Published in final edited form as:

J Microbiol Methods. 2015 December ; 119: 163–167. doi:10.1016/j.mimet.2015.10.019.

A new mass spectrometry based bioassay for the direct assessment of hyaluronidase activity and inhibition

Emily R. Britton^a, Carolyn B. Ibberson^b, Alexander R. Horswill^b, and Nadja B. Cech.^{a,*}

^aDepartment of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro, North Carolina, USA

^bDepartment of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

Abstract

The development of drug resistance by bacterial pathogens is a growing threat. Drug resistant infections have high morbidity and mortality rates, and treatment of these infections is a major burden on the health care system. One potential strategy to prevent the development of drug resistance would be the application of therapeutic strategies that target bacterial virulence. Hyaluronidase is virulence factor that plays a role in the ability of Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus agalactiae* to spread in tissue. As such, this enzyme could be a target for the development of future anti-virulence therapies. To facilitate the identification of hyaluronidase inhibitors, quantitative and reproducible assays of hyaluronidase activity are required. In the present study, we developed a new mass spectrometry based bioassay for this purpose. This assay directly measures the quantity of a degradation product (3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine) produced by the hyaluronidase enzyme. Validation parameters for the new assay are as follows: repeatability, <7%; intermediate precision, <10%; range, 0.78-50 μ M; limit of detection, 0.29 μ M ; and limit of quantification, 0.78 μ M. Using the new assay, the IC₅₀ value for a published inhibitor of *S. agalactiae* hyaluronidase, ascorbic acyl 6-palmitate, was 8.0 ± 1.0 μ M. We also identified a new hyaluronidase inhibitor, n-cyclohexanecarbonylpentadecylamine, with an IC₅₀ of 30.4 ± 9.8 μ M. In conclusion, we describe a new, direct, and reproducible method for assessing hyaluronidase activity using mass spectrometry that can facilitate the discovery of inhibitors.

Keywords

Hyaluronidase; *Streptococcus agalactiae*; mass spectrometry; bioassay; inhibitor

*Corresponding author. Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, 301 McIver St, Greensboro, North Carolina, USA. Tel: +1 336 334 3017; fax: +1 336 334 5402. nbcech@uncg.edu.

Conflict of interest: The authors declare that they have no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The development of resistance by bacterial pathogens is a growing threat (Centers for Disease Control, 2013). The overuse of antibiotics accelerates resistance development, and there is an urgent need for alternative therapeutic strategies against bacterial infections (Aziz, 2013). One potential alternative target is hyaluronidase, a virulence factor secreted by Gram-positive bacterial pathogens such as *Staphylococcus aureus* (Ibberson et al., 2014) and *Streptococcus agalactiae* (Li and Jedrzejewski, 2001, Wang et al., 2014). Both of these pathogens are considered by the Centers for Disease Control to be of concerning and serious threat for resistance development (Centers for Disease Control, 2013). Hyaluronidase degrades hyaluronan, a polymer found in the skin and other tissues. The resulting disaccharide product serves as an energy source for the bacteria, enabling the infection to spread through tissues (Starr and Engleberg, 2006, Wang et al., 2014). Thus, anti-virulence therapeutic agents could be developed against bacterial infections by specifically targeting hyaluronidase (Hynes and Walton, 2000, Ibberson et al., 2014, Spickenreither et al., 2006).

Only a few studies have investigated the inhibition of hyaluronidase by chemical means. It has been shown that ascorbic acyl 6-palmitate and other vitamin C derivatives have low micromolar potency against *Streptococcus pneumoniae* and *Streptococcus agalactiae* hyaluronidases (Botzki et al., 2004, Spickenreither et al., 2006). These studies show the potential of hyaluronidase inhibition as a therapeutic target. Further efforts towards the identification and development of more potent hyaluronidase inhibitors will require a robust screening assay that provides quantitative comparisons of inhibitor potency.

Several methods have been developed to measure the activity of the hyaluronidase enzymes. Qualitative measurements can be obtained using agar plate assays. These assays rely on measuring clearance zones in solid media containing hyaluronan after addition of the hyaluronidase enzyme (Ibberson et al., 2014, Richman and Baer, 1980, Tung et al., 1994). More quantitative measurements can be obtained using spectrophotometric approaches, which rely on a reaction between the disaccharide and a substrate that in turn produces a colored product (Takahashi et al., 2003). For example, the Morgan-Elson reaction employs the transformation of the N-acetyl glucosamine reducing end of the disaccharide to a series of products using varied pH and temperature environments. The final product is subsequently reacted with p-dimethylaminobenzaldehyde to produce a red complex that can be detected by monitoring absorbance at 585 nm (Reissig et al., 1955). This method was improved upon by measuring fluorescent intensity after excitation at 545 nm, resulting in an increase in sensitivity (Takahashi et al., 2003). Finally, turbidimetric measurements can be utilized to assess hyaluronidase inhibition, in which hyaluronidase is incubated with hyaluronan, and subsequently treated with cetylpyridinium to precipitate undigested hyaluronan. An increase in light transmittance correlates with increasing disaccharide concentration (Tung et al., 1994). The aforementioned assays have important utility. For example, agar based assays and turbidimetric measurements can serve as a simple preliminary screen to identify potential inhibitors, and spectrophotometric assays have demonstrated utility for measuring enzyme kinetics parameters (Vercruysse et al., 1995). However, the inherent indirect nature of these assays means that they are time-consuming and subject to interference. Our laboratory has designed an assay that utilizes mass

spectrometry to reproducibly measure the production of disaccharides by hyaluronidase enzymes. In contrast to spectrophotometric techniques, our design requires fewer steps. The new assay is also sensitive and robust, providing excellent repeatability from assay to assay, even across multiple days. Here we present the new methodology and show its applicability to measuring the activity of hyaluronidase inhibitors.

2. Materials and Methods

2.1. Purification of *S. aureus* hyaluronidase

To generate the hyaluronidase overexpression construct pCR03, the *hysA* gene without the encoded signal sequence was amplified from AH 1263 genomic DNA using primers CBR41 (5'GTTGTTGCTAGCGATACGAATGTTCAAACGCCAG3') and CBR42 (5'GTTGTTCTCGAGGTGTCGAGATTTTCTTGCATT3'), which contain *NheI* and *XhoI* sites at their 5' ends, respectively (Ibberson et al., 2014). The resulting PCR product was ligated into pET28a linearized with *NheI* and *XhoI* and electroporated into *E. coli* strain ER2566 to generate strain AH2856. To purify hyaluronidase, AH2856 was grown overnight at 37°C in luria broth (LB) (Sigma, St. Louis, MO) containing kanamycin (50 µg/mL), and subcultured into 1 L of fresh LB at a ratio of 1:250. The culture was grown at 30°C to mid-logarithmic phase, IPTG was added to 1 mM final concentration, and the culture was allowed to grow for an additional 4 h. Cells were harvested by centrifugation, washed once with water, pelleted by centrifugation, and frozen at -20°C. Cells were mechanically lysed by two passages through a Microfluidics Microfluidizer model LV1 (Newton, MA) at 25,000 psi. Cell lysate was clarified by centrifugation for 20 min at 15,000 × *g* at 4 °C. Protein was purified in the presence of 1 mM Tris(hydroxypropyl)phosphine (THP) using Fractogel His-Bind Resin (EMD Millipore, Darmstadt, Germany) as per the manufacturer's instructions. The protein was dialyzed into phosphate buffered saline (PBS) containing 1 mM THP, concentrated using Millipore Centrifugal Filter Unit (Temecula, CA, 30,000 molecular weight cut-off), and brought to 10 mg/mL in 1× PBS containing 1 mM THP. The protein suspension was frozen in 20% glycerol at -20°C until use.

2.2. Bioassay procedure

Concentrated stock solutions of hyaluronidase from *S. agalactiae* (R & D Systems, Minneapolis, MN) and *S. aureus* (isolated as described in 2.1) were prepared with PBS with 10% glycerol and stored at -80°C in Lo-binding Eppendorf tubes. Aliquots were thawed at room temperature and diluted with PBS to a concentration of 1.25 µg/mL. A stock solution of hyaluronic acid sodium salt from *Streptococcus equi* (Sigma, St. Louis, MO) was prepared at 5 mg/mL with deionized water (EMD Millipore, Temecula, CA). To each Lo-binding Eppendorf tube, 1.25 µg/mL hyaluronidase and 1 mg/mL extract in dimethylsulfoxide (DMSO) were added and incubated at 37°C. After incubation, 5 mg/mL hyaluronan was added to each tube and thoroughly mixed before incubation at 37°C for 15 min. and shaking at 400 rpm. The final assay concentrations of hyaluronidase, extract and hyaluronan were 0.61 µg/mL, 24.4 µg/mL and 2.44 mg/mL, respectively. To quench the reaction, an aliquot from the reaction mixture was diluted 50-fold with acetonitrile (Fisher Scientific, Pittsburgh, PA) containing 0.1% formic acid (Fisher Scientific, Pittsburgh, PA). The diluted reaction was then submitted to ultraperformance liquid chromatography-mass

spectrometry (UPLC-MS) analysis via a Waters Acquity UPLC with an Acquity UPLC column (BEH HILIC, 1.7 μm , 2.1 $\times$ 50 mm, Waters Corporation, Milford, MA) and a Thermo Quantum Access triple stage quadrupole (TSQ) mass spectrometer with electrospray ionization source (Thermo Fisher Scientific, MA, USA). Ascorbic acyl 6-palmitate (also referred to as ascorbic acid 6-palmitate) (Sigma, St. Louis, MO) served as the positive control. The identity of the disaccharide (3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine, Fig. 1) was confirmed by accurate mass measurements (Thermo LTQ Orbitrap, Thermo Fisher Scientific, MA, USA), its absence in assays with inactive enzyme, and by comparison with a standard (Carbosynth, UK). The Thermo LTQ Orbitrap was operated at the following settings: spray voltage, 4000 V; sheath gas, ion sweep gas, and auxiliary gas pressure, 30, 0, and 20, respectively; capillary temperature, 300°C; tube lens offset, 100 V.

2.3. ESI-MS analysis

Hyaluronan was degraded by *S. agalactiae* hyaluronidase and used to optimize MS settings. All solvents used in the present study were Optima LC-MS grade (Fisher Scientific, Pittsburgh, PA). Diluted reaction mixtures (3 μL) were eluted from the column at a flow rate of 0.2 mL/min using the following binary gradient with solvent A consisting of water with 0.1% formic acid additive and solvent B consisting of acetonitrile with 0.1% formic acid additive. The gradient initiated at an isocratic composition of 15:85 (A:B) for 1.5 min., increasing linearly to 100:0 (A:B) from 1.5 to 2.5 min., followed by an isocratic hold at 100:0 (A:B) from 2.5 to 4.4 min., gradient returned to starting conditions of 15:85 (A:B) from 4.4 to 4.5 min., and was held from 4.5 to 5 min. The Thermo Quantum Access triple stage quadrupole mass spectrometer was operated at the following settings: spray voltage, 4000 V; vaporizer temperature, 384°C; sheath gas, ion sweep gas, and auxiliary gas pressure, 60, 2.0, and 5, respectively; capillary temperature, 202°C; tube lens offset, 181 V.

Percent inhibitions were based on a comparison of relative peak areas associated with the protonated m/z of the disaccharide units. Standard error was calculated for each triplicate set of measurements, and IC_{50} values were calculated using a four parameter logistic standard curve analysis function in SigmaPlot (v12.5).

2.4. Method validation for the detection of 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine

A stock solution of 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine (99% pure, Carbosynth, West Berkshire, UK) was serially diluted to make seven concentrations of 0.78, 1.6, 3.1, 6.2, 12.5, 25 and 50 μM . A calibration curve was generated using the UPLC-MS as described above, and the log of the peak areas of the selected ion chromatograms for 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine ions were plotted versus the log of the concentration.

Method validation was conducted according to the guidelines of the International Committee of Harmonisation (ICH) section Q2 (R1) (International Committee of Harmonisation, 2005) with external calibration curves. The linear dynamic range was determined by analyzing samples ranging in concentration from 0.39 to 500 μM . Repeatability was evaluated by

analyzing the seven standards that fell within the linear range (0.78–50 μM) in triplicate and determining the standard deviation and relative standard deviation of the back-calculated 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine concentration for those analyses. Intermediate precision was evaluated by conducting the repeatability study three times on three different days. The standard deviation and relative standard deviation for the intermediate precision were calculated using the average back-calculated concentration for each of the three days for each standard concentration. Limit of detection (LOD) was defined as the lowest concentration of 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine that provided a signal-to-noise ratio of 3. Limit of quantitation (LOQ) was defined as the lowest concentration that provided a residual of less than 15%.

3. Results

3.1. Activity of *S. agalactiae* and *S. aureus* hyaluronidases

We first sought to confirm the ability to detect the disaccharide product (Fig. 1) produced by action of hyaluronidase on the hyaluronan polymer. LC-MS analysis of the reaction mixture in which *S. agalactiae* or *S. aureus* hyaluronidase enzymes were incubated with hyaluronan resulted in production of two slightly resolved ions detected with m/z 380.1192. The identity of the ions as products of hyaluronidase was supported by their observed loss after boiling the *S. aureus* and *S. agalactiae* reaction mixtures to deactivate the enzyme (Fig. 2). Additional confirmation was provided by measurements with high resolving power mass spectrometry, in which the observed mass of the observed ions (380.1182) matched the calculated monoisotopic mass for protonated 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine (380.1192) to within 2.6 ppm. Finally, we monitored time-dependent production of the disaccharide products (Supplementary Figure S1), and determined that disaccharide production increased over the range of 10 to 60 min. Thus, 15 min was chosen as the incubation time for the reactions.

3.2. Use of the HILIC-ESI-MS assay to evaluate inhibition

We also endeavored to evaluate the effectiveness of the method demonstrated in *Section 3.1* to monitor enzyme inhibition. Ascorbic acyl 6-palmitate (compound **1**, Fig. 3), has a published IC_{50} value of 4 μM against *S. agalactiae* hyaluronidase (Botzki et al., 2004). This literature value is in agreement with the IC_{50} value of $8.0 \pm 1.0 \mu\text{M}$ that was measured for a dose-response study of hyaluronidase inhibition by ascorbic acyl 6-palmitate monitored with UPLC-MS detection (Fig. 4A). On the basis of structural similarity to ascorbic acyl 6-palmitate, we predicted that n-cyclohexanecarbonylpentadecylamine (Cayman Chemical, Ann Arbor, MI, compound **2**, Fig. 3) would also act as a hyaluronidase inhibitor. This compound was also observed to inhibit the activity of hyaluronidase, although with somewhat less potency (IC_{50} $30.4 \pm 9.8 \mu\text{M}$, Fig. 4).

3.3. Method validation

A number of parameters were measured to validate the new method. From these validation studies, the linear dynamic range was determined to be from 0.78 to 50 μM 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine (Table 1). A calibration curve generated over this concentration range had a correlation coefficient of 0.997. With the new method, the

limit of detection (LOD) and limit of quantitation (LOQ) were 0.29 μM and 0.78 μM 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine, respectively. The LOD reported here lower than that reported by Reissig et al. using the Morgan-Elson colorimetric assay (1955). These investigators reported a limit of detection of 3×10^{-10} moles of N-acetyl-D-glucosamine (the reducing end of the disaccharide product), which is equivalent to 1.32 $\mu\text{g/mL}$ N-acetyl-D-glucosamine or 6.0 μM 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine. The LOD for the fluorometric version of the Morgan-Elson assay described by Takahashi et al. (2003) was 0.016 $\mu\text{g/mL}$ N-acetyl-D-glucosamine, equivalent to 0.071 μM 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine. This limit of detection is approximately 4 times lower than the LOD reported here. Thus, the new mass spectrometric assay appears to be more sensitive than the published colorimetric assay, but less sensitive than the fluorometric assay.

As a measure of the accuracy of the new method, the back calculated concentrations of the standards (residuals) differed from their nominal concentrations by 11% across the linear dynamic range (Table 2). The new method also demonstrated excellent precision. As evidence of this, the relative standard deviation values for both repeatability (precision between injections) and intermediate precision (precision across multiple days) were less than or equal to 10% (Table 2).

4. Discussion

Mass spectrometric analysis of sugars in biological matrices has often been complicated by the high polarity of these compounds, which prevents their retention on the reversed phase C-18 columns that are commonly employed for LC-MS analyses (Nguyen and Schug, 2008). With this study, we employed instead hydrophilic interaction liquid chromatography (HILIC), a chromatographic approach that has recently gained popularity for the analysis of polar species (Hemstrom and Irgum, 2006, Nguyen and Schug, 2008). The application of HILIC enabled the disaccharide product to be retained effectively (1.4 min, Fig. 2, panels A and C). This product was not retained with reverse phase chromatography (data not shown). Using HILIC coupled to mass spectrometry, it was possible to quantitatively monitor the activity of the hyaluronidase enzyme, and to replicate the published IC_{50} value of a known hyaluronidase inhibitor. Unlike previously published spectrophotometric methods (Reissig et al., 1955, Takahashi et al., 2003, Tung et al., 1994), the new MS based assay directly measures the product of the enzyme. Another advantage of this approach is that it will not be subject to interference by compounds that absorb or scatter light. Thus, although it is somewhat less sensitive than the published fluorometric method, it could be a useful alternative approach for situations where interference is observed with fluorometric analyses.

The detection of two ions in the active enzyme cultures was unexpected. These ions were partially resolved chromatographically (Figure 2), which indicates that they are both present in the solution prior to analysis, and not an artifact of analysis by the mass spectrometer. Given that both of these ions match the predicted mass of the 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine product, it is presumed that they are isomers of the same molecule. The configuration of these isomers could not be confirmed with mass

spectrometry, however, their presence and absence, respectively, in mixtures with and without active enzyme made them a useful product on which to base the biological assay.

Numerous pathogenic bacteria secrete hyaluronidase, including *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Peptostreptococcus*, *Streptomyces*, *Clostridium*, and *Propionibacterium* (Hynes and Walton, 2000). Hyaluronidases from *S. agalactiae*, *S. pneumoniae* and *S. aureus* have been found to have a high degree of homology, with greater than 35% amino acid identity across the entire enzyme and complete conservation of catalytic residues (Botzki et al. 2004, Hynes and Walton, 2000). Thus, it is possible that a hyaluronidase inhibitor could have broad-spectrum activity against multiple pathogens that secrete hyaluronidase. Therapeutic agents targeting hyaluronidase would have the advantage of selectively targeting pathogenic bacteria, leaving beneficial bacterial flora intact (Hynes and Walton, 2000). The bioassay developed here could enable screening for new hyaluronidase inhibitors against a range of bacteria pathogens, and, as such, could contribute importantly towards the development of new therapies based on hyaluronidase inhibition.

5. Conclusion

This work demonstrates the effectiveness of a mass spectrometric method for monitoring activity and inhibition of hyaluronidase. Here we employed the assay to monitor activity of *S. aureus* and *S. agalactiae* hyaluronidases, and to identify a new hyaluronidase inhibitor (n-cyclohexanecarbonylpentadecylamine). Future studies could employ this mass spectrometric approach in more extensive screening efforts, or to study enzyme kinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Partial funding for this research was provided by the National Center for Complementary and Integrative Medicine, a component of the National Institutes of Health (grant number 1R01 AT006860-01).

References

- CDC. Antibiotic Resistance Threats in the United States, 2013. Centers for Disease Control and Prevention; 2013.
- Aziz AM. The role of healthcare strategies in controlling antibiotic resistance. *Brit J Nutr.* 2013; 22:1066–1074.
- Botzki A, Rigden DJ, Braun S, Nukui M, Salmen S, Hoechstetter J, Bernhardt G, Dove S, Jedrzejewski MJ, Buschauer A. L-Ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor. X-ray structure and molecular modeling of enzyme-inhibitor complexes. *J Biol Chem.* 2004; 279:45990–45997. [PubMed: 15322107]
- Hemstrom P, Irgum K. Hydrophilic interaction chromatography. *J Separ Sci.* 2006; 29:1784–1821.
- Hynes WL, Walton SL. Hyaluronidases of Gram-positive bacteria. *FEMS Microbiol Lett.* 2000; 183:201–207. [PubMed: 10675584]
- Ibberson CB, Jones CL, Singh S, Wise MC, Hart ME, Zurawski DV, Horswill AR. *Staphylococcus aureus* hyaluronidase is a CodY-regulated virulence factor. *Infect Immun.* 2014; 82:4253–4264. [PubMed: 25069977]

- International Committee of Harmonisation. Validation of analytical procedures: text and methodology Q2(R1). 2005:1–13.
- Li S, Jedrzejewski MJ. Hyaluronan binding and degradation by *Streptococcus agalactiae* hyaluronate lyase. *J Biol Chem*. 2001; 276:41407–41416. [PubMed: 11527972]
- Nguyen HP, Schug KA. The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications. *J Separ Sci*. 2008; 31:1465–1480.
- Reissig JL, Storminger JL, Leloir LF. A modified colorimetric method for the estimation of N-acetylamino sugars. *J Biol Chem*. 1955; 217(2):959–966. [PubMed: 13271455]
- Richman PG, Baer H. A convenient plate assay for the quantitation of hyaluronidase in Hymenoptera venoms. *Anal Biochem*. 1980; 109:376–381. [PubMed: 7224162]
- Spickenreither M, Braun S, Bernhardt G, Dove S, Buschauer A. Novel 6-O-acylated vitamin C derivatives as hyaluronidase inhibitors with selectivity for bacterial lyases. *Bioorg Med Chem Lett*. 2006; 16:5313–5316.
- Starr CR, Engleberg NC. Role of hyaluronidase in subcutaneous spread and growth of group A streptococcus. *Infect Immun*. 2006; 74:40–48. [PubMed: 16368955]
- Takahashi T, Ikegami-Kawai M, Okuda R, Suzuki K. A fluorimetric Morgan–Elson assay method for hyaluronidase activity. *Anal Biochem*. 2003; 322:257–263. [PubMed: 14596836]
- Tung JS, Mark GE, Hollis GF. A microplate assay for hyaluronidase and hyaluronidase inhibitors. *Anal Biochem*. 1994; 223:149–152. [PubMed: 7695091]
- Vercruysse KP, Lauwers AR, Demeester JM. Kinetic investigation of the action of hyaluronidase on hyaluronan using the Morgan-Elson and neocuprine assays. *Biochem J*. 1995; 310:55–59. [PubMed: 7646472]
- Wang Z, Guo C, Xu Y, Liu G, Lu C, Liu Y. Two novel functions of hyaluronidase from *Streptococcus agalactiae* are enhanced intracellular survival and inhibition of proinflammatory cytokine expression. *Infect Immun*. 2014; 82:2615–2625. [PubMed: 24711564]

Highlights

- We designed a new bioassay to measure hyaluronidase inhibition
- The assay relies on mass spectrometry to directly quantify disaccharide production
- Ascorbic acid 6-palmitate was used as a control inhibitor
- This compound demonstrated an IC_{50} similar to the published value
- n-cyclohexanecarbonylpentadecylamine was identified as a hyaluronidase inhibitor

Fig. 1. Reaction between hyaluronan polymer and hyaluronidase. 3-(4-deoxy-β-D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine units are produced via β elimination of the 1→4 glycosidic bond.

Fig. 2.

Selected ion chromatograms representing two parallel hyaluronidase bioassays. A reaction performed with 0.61 $\mu\text{g/mL}$ *S. agalactiae* hyaluronidase and 2.44 mg/mL hyaluronan is shown in panel (A), and another reaction with identical conditions except that the enzyme was boiled for 15 min. prior to incubation is shown in (B). The experiment performed under the same conditions using active and denatured *S. aureus* hyaluronidase is shown in panels C and D, respectively. Peaks for m/z 380.0 were observed at 1.3 min for the active enzyme (A, C), and not for the denatured hyaluronidase (D, E). The experiment was performed in triplicate and the same results in the representative chromatograms above were observed for all replicates. NL indicates the level of signal (counts detected by the mass spectrometer) used to normalize each chromatogram.

Fig. 3.
Ascorbic acyl 6-palmitate (**1**) and n-cyclohexanecarbonylpentadecylamine (**2**).

Fig. 4.

Panel A represents a dose response curve of *S. agalactiae* hyaluronidase versus ascorbic acyl 6-palmitate (conditions identical to Fig. 2A). The measured IC_{50} was $8.0 \pm 1.0 \mu M$. Panel B represents a dose response curve of *S. agalactiae* hyaluronidase versus n-cyclohexanecarbonylpentadecylamine, for which the IC_{50} was $30.4 \pm 9.8 \mu M$. Each data point is an average of triplicate measurements of disaccharide peak area, with error bars corresponding to standard error.

Table 1
Regression parameters, detection limit, and limit of quantification for 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine

Validation parameter	Result ^a
Correlation coefficient	0.997
Standard error	0.040
y-Intercept	5.886 $\pm$ 0.025
Slope of regression line	1.006 $\pm$ 0.025
Number of data points	21
Limit of detection (LOD)	0.29 μ M ^b
Lower limit of quantitation (LLOQ)	0.78 μ M ^c
Upper limit of quantitation (ULOQ)	50 μ M

^a Calculations were based on triplicate analyses of seven standards of 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine using UPLC-MS.

^b LOD was defined as the concentration that provided a signal to noise ratio of 3:1 with noise determined by injection of a solvent blank (acetonitrile with 0.1% formic acid).

^c LOQ was defined as the lowest concentration of standard in the calibration curve that provided a residual of less than 15% (Table 2).

Table 2
Precision data for 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine

Theoretical concentration (μ M)	Measured concentration ^a (μ M)	Residuals ^b (%)	Repeatability ^c (%)	Intermediate precision ^d (%)
0.78	0.82	5	2	10
1.6	1.4	-11	7	6
3.1	3.2	1	2	4
6.2	6.2	0	2	10
12.5	13.8	10	3	4
25	27	7	5	5
50	46	-7	5	7

^a The measured concentration is an average of back calculated concentration of 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine obtained from triplicate analyses on three different days.

^b (Measured concentration-theoretical concentration) / theoretical concentration $\times$ 100.

^c Repeatability is expressed as the percentage relative standard deviation for triplicate analyses conducted on a single day.

^d Intermediate precision is expressed as the percentage relative standard deviation of the three back-calculated 3-(4-deoxy- β -D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine concentrations (each an average of triplicate measurements) determined on three separate days.