

## ORIGINAL CONTRIBUTION

# Evaluation of alternative diluents for clinical use of collagenase clostridium histolyticum (CCH-aaes)

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**Funding information**

Funding for this research was provided By Endo Aesthetics LLC.

**Abstract**

**Background:** Collagenase clostridium histolyticum (CCH-aesthetic formulation [CCH-aaes]; QWO™ [Endo Aesthetics, Malvern PA, USA] is approved as a subcutaneous injection for treatment of cellulite. In the aesthetic practice, dilution of marketed products is commonly employed to tailor treatments to individual patients or off-label locations. Dilution beyond the 0.23 mg/ml achievable with the proprietary diluent supplied with the CCH-aaes lyophilized powder requires diluents readily available in clinic.

**Aim:** To characterize the functionality and stability of CCH-aaes when reconstituted and/or diluted with alternative diluents, including normal saline, bacteriostatic saline, and/or proprietary diluent.

**Patients/Methods:** Each dilution was assessed for purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), activity using collagenase (AUX-I) and gelatinase (AUX-II) assays, and aggregation using size-exclusion chromatography.

**Results:** When reconstituted with either saline or proprietary diluent, and diluted with proprietary diluent or saline, purity, activity, and stability of CCH-aaes is maintained for up to 24 h at 5°C or 25°C. In contrast, use of bacteriostatic saline to reconstitute and/or dilute CCH-aaes results in up to a 40% decrease in activity and aggregation of 5.3% of CCH-aaes protein. Importantly, inclusion of 2% lidocaine and 1:200 000 epinephrine does not negatively impact CCH-aaes purity, concentration, or activity for up to 24 h at 5°C or 25°C.

**Conclusions:** From an efficacy and safety perspective, CCH-aaes must not be/should not be reconstituted and/or diluted with bacteriostatic saline to avoid injection of protein aggregates. Ideally, CCH-aaes should be reconstituted in proprietary diluent: further dilution with normal saline and addition of lidocaine and epinephrine is acceptable.

**KEYWORDS**

CCH-aaes, cellulite, collagenase, collagenase clostridium histolyticum, diluent, dilution

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## 1 | INTRODUCTION

Collagenase clostridium histolyticum (CCH), isolated and purified from fermentation of *Clostridium histolyticum* (CCH-aesthetic formulation [CCH-aes]; QWO™ [Endo Aesthetics, Malvern PA, USA], is recently approved as a subcutaneous injection for the treatment of moderate to severe cellulite in the buttocks of adult women.<sup>1</sup> CCH-aes comprises 2 collagenases: Collagenase I (AUX-I, Clostridial class I collagenase) and Collagenase II (AUX-II; Clostridial class II collagenase) in a 1:1 mass ratio. These collagenases have a high specificity for Type I and III collagen, and because each class of collagenase acts upon different sites within the collagen protein, the hydrolyzing capacity of CCH-aes is synergistic.<sup>2,3</sup> Once injected, CCH-aes targets local fibrous septae, and enzymatic disruption of these septae leads to release of the tension thought to underpin the unevenness in skin surface characteristic of cellulite.<sup>4-6</sup>

In the aesthetic practice, where CCH-aes for the treatment of cellulite is most likely to be used, clinicians frequently adapt on-label treatments for both off-label areas and in order to tailor treatment to individual patient needs. For injectable treatments, including dermal fillers and botulinum toxin (i.e., Botox), dilution is a commonly employed way to control treatment outcome and/or tailor treatments to off-label locations.<sup>7-9</sup> CCH-aes for the treatment of cellulite is packaged as either a lyophilized powder (0.92 mg), which is reconstituted with 4 ml of proprietary diluent or a lyophilized powder (1.84 mg) which is reconstituted with 8 ml of proprietary diluent, to achieve a final concentration of 0.23 mg/ml. Treatment of a single cellulite dimple in the buttock generally requires one injection of 0.3 ml of reconstituted product; although an elongated dimple may require more than one injection spaced approximately 2 cm apart.<sup>10</sup> The proprietary diluent contains both 0.6% sodium chloride (NaCl) and 0.03% calcium chloride dihydrate (CaCl<sub>2</sub>), and was developed to maintain protein stability in an isotonic solution.<sup>1,11</sup> In clinical practice, if the clinician wishes to further dilute the product beyond 0.23 mg/ml, there will be an insufficient quantity of proprietary diluent to do so. The most widely used diluents in clinical practice are normal saline (0.9% NaCl) and bacteriostatic saline (0.9% NaCl and 0.9% benzyl alcohol), and the addition of lidocaine and epinephrine to injections just prior to treatment is also common practice. The aim of this study was to characterize the functionality and stability of CCH-aes when reconstituted from a lyophilized state and diluted with these alternative diluents. The impact of dilution within a glass versus plastic vessel was also assessed.

## 2 | METHODS

### 2.1 | Diluents and conditions tested

Following reconstitution of 1.84 mg lyophilized CCH-aes (QWO™ [Endo Aesthetics; Malvern PA, USA]) with 7.6 ml of proprietary diluent (0.03% calcium chloride dihydrate in 0.6% sodium chloride,

and Water for Injection) in a glass vial, the solution was diluted with 30 ml of one of the following: (1a) bacteriostatic saline (0.9% NaCl and 0.9% benzyl alcohol) in glass, (1b) 0.9% NaCl (normal saline) in plastic or (1c) 0.9% NaCl in glass. An additional three conditions were evaluated with a 1.84 mg lyophilized CCH-aes vial by reconstituting and then diluting it using the same solution for both steps: (2a) bacteriostatic saline (in glass), (2b) 0.9% NaCl (in glass) or (2c) the proprietary diluent (in glass). In addition, CCH-aes reconstituted in proprietary diluent and diluted with saline was tested following addition of 4 ml of lidocaine (2%) and epinephrine (1:200 000) to in 37.6 ml of sample. Initial reconstitution of CCH-aes was done with 7.6 ml, rather than the 8 ml recommended because in internally conducted quality control experiments, reconstitution with 8 ml of diluent consistently yields 7.6 ml of solution when withdrawn into a syringe.

For each of these samples, stability, purity, and enzyme activity were assessed at baseline, after 24 h at both 5°C and 25°C, and after 120 h at 5°C. The control in each set of experiments was the baseline data from each of the sample conditions described above.

### 2.2 | SDS page purity assay

Sample purity was assessed by densitometry and integration of bands observed following reduced SDS-PAGE. SDS-PAGE conditions utilizing a NuPage 4-12% Sodium Dodecyl Sulfate Bis-Tris Polyacrylamide Gel (Invitrogen #NP0322-BOX) with Coomassie Blue staining. Changes reported for each condition are relative to its respective baseline sample ( $t_0$ ).

### 2.3 | Size-exclusion chromatography for determining aggregation and stability

Sample AUX-I and AUX-II content and any protein aggregation were determined using size-exclusion high performance liquid chromatography at 280 nm (HPLC, Agilent 1100 System with a Superdex 200 10/300 GL column, Cat. No. 17-5175-01). Protein aggregation was determined by peak area integration for each sample relative to reconstitution with proprietary diluent plus a saline diluent in glass at  $t_0$ .

### 2.4 | Enzyme activity assays

Collagenase (AUX-I) enzyme activity was evaluated utilizing serial dilutions of a commercially available peptide substrate (Glycine-Proline-Alanine) and Gelatinase (AUX-II) enzyme activity was evaluated utilizing serial dilutions of a commercially available soluble rat collagen as substrate as previously described.<sup>12</sup> Enzyme activity was determined relative to a reference standard and any changes in activity are reported for each condition relative to reconstitution with proprietary diluent plus a saline diluent in Glass at  $t_0$ .

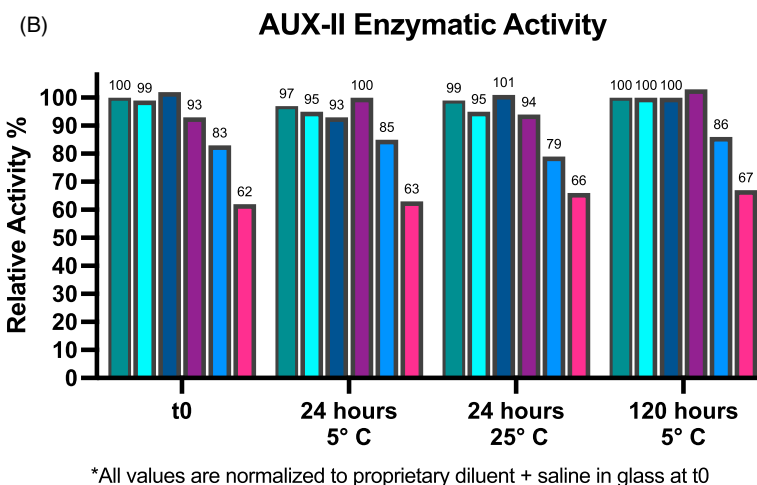
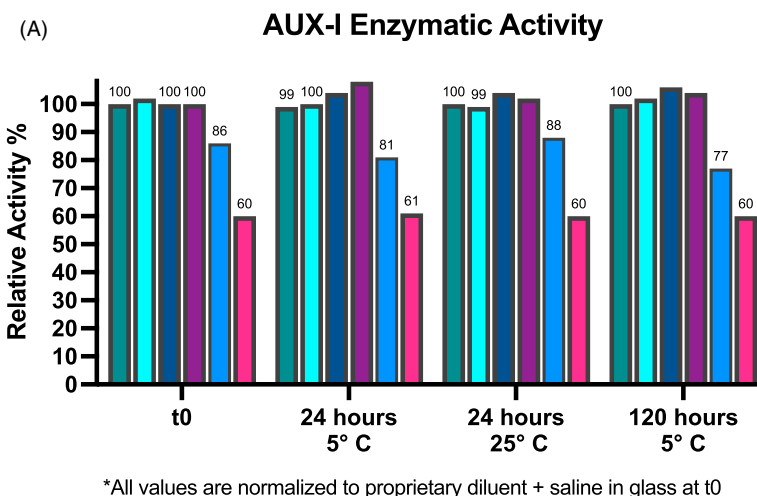
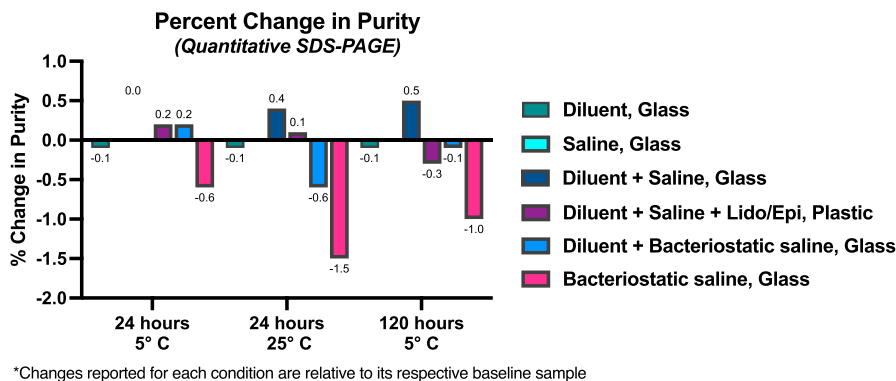
### 3 | RESULTS AND DISCUSSION

Overall, there were only minimal effects on enzyme purity but the greatest decrease in purity and enzyme activity was observed when bacteriostatic saline was used as the diluent, irrespective of the reconstitution solution. As seen in Figure 1, when bacteriostatic saline comprised the majority of the final solution a substantial

decrease (1–1.5%) in purity is observed after 24 h at 25°C or 120 h at 5°C, relative to the same conditions at baseline.

The relative impact on AUX-I and AUX-II activity was comparable across all conditions evaluated (compare Figure 2A,B), with the exception of solutions containing bacteriostatic saline. The greatest loss of activity was observed for both AUX-1 and AUX-II when bacteriostatic saline was used for both reconstitution and dilution

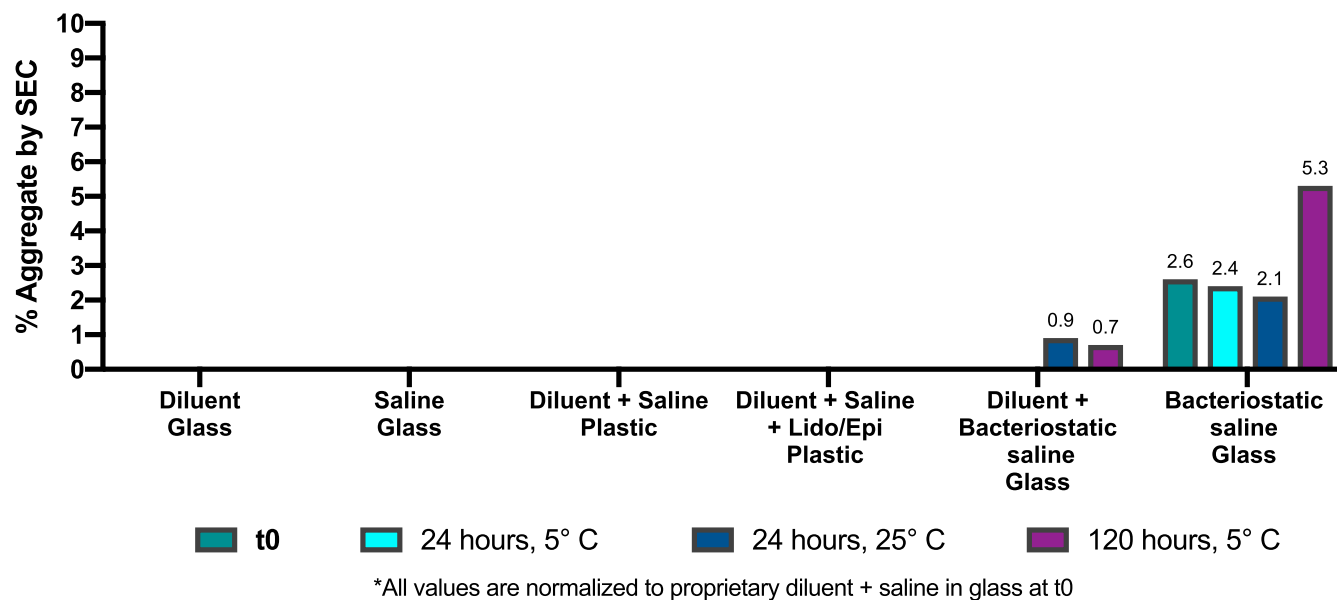
**FIGURE 1** Changes in sample purity are shown as measured by quantitative SDS PAGE



**FIGURE 2** Enzymatic activity of AUX-I (SRC Assay, Panel A) and AUX-II (GPA Assay, Panel B) after 24 h at 5°C and 25°C and after 120 h at 5°C following reconstitution and dilution with proprietary diluent, bacteriostatic saline, and regular saline in glass or plastic containers



## Protein Aggregation



**FIGURE 3** Percentage of CCH-aaes present as protein aggregate at t0 (blue), after 24 h at 5°C (red) and 25°C (green), and after 120 h at 5°C (purple) following reconstitution and dilution with proprietary diluent, bacteriostatic saline, and regular saline in glass or plastic containers (listed on x-axis)

(~40% enzyme activity loss). This effect was somewhat ameliorated by initial reconstitution in proprietary diluent with dilution in bacteriostatic saline, although that still resulted in a 20% loss in enzyme activity observable within minutes of dilution. In both instances, the observed loss in enzyme activity did not worsen over time.

Importantly, both AUX-I and AUX-II retained full enzyme activity when reconstituted and diluted in normal saline or proprietary diluent alone or in the combinations, even after 24 h at 25°C or 120 h at 5°C. The use of different container materials (plastic vs. glass) for dilution did not appear to impact enzyme activity. Additionally, the use of saline containing 2% lidocaine with 1:200 000 epinephrine as a diluent did not diminish enzyme activity.

Taken together, these findings indicate that the use of plastic or glass vessels for dilution is acceptable for maintaining CCH-aaes activity when proprietary diluent or normal saline is used to reconstitute CCH-aaes and saline used to dilute the product. Further the use of bacteriostatic saline to dilute and/or reconstitute CCH-aaes results in a near immediate ~40% reduction in activity. Importantly, the addition of lidocaine and epinephrine does not negatively impact enzyme activity, even after 120 h at 5°C, a finding with clinical implications, given that these ingredients are often used for both dermal filler and botulinum toxin injections to minimize patient discomfort and injection-site swelling/redness.

### 3.1 | Diluent effect on aggregation and protein stability

Size-exclusion chromatography revealed that the use of bacteriostatic saline in either the reconstitution or dilution step resulted in

protein aggregation (Figure 3). This effect was amplified when bacteriostatic saline was used for both reconstitution and dilution, with an immediate formation of 2.6% aggregate which nearly doubled to 5.3% after storage for 120 h at 5°C.

Since both of the samples containing bacteriostatic saline were prepared in glass vessels, any further impact of plastic on aggregation is unknown.

No aggregation was observed at any time point (or in glass or plastic) when CCH-aaes was reconstituted in proprietary diluent and diluted in either saline or proprietary diluent, or in CCH-aaes reconstituted and diluted in normal saline. Since the size exclusion method provided excellent resolution of the AUX-I and AUX-II enzymes and was not affected by any of the matrices, it was utilized to evaluate their concentrations in solution.

## 4 | CONCLUSION

When reconstituted with either saline or proprietary diluent, and diluted with either proprietary diluent or saline, the purity, activity, and stability of CCH-aaes is maintained for up to 24 h at either 5°C or 25°C. These results indicate that in the clinic, if the proprietary diluent is somehow lost or is present in insufficient quantities for CCH-aaes dilution, dilution with saline to a concentration of 0.047 mg/ml allows the final reconstituted, dilute solution to retain activity, purity, and solubility. However, the greater loss in enzyme activity and higher degree of aggregation observed for the sample reconstituted and diluted with bacteriostatic saline in comparison to the sample reconstituted with proprietary diluent and diluted with bacteriostatic saline indicates that  $\text{CaCl}_2$  does have a supportive effect on CCH-aaes stability and enzyme activity. Indeed, laboratory data

show that  $\text{CaCl}_2$  is critical for maintaining CCH-aes enzymatic activity and conformation.<sup>11,13</sup> The presence of  $\text{CaCl}_2$  in the proprietary diluent is intended to keep AUX-I and AUX-II in an active and open conformation, and should be used whenever possible to reconstitute CCH-aes. Importantly, the addition of lidocaine and epinephrine to CCH-aes by adding it to the saline diluent, does not negatively impact CCH-aes purity (including aggregate formation), concentration, or enzyme activity for up to 24 h at either 5°C or 25°C.

The use of bacteriostatic saline (a mainstay for dilution of toxin and fillers) causes a substantial loss of activity and full-length protein content as well as the formation of aggregates. Thus, from a potential efficacy and safety perspective, CCH-aes should not be reconstituted and/or diluted with bacteriostatic saline to avoid injection of protein aggregates. In addition to the loss of functional protein in the injection, aggregates may prompt an immunologic response that leads to loss of treatment benefit.<sup>14</sup> Beyond the potential for reduced treatment benefit, injection of protein aggregates may generate cross-reactivity to endogenous proteins, allergic reactions, or hypersensitivity.<sup>14,15</sup> Therefore, preventing protein aggregation is important for optimal treatment outcome and patient safety.

Together, these findings provide clinicians with the information needed to reconstitute and dilute CCH-aes without compromising protein purity, concentration or enzyme activity. As most aesthetic practices reconstitute toxin and prepare syringes for use ahead of the time of injection, the findings presented here showing that this is not appropriate for CCH-aes may prompt a change in workflow for many offices.

## ACKNOWLEDGMENTS

Technical editorial and medical writing assistance was provided by Ginny Vachon, PhD, Principal Medvantage, LLC, Atlanta, Georgia under the direction of the authors. Funding for this support was provided by Endo Pharmaceuticals Inc., Malvern, PA.

## CONFLICT OF INTEREST

HW, KC, and MM are employees of Endo Pharmaceuticals, Inc, JE is an employee of Endo Aesthetics LLC, and ER is an employee of Endo Aesthetics LLC and a consultant at InMode.

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**How to cite this article:** Wolfe HR, Rosenberg E, Ciftci K, Edgecombe J, McLane MP. Evaluation of alternative diluents for clinical use of collagenase clostridium histolyticum (CCH-aes). *J Cosmet Dermatol*. 2021;20:1643-1647. <https://doi.org/10.1111/jocd.14078>