nm and from 99.30 % to 120.62 % at 640 nm. These values exceed the specified acceptance range of 80–110 %, as outlined in Supplementary File 9, indicating inconsistencies in the assay. We suspected that this variation may be due to the HS, as it contains various types of proteins that can form complexes with hyaluronic acid. Therefore, further improvements were necessary to enhance accuracy of the method.

3.4.4. Precision of the USP46-NF41 HAse Assay Protocol

The precision of an analytical procedure expresses how the observed

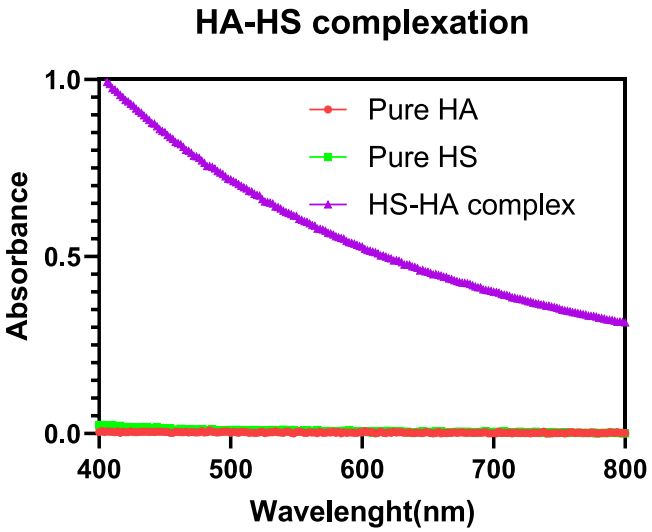


Fig. 5. Spectrum of HA solution (MW 757 kDa, 250 μg/mL), HS solution (pH 4.2, 1.63 mg/mL), and HA-HS complex (pH 4.1, HA 250 μg/mL reacted with HS solution 1.63 mg/mL) at 22°C between 400 and 800 nm (Mean ± SD). HA and HS solution were directly scanned from the wavelength 400–800 nm. While HA-HS complex was performed by dissolving HA in PBS, then incubated at 37°C for 30 min [19]. Subsequently, a horse serum (pH 4.2) was added in tubes and left at 22 °C for 30 min. The absorbance of the mixture was then scanned from the wavelength 400–800 nm. Each sample was run triplicates (n = 3) and the absorbance was reported as Mean ± SD.

Table 2
Accuracy Results Using the USP46-NF41 Hyaluronidase Assay Protocol (the mean of %recovery ± SD).

Accuracy	% Recovery	
	400 nm	640 nm
0.6 IU/mL	117.67 ± 9.90	119.65 ± 11.15
1.0 IU/mL	114.88 ± 12.42	120.62 ± 13.63
1.4 IU/mL	98.23 ± 5.82	99.30 ± 5.35

values scatter across a series of measurements obtained from multiple samples of the same homogeneous material under the specified conditions. In this assay, both repeatability and intermediate precision were evaluated to assess precision. The SD calculation is provided in the Supplementary File 18, and the results are outlined in Table 3. The % RSD for repeatability at 400 nm ranged from 8.92 % to 11.92 %, and at 640 nm, it ranged from 9.06 % to 21.13 %. Repeatability at both

Table 3
Repeatability and intermediate precision analysis of turbidity measurements obtained by the USP46-NF41 hyaluronidase assay protocol. The results are reported as % recovery and %RSD.

Sample	400 nm			640 nm		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
0.5 IU−1	98.95	111.99	88.51	102.85	117.23	95.64
0.5 IU−2	118.98	94.76	96.98	126.19	104.10	93.29
0.5 IU−3	126.72	118.70	67.15	132.18	123.09	55.99
0.5 IU−4	121.60	108.18	85.89	125.24	112.48	62.66
0.5 IU−5	120.58	106.18	87.91	126.19	109.13	79.54
0.5 IU−6	107.85	87.50	92.95	112.47	94.05	70.91
Mean	115.78	104.55	86.56	120.86	110.01	76.34
%RSD of Repeatability (acceptance criteria ≤ 11 %)	8.92	10.97	11.92	9.06	9.26	21.13
%RSD of Intermediate precision (acceptance criteria ≤ 16 %)	14.41			22.67		

wavelengths failed to meet the acceptance criteria outlined in Supplementary File 9, as it exceeded the 11 % threshold.

For intermediate precision, the %RSD was 14.41 % at 400 nm and 22.67 % at 640 nm, as detailed in Table 3. Only the result at 400 nm met the acceptance criteria (<16 %), while at 640 nm, it exceeded the threshold. Like accuracy, we suspected that this variation may stem from the different composition of horse serum, which can form heterogeneous complexes with HA, contributing to the variation. At this point, we concluded that the HAse USP46-NF41 Assay Protocol was insufficient in both accuracy and precision. Nevertheless, the optimized conditions using a detection wavelength of 400 nm, a horse serum protein concentration of 1.62 mg/mL, a pH of 4.2, and a temperature of 22 °C can achieve turbidity formation necessary to provide an absorbance value of 1, which is a good starting point for this assay since the absorbance decreases upon enzyme addition. Therefore, these conditions were chosen for further study to improve the protocol. Moreover, we knew that the reading time might be flexible between 10 and 45 mins, not fixed at 30 mins as described in the USP monograph. Additionally, salt concentration could affect turbidity formation, resulting in interference with the assay, except at low concentrations (<20 mmol/L). To improve accuracy and precision, BSA was selected to be evaluated against horse serum due to its purity. BSA contains only one type of protein, albumin. Additionally, the enzyme concentration range was expanded from 0–1.5 IU/mL to 0–5 IU/mL, as even a small change in absorbance can affect accuracy. This expansion is expected to improve robustness.

3.5. Optimization of substrate concentration for improved USP assay protocol

Increasing the enzyme concentration within the new assay range led to a decrease in absorbance values (i.e., turbidity). This occurred because the remaining substrate (HA), after enzyme digestion, decreased and formed complexes with the protein agent to generate turbidity. As a result, there was uncertainty about whether enough substrate would remain available for the enzyme at the new concentration range to produce a reliable range and linearity. Optimising the substrate concentration was, therefore, essential to establish a suitable range for accurate measurement.

Both HS and BSA were evaluated with four different concentrations of HA: 250 μg/mL, 300 μg/mL, 400 μg/mL, and 500 μg/mL. The expected range should start with an absorbance value of around 1, and the endpoint (highest enzyme concentration) should remain above 0, leaving room for variation. This ensures this, even if variation occurred, the ending absorbance would not fall below zero. The results of HS reacting with different concentrations of HA, as outlined in Fig. 6A, showed that at the highest enzyme concentration (5 IU/mL), the absorbance for 250 μg/mL HA was close to 0, which was not ideal. Other concentrations of HA reacted with HS and produced the expected range, with absorbance values starting around 1 and ending above zero. Therefore, 300 μg/mL of HA was chosen for further study in the improved USP assay protocol, as it used the least amount of substrate to produce the expected absorption range. The results of BSA reacting with different concentrations of HA, as outlined in Fig. 6B, demonstrate that all concentrations of HA reacted with BSA could produce the expected absorption range, with absorbance values starting around 1 and ending above zero. However, to facilitate comparison with horse serum in subsequent experiments, 300 μg/mL was selected for further study.

3.6. Method validation for an improved USP assay

3.6.1. Specificity of improved USP assay

The spectrum of the HA-HS complex, HA-BSA complex, HA solution (MW 2 MDa, 300 μg/mL), 1.63 mg/mL HS solution, and 3.75 mg/mL BSA solution were evaluated by measuring the absorbance between 400 and 800 nm to assess the specificity of the improved USP assay. The results showed that the spectra of both complexes were not affected by

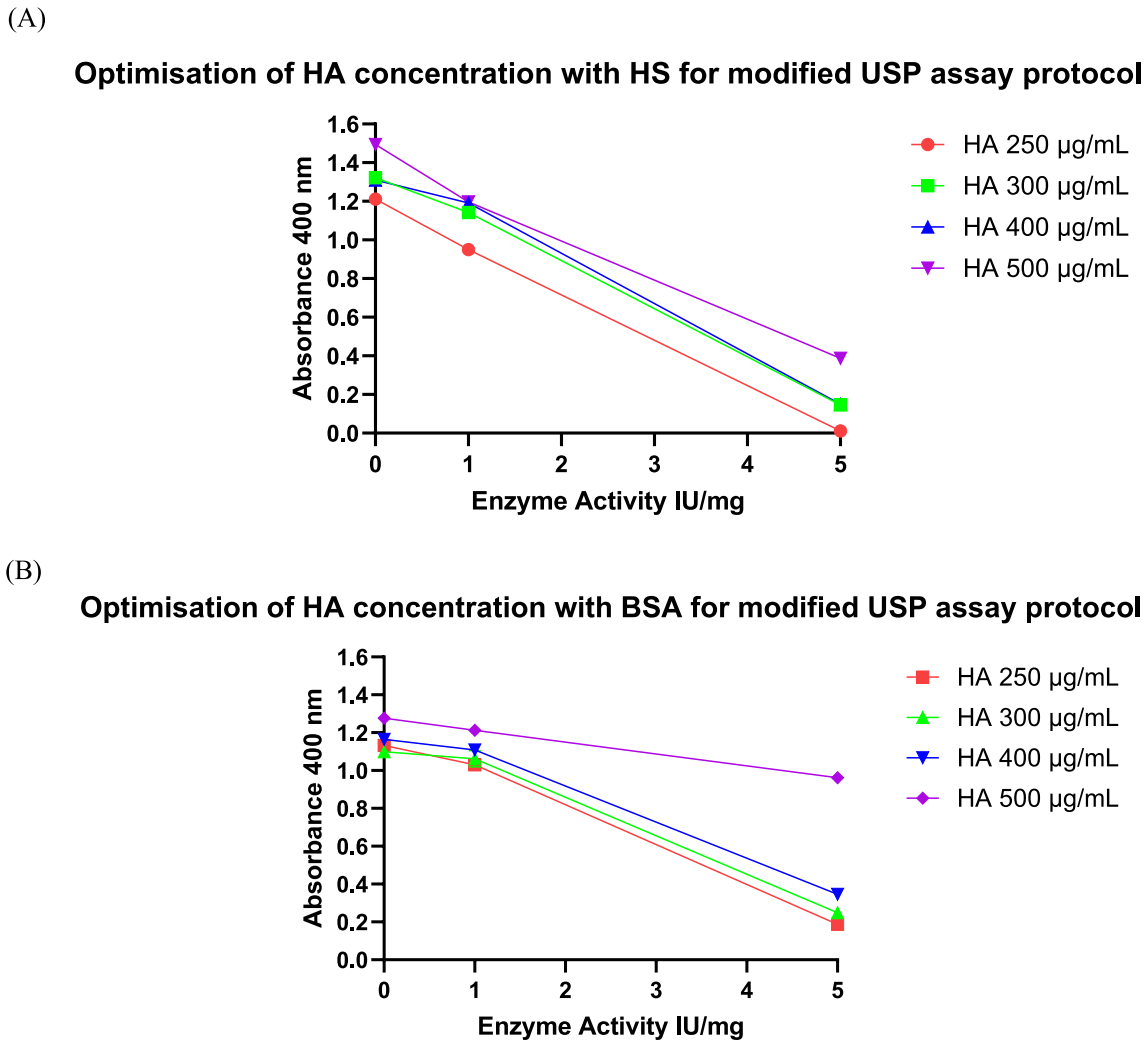


Fig. 6. Comparison of turbidity development at varying HA (MW 2MDa) concentrations from 250 to 500 µg/mL, mixed with either 3.75 mg/mL BSA (A) or 1.63 mg/mL horse serum (HS) (B). Enzyme activities of 0, 1, and 5 IU/mL were used. Absorbance was measured at 400 nm. Each sample was run in triplicate (n = 3), and absorbance values are reported as Mean ± SD.

other substances present in the assay mixture, as illustrated in Fig. 7, confirming that the specificity of the method meets the acceptance criteria outlined in Table 1. These findings aligned with the specificity results obtained from the USP 46-NF 41 HAse Assay Protocol, where the spectrum of the HA-HS complex can be detected without interference from other substances in the assay. Lenormand et al. also obtained similar results, that showed formation of the HA-BSA complex in a turbidimetric assay when measured at 400 nm was not influenced by other substances in the reaction mixture [32].

3.6.2. *Range and Linearity of improved USP assay*

In the subsequent phase of method validation, the assay range was evaluated to ensure reliability and adherence to the acceptance criteria for linearity ($R \geq 0.995$) as outlined in Table 1. HAse standard enzyme concentrations ranging from 0 to 5 IU/mL were tested under optimized conditions (pH 4.1 and 22 °C), with absorbance measured at 400 nm. Calibration curves were plotted for both HS and BSA. The linear regression equations obtained were $y = -0.2783x + 1.4098$ with a correlation coefficient (R) of 0.9925 for HS and $y = -0.1433x + 0.8492$ with an R of 0.9965 for BSA, as shown in Fig. 8. The HS calibration curve failed to meet the acceptance criteria ($R < 0.995$), indicating insufficient linearity within the tested range. Conversely, BSA satisfied the linearity requirement ($R \geq 0.995$), demonstrating a more reliable response for this assay. The coefficient of determination (R) reflects the goodness of fit of the data to the regression line, with values closer to 1 signifying stronger linearity. The BSA calibration curve, with an R of 0.9965, not only met the acceptance criteria but also outperformed HS, which exhibited an R of 0.9925. These findings supported that BSA is a more suitable alternative to HS in this assay, offering superior linearity and consistency within the specified concentration range. Additionally, the use of BSA may simplify standardization in future applications by addressing the variability associated with using HS as the protein agent in this method.

3.6.3. *Accuracy of improved USP assay*

Accuracy refers to the degree to which the observed value aligns with the true or reference value, typically expressed as percentage recovery. This section compares the accuracy of the improved assay protocol using BSA as the protein agent against HS, which is commonly used in the USP46-NF41 HAse Assay Protocol, to evaluate whether BSA can reduce variability and improve accuracy. The accuracy results of the improved assay protocol, presented in Table 4, showed that the percentage recovery ranged from 113.23 % to 130.09 % when HS was used as the protein agent to form a complex with the substrate (HA), and from 86.09 % to 96.34 % when BSA was used. These results indicated that the use of HS introduced variability into the improved method, as the

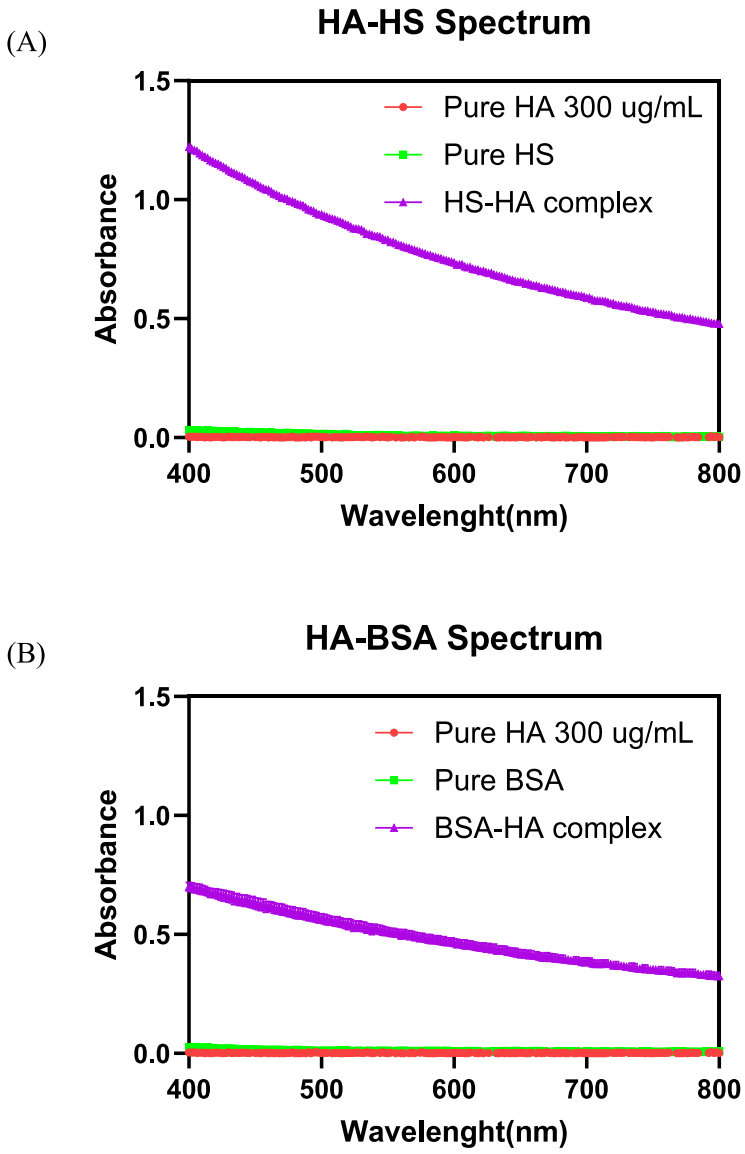


Fig. 7. Comparison of the spectrum of the HA-HS complex, HA-BSA complex, HA solution (MW 2MDa,300 μg/mL), 1.63 mg/mL HS solution, and 3.75 mg/mL BSA solution was evaluated between 400 and 800 nm. Each sample was run in triplicate (n = 3), with absorbance values reported as Mean ± SD.

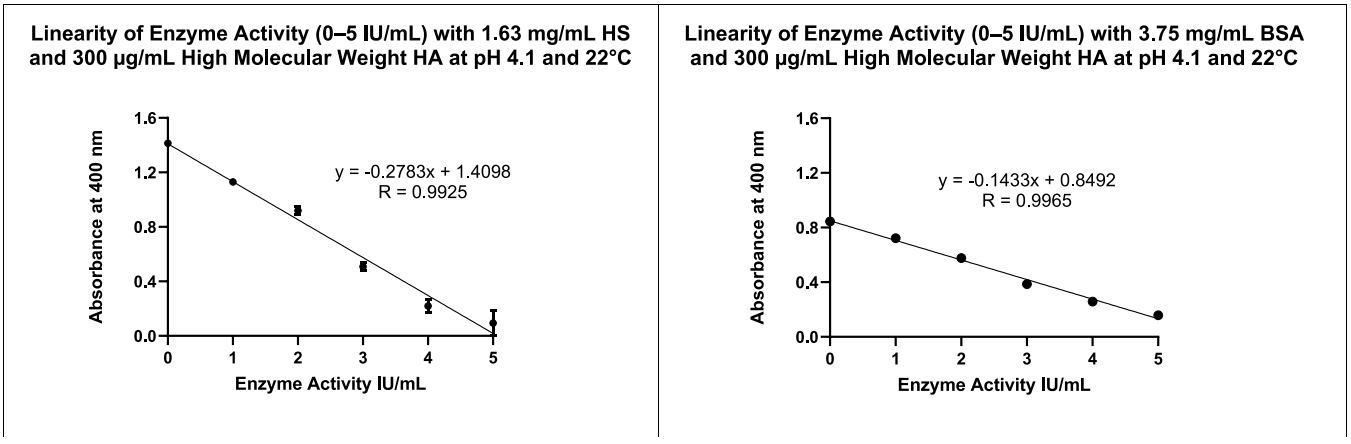


Fig. 8. Comparative linearity of the improved hyaluronidase turbidimetric assay using 1.63 mg/mL horse serum (HS) and 3.75 mg/mL bovine serum albumin (BSA) at pH 4.1 and 22°C. The assay was validated with hyaluronidase enzyme concentrations ranging from 0 to 5 IU/mL, measured at 400 nm. Linear regression for HS yielded $y = -0.2783x + 1.4098$ with an R value of 0.9925, which did not meet the acceptance criteria ($R \geq 0.995$). For BSA, the linear regression equation was $y = -0.1433x + 0.8492$ with an R value of 0.9965, meeting the acceptance criteria. Absorbance values are reported as Mean ± SD.

Table 4
Accuracy results by improved USP assay.
(the mean of %recovery ± SD).

Accuracy(acceptance criteria 80–110 %)	% Recovery	
	Horse serum	BSA
1.5 IU/mL	115.4 ± 5.67	86.09 ± 2.35
2.5 IU/mL	130.09 ± 1.02	94.28 ± 1.53
3.5 IU/mL	113.23 ± 1.58	96.34 ± 3.54

recovery values failed to meet the acceptance criteria of 80–110 % outlined in Table 1. This finding was consistent with the accuracy results of the USP46-NF41 HAse Assay Protocol that uses HS, as outlined in Table 2. In contrast, using BSA resulted in a % recovery within the acceptable range (80–110 %) as outlined in Table 1, confirming that BSA is a suitable alternative to HS for this assay, offering improved accuracy and reduced variability. The reduction in variability is likely due to BSA consisting of only one type of protein, albumin, which forms a complex with the substrate (HA) to produce turbidity. In contrast, HS is composed of various proteins, including albumin, alpha-1 globulin, alpha-2 globulin, beta-globulin, and gamma-globulin, each of which may bind to HA, potentially introducing variability. HA can interact with a variety of proteins, not just albumin. For instance, Bełdowski et al. (2021) described how HA can interact with proteins like albumin and γ-globulin in synovial fluid. This variability in protein interactions may lead to the formation of different complexes with HA, contributing to the observed variability in turbidity [30].

3.6.4. Precision of improved USP assay

The SD calculation is provided in the Supplementary File 19. Repeatability and intermediate precision results are provided in Table 5 and indicate that repeatability ranged from 1.13 % to 7.71 % for HS, and from 1.48 % to 3.61 % for BSA. Intermediate precision was calculated at 3.23 % for HS and 7.92 % for BSA. The acceptance criteria for repeatability and intermediate precision are ≤ 7.3 % and ≤ 11 %, respectively, as outlined in Table 1. The results showed that only the repeatability on day 2, when using HS as the protein agent in the improved USP assay (7.71 %), as highlighted in yellow in Table 5, exceeded the 7.3 % threshold. Unfortunately, this single-day failure caused the repeatability to not meet the acceptance criteria, as outlined in Table 1. While the intermediate precision using HS met the criteria, as highlighted in green in Table 5, the overall precision did not pass. Conversely, all other results met the acceptance criteria for both intermediate precision and repeatability when using BSA as the protein agent in the improved assay, as highlighted in green in Table 5. Using BSA also demonstrated better performance in terms of precision, fulfilling the acceptance criteria for both repeatability and intermediate precision.

Although HS generated higher turbidity and appeared more sensitive to HAse activity, as shown in Figs. 6 and 8, this increased sensitivity came with greater variability, likely due to the diverse protein components in HS, which may interact with the substrate in various ways, leading to fluctuations in both accuracy and reproducibility. In contrast, BSA showed lower turbidity but offered better performance in terms of precision and accuracy. This trade-off between sensitivity and precision is a reasonable and beneficial compromise that enables more accurate and reproducible results. This is likely because BSA contains only albumin, eliminating the variability introduced by the multiple proteins in HS. Additionally, the use of BSA ensures greater consistency between batches, as the protein composition of HS may vary significantly between batches. Furthermore, BSA offers practical advantages, including easier preparation and storage. Unlike HS, which is liquid and requires storage at −20 °C with risks of freeze-thaw degradation, BSA is available in powder form, storable at 2–8 °C. The affordability and widespread availability of BSA from various suppliers make it a more reliable and efficient alternative for the assay.

Table 5
Comparative repeatability and intermediate precision of Improved USP Assay Using HS (1.63 mg/mL) vs. BSA (3.75 mg/mL) at pH 4.1 and 22 °C. The result are reported as % recovery and %RSD.

Sample	BSA			HS		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
2.5 IU−1	86.70	107.82	99.75	129.27	125.21	127.26
2.5 IU−2	94.84	108.93	102.13	131.29	131.99	128.40
2.5 IU−3	94.24	107.14	102.82	140.15	131.87	128.87
2.5 IU−4	93.76	105.24	103.57	138.79	128.05	126.68
2.5 IU−5	88.74	105.13	101.06	129.71	131.41	130.56
2.5 IU−6	91.07	108.30	103.85	134.69	106.85	127.13
Mean	91.56	107.09	102.20	133.98	126.07	128.15
%RSD of Repeatability (acceptance criteria ≤ 7.3 %)	3.61	1.48	1.53	3.49	7.71	1.13
%RSD of Intermediate precision (acceptance criteria ≤ 11 %)	7.92			3.23		

3.7. Influence of storage time on turbidity formation in the improved USP Assay

Storage time for turbidity formation is an important factor in this assay. The original USP monograph specified that measurements should be taken 30 mins after adding the protein substrate (i.e., HS) to form the complex. We recommend measuring within the time window of between 30 mins and 1 h, as our results show (Fig. 9) no significant differences in absorbance after 30 mins across all hyaluronidase concentrations tested. This suggested that turbidity reaches a stable state during this period. Before 30 mins, we observed greater variability in absorbance at lower enzyme concentrations (control and 1 IU/mL). This was likely due to incomplete binding between the high amount of HA and BSA, resulting in unstable turbidity formation. In contrast, at higher enzyme concentrations (2.5 and 5 IU/mL), more substrate is hydrolyzed, leaving less residual HA available to form the turbidity complex. As a result, turbidity stabilizes earlier, within the first 5 mins, for these concentrations. Therefore, selecting the 30 mins to 1 h time window ensures consistent and reliable absorbance readings, particularly for comparative analysis across different enzyme activity levels and affording greater timing flexibility compared to the original USP monograph method, ideal especially when measuring many samples. During the experiments, supply limitations led to a switch to high molecular weight HA (2 MDa). This substitution is still appropriate, as the enzymatic degradation of high molecular weight HA into specific lower molecular weights is highly relevant for industrial applications. However, the molecular weight of HA does affect turbidity formation, with an increase in HA molecular weight leading to greater turbidity because longer HA chains tend to form larger aggregates (Supplementary File 20). With higher turbidity as a starting point offers an advantage when measuring the loss of turbidity using absorbance changes. This is because a larger initial absorbance value allows for more precise detection of even small decreases in turbidity as the sample becomes clearer.

3.8. Influence of salts on turbidity measurements

Various types of salts that are present in microbial cultivation media and buffers used for protein purification could interfere with the measurement of enzyme activity by neutralizing the electrostatic interactions between HA and proteins responsible for turbidity formation. To assess the effects of these possible inferences on our improvement to the USP monograph, various salts were screened at 200 mmol/L. The screening results, as shown in Fig. 10A, demonstrated that only $(NH_4)_2SO_4$ and Na_2HPO_4 significantly interfered with the improved USP assay, whereas other salts did not. $(NH_4)_2SO_4$ and Na_2HPO_4 were further studied across a broader range of concentrations from 0, 50, 100, 150,

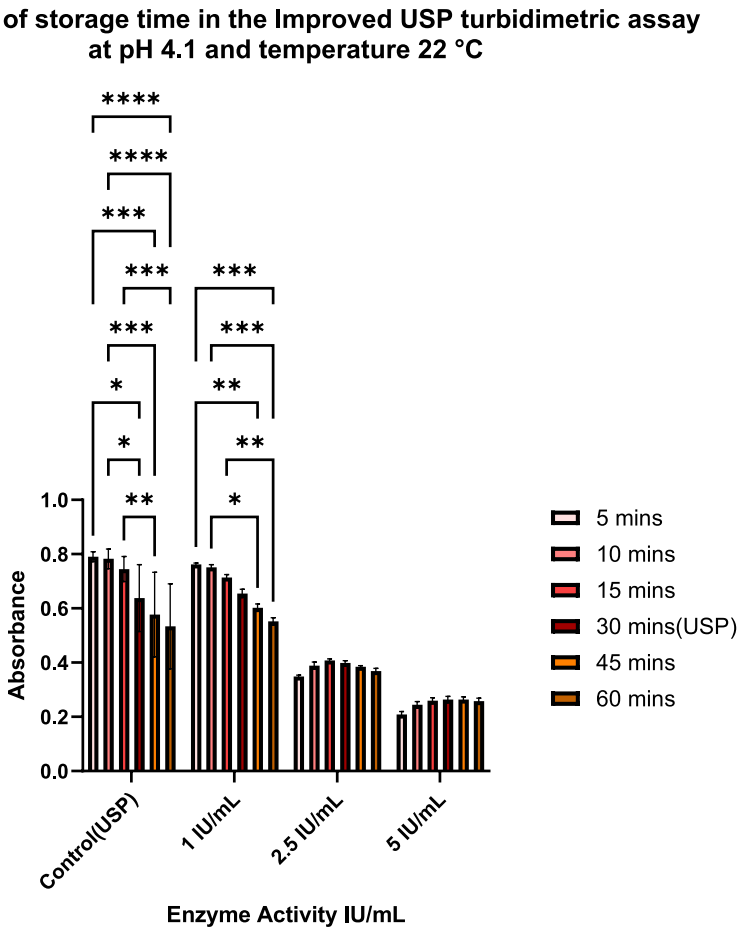


Fig. 9. The effect of storage time in the improved USP turbidimetric assay at pH 4.1 and 22 °C. Each sample was run in triplicate (n = 3) and the absorbance is reported as mean ± SD. *indicates a significant difference in absorbance for each sample compared to a different time point. ($P \leq 0.05$, *$P \leq 0.01$, **$P \leq 0.001$, and **$P \leq 0.0001$).

and 200 mmol/L to determine how tolerant the method was to the effects of these salts. The results, shown in Fig. 10B, indicated however that both salts impacted the method at the highest concentration (200 mmol/L). However, this is unlikely to be an issue in practice, as the actual concentrations used in solutions, shown in Supplementary File 20, are considerably lower. In addition, some metal ions, such as Cu^{2+}, Mn^{2+}, and Fe^{3+}, have been reported to significantly inhibit HAse enzyme activity [33], which was next assessed using our improved protocol.

3.9. Influence of salts on HAse enzyme activity

Various salts were screened at concentrations of 0, 25, 50, 100, and 200 mmol/L to evaluate the potential inference on enzyme activity. The screening results indicated that all salts, except for $MgCl_2$, significantly inhibited enzyme activity at 200 mmol/L, as presented in Fig. 11A. Specifically, NaCl and NaH_2PO_4 were found to inhibit enzyme activity at concentrations ranging from 50 to 200 mmol/L, $(NH_4)_2SO_4$ inhibited enzyme activity at 100–200 mmol/L, and CH_3COONa inhibited enzyme activity but only at the highest concentration tested of 200 mmol/L. Tris-HCl and Na_2HPO_4 inhibited enzyme activity at all concentrations tested, as illustrated in Fig. 11B. However, in practical applications, only Tris-HCl and Na_2HPO_4 would be expected to affect enzyme activity, as the concentrations of other salts in commonly used reagents are significantly lower, as shown in Supplementary File 21.The key point to highlight from experiments 3.7 and 3.8 is that salts can affect both turbidity formation and enzyme activity measurement, leading to inaccurate results. Therefore, it is important to carefully consider the type of salt and its concentration when using this measurement method. Additionally, desalting the enzyme prior to testing may be necessary to avoid interference from salts.

4. Conclusion

HAses have received approval by the US Food and Drug Administration for a variety of clinical applications, including: (I) subcutaneous fluid infusion (hypodermoclysis), (II) as an adjuvant to enhance the absorption and dispersion of drugs in subcutaneous tissue or to manage extravasation, and (III) as an adjunct to facilitate the absorption of contrast media in subcutaneous urography. Additionally, HAses are also used off-label to correct complications and unsatisfactory results following cosmetic filler injections [9]. Hence, a method that ensures rigorous quality assurance of enzyme activity is absolutely essential when these enzymes are used in pharmaceutical products or processes. Unfortunately, the USP monograph USP46-NF41 for measuring specific HAse enzyme activity was reported herein to be invalid because the protocol failed to meet acceptance criteria for accuracy and precision. However, several modifications were made that consistently resulted in test results meeting the required quality assurance parameters. Regarding salt interference, these only significantly impacted turbidity formation at high concentrations and are unlikely to pose practical issues. However, Tris-HCl and Na_2HPO_4 surprisingly inhibited enzyme activity at all concentrations tested, precluding these commonly used buffers in downstream unit operations. We offer the following step-by-step assay protocol:

Firstly, a standard concentration-response curve is prepared using six different concentrations of standard HAse enzyme (0, 1, 2, 3, 4, and 5

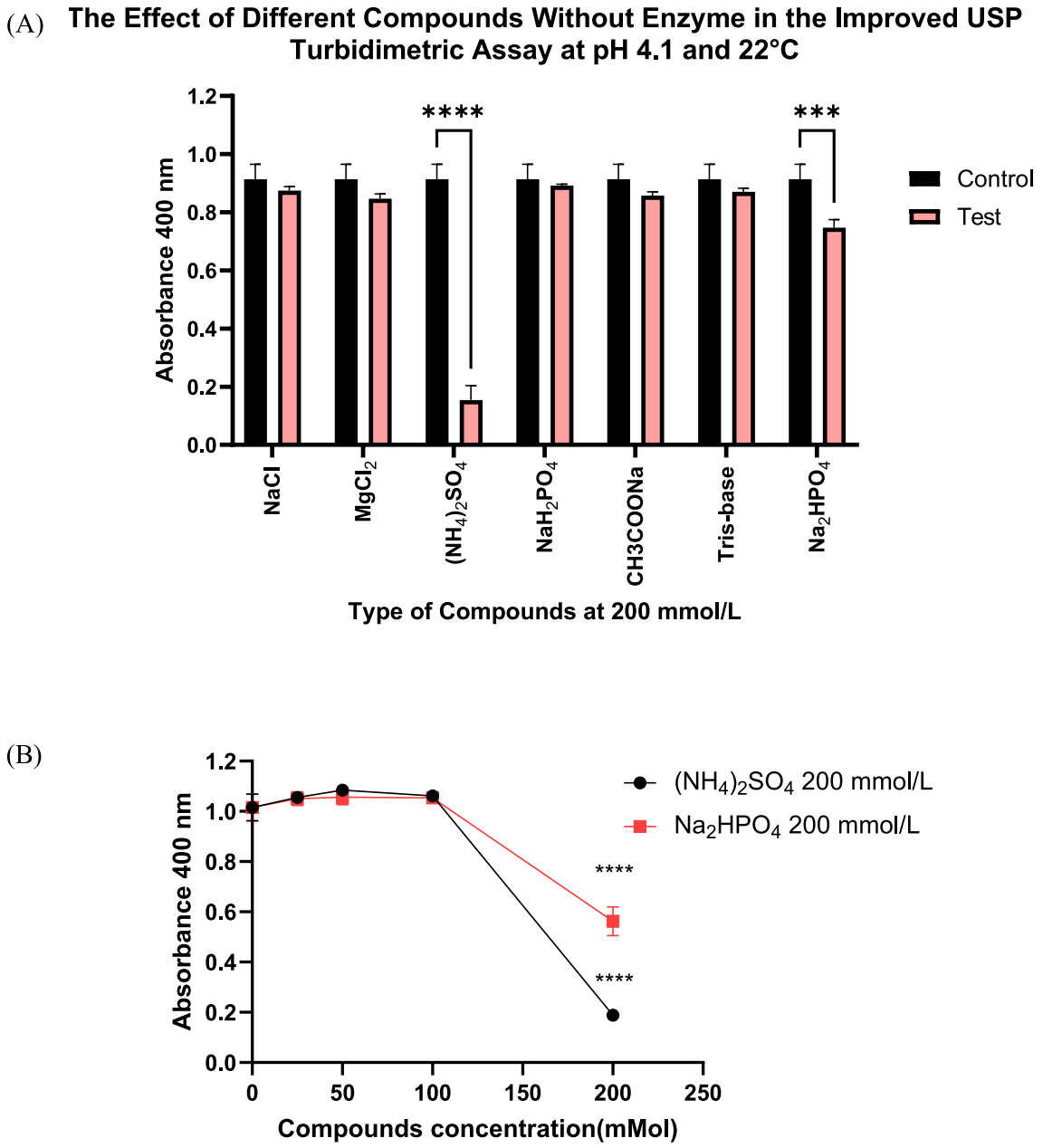
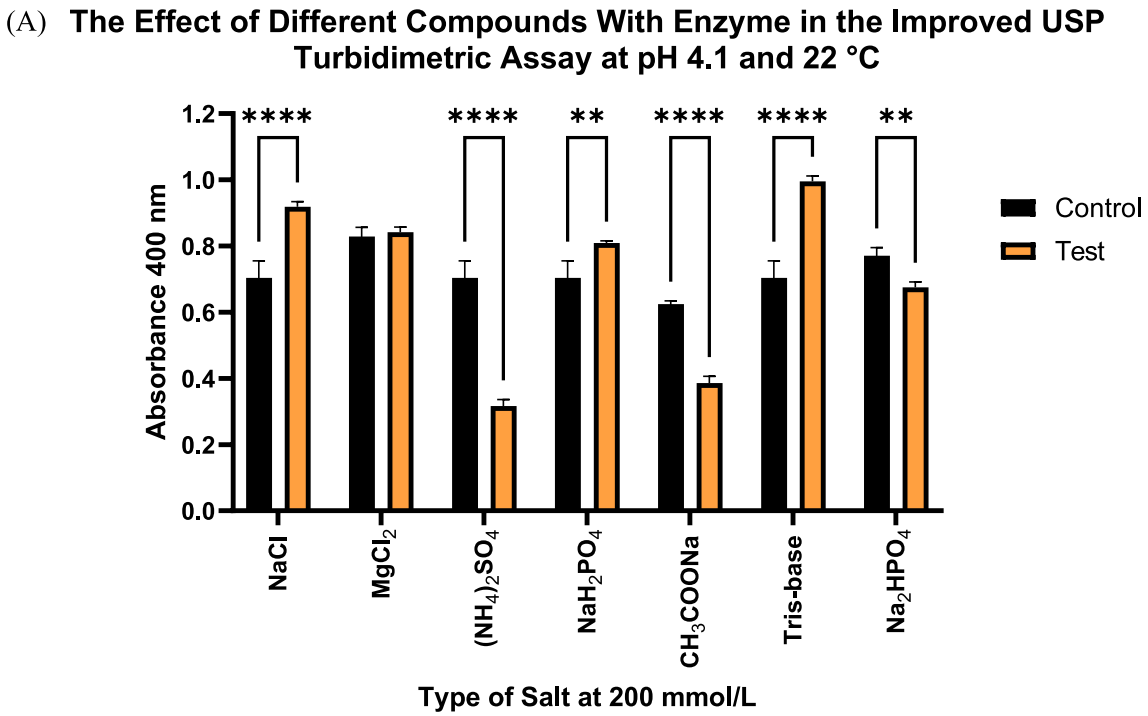


Fig. 10. Influence of different compounds on turbidity measurement in the improved method at pH 4.1 and 22 °C. (A) Screening results of various salts at 200 mmol/L, demonstrating significant interference from $(NH_4)_2SO_4$ and Na_2HPO_4. (B) Concentration-dependent effects of $(NH_4)_2SO_4$ and Na_2HPO_4 at 0, 50, 100, 150, and 200 mmol/L, showing interference only at the highest concentration. This is unlikely to pose a practical concern, as actual concentrations in typical experimental conditions are expected to be much lower. Each sample was analysed in triplicate (n = 3), with absorbance values expressed as mean ± SD. * indicates a significant difference in absorbance compared to the control (****$P \leq 0.0001$).

IU/mL), each concentration tested in duplicate to establish the calibration curve. To begin, add 0.5 mL of hyaluronic acid (HA) solution (MW: 2 MDa) at a concentration of 300 μg/mL to each test tube. For the blank test tube, add 0.5 mL of PBS instead. Next, the enzyme diluent is added in specific volumes to each test tube. The volumes used are 0.5 mL, 0.4 mL, 0.3 mL, 0.2 mL, 0.1 mL, and 0 mL. For the blank test tube, add 0.5 mL of enzyme diluent. The corresponding volume of standard enzyme is then introduced into each test tube to achieve a final volume of 1 mL, with enzyme volumes being 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mL. A 30-second gap should be maintained between each enzyme addition to allow time for the subsequent addition of BSA in the future step. The contents of each test tube are gently mixed, and the test tubes are incubated in a water bath set to 37 °C for 30 mins to allow the HAse enzyme to break down the hyaluronic acid. After incubation, each tube is sequentially removed from the water bath at 30-second intervals, and immediately 4 mL of 3.75 mg/mL BSA solution is added to each test tube to allow BSA to form a complex with the remaining hyaluronic acid, after enzyme digestion, to produce turbidity. After adding the BSA solution, the final mixture is shaken thoroughly and allowed to stand at room temperature (22 °C) for 30 mins to ensure proper complex formation. Finally, the absorbance of each mixture is measured at 400 nm. Prior to measuring the absorbance, each mixture is shaken for 5 s to ensure uniformity.

Overall, this improvement to the USP46-NF41 monograph provides a more reliable and adaptable approach for measuring HAse activity. However, potential salt inferences should be considered when using buffers during unit operations in manufacturing processes involving HAse.



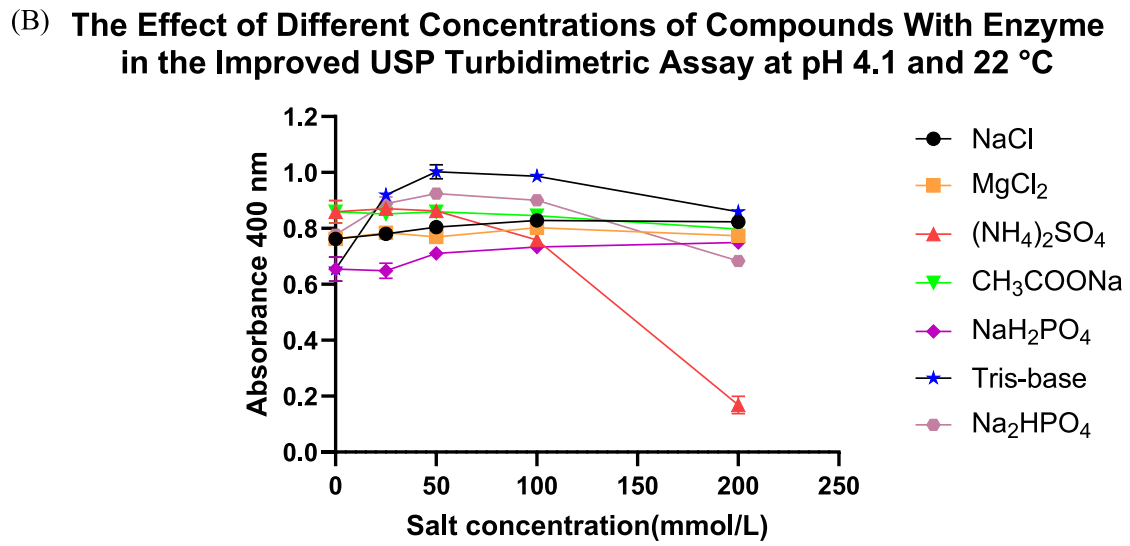


Fig. 11. Influence of different compounds on hyaluronidase enzyme activity in the improved method at pH 4.1 and 22 °C. (A) Screening results of various salts at 200 mmol/L, showing significant interference from all salts, except for $MgCl_2$. (B) Concentration-dependent effects of NaCl, NaH_2PO_4, $(NH_4)_2SO_4$, CH_3COONa, Tris, and Na_2HPO_4 at concentrations of 0, 25, 50, 100, 150, and 200 mmol/L. NaCl and NaH_2PO_4 interfered with enzyme activity at concentrations ranging from 50 to 200 mmol/L, while $(NH_4)_2SO_4$ affected activity at 100–200 mmol/L. CH_3COONa caused interference only at 200 mmol/L, and Tris and Na_2HPO_4 interfered with enzyme activity at all tested concentrations. In practical applications, however, only Tris and Na_2HPO_4 are expected to impact enzyme activity, as the concentrations of other salts in the medium or buffer are significantly lower. Each sample was analysed in triplicate (n = 3), with absorbance values expressed as mean ± SD. *Indicates a significant difference in absorbance compared to the control (****P ≤ 0.0001).

CRediT authorship contribution statement

Renanta B. Fernandes: Writing – review & editing, Validation, Resources, Methodology, Formal analysis, Conceptualization. **Carlos A. Breyer:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Formal analysis, Conceptualization. **Amornrid Tanpipat:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Júnior Adalberto Pessoa:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Richard B. Parsons:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Leticia M. Silva:** Writing – review & editing, Validation, Resources, Project administration, Investigation, Formal analysis, Conceptualization. **Milton V. Gomes:** Writing – review & editing,

Validation, Resources, Investigation, Formal analysis, Conceptualization. **Long Paul F:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

Carlos A. Breyer, Renanta B. Fernandes, Milton V. Gomes and Leticia M. Silva are employees of BioBreyer R&D Ltda. Adalberto Pessoa Júnior is a consultant for BioBreyer R&D Ltda. All declare a financial interest in the subject matter discussed in this manuscript but certify that they have not provided any direct or indirect financial support for the subject matter or materials discussed in this manuscript. All other authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.117032.

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