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Note

Determination of Pulmozyme® (dornase alpha) stability using a kinetic colorimetric DNase I activity assay

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Abstract

An enzymatic activity assay was developed for the determination of *dornase alpha* human recombinant desoxyribonuclease (DNase I) stability. The method was adapted from a colorimetric endpoint enzyme activity assay for DNase I based on the degradation of a DNA/methyl green complex. With the described modifications the kinetic measurement of enzyme activity is feasible on an automated analyzer system within a rather short time. The development of this assay was based on the need for reliable detection of a possible loss of enzyme activity after transferring the commercial therapeutic agent into sealed glass vials required for a placebo-controlled study. The measuring range of this stability test was from 0 to 3000 U/L corresponding to 0–120% of the original enzyme activity; CV values of control solutions inside the measuring range were between 3% and 5%. The enzyme activity decreased less than 15% during the observation period of 180 days. In conclusion the current kinetic assay is a reliable method for a simple time-saving determination of DNase I activity to test Pulmozyme® stability as required for quality control. As *dornase alpha* is used for inhalation, this method also proved its reliability in testing DNase stability during aerosolization with new inhalation devices (*e-flow*).

Keywords: Dornase alpha; Pulmozyme; DNase I; Methyl green; Enzyme activity; Nebulizer; Inhaler system; Cystic fibrosis; CF

1. Introduction

Pulmozyme® (human recombinant DNase, *dornase alpha*) was cloned and expressed in Chinese hamster ovary cells at Genentech for the treatment of patients with cystic fibrosis (CF) [1]. Chronic pulmonary infections are the major cause of morbidity and mortality in CF. The viscoelasticity of the sputum has been shown to be reduced by *dornase alpha* treatment in CF patients, because it contains high amounts of extracellular DNA derived from dead leukocytes. The effects of this excess of free DNA in pulmonary secretions of CF patients are an increase of sputum viscosity and a decrease in antimicrobial efficacy due to binding of DNA to aminoglycosides [2]. Until now several placebo-controlled double-blind studies showed not only an improvement in pulmonary function and a reduction of respiratory exacerbations in CF patients under treatment with aerosolized *dornase alpha* [3–5], but also an indirect anti-inflammatory effect [6]. As *dornase alpha* now is well established for CF, other possible indications and new inhalation devices arise, which need to be investigated for clinical benefit. The aim of our work was the development of an inexpensive, reliable and automated method for *dornase alpha* stability testing, which may support quality control of Pulmozyme® stability and may be helpful in the development of therapeutic strategies and devices for the nebulization treatment of CF patients. In the following short report, the stability of *dornase alpha* is presented after transferring it into glass vials prior to a placebo-controlled study.

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2. Materials and methods

2.1. Generation of the colorimetric substrate solution

Ten milliliters of a DNA solution (2 mg/ml) in buffer A (25 mmol/L Hepes, 1 mmol/L EDTA, pH 7.5) was added to 600 μl of 0.4% methyl green solution in buffer B (20 mmol/L acetate–NaOH, pH 4.2) and 2.75 ml buffer C (25 mmol/L Hepes, pH 7.5, 4 mmol/L $CaCl_2$, 4 mmol/L $MgCl_2$, 0.1% bovine serum albumin, 0.01% Thimerosal, 0.05% Tween 20). In contrast to the method of Sinicropi et al. [7], to remove free methyl green in the freshly prepared substrate solution we added 1 and 10 μl hydrogen peroxide (35%), respectively, and preincubated the mixture overnight at room temperature in a 15 ml polypropylene tube (Sarstedt, Germany) under slight continuous rotation.

2.1.1. Samples

Dornase alpha (Pulmozyme®, 1,000,000 U/L, Hoffmann-La Roche, Basel, Switzerland) was made available by the manufacturer. The transfer of the original commercial drug into sealed glass vials was performed under sterile conditions by the pharmacy of the Medizinische Hochschule Hannover.

2.2. Assay conditions

Samples were diluted with buffer C (1:400) and transferred into a sample tube of a *Cobas mira* automated analyzer (Roche Diagnostics, Mannheim, Germany) containing 100 μl of buffer C, and 90 μl substrate solution. The reaction was started by addition of 90 μl of the diluted sample. Calibration was performed with a duplicate series of dilutions of *dornase alpha* (Pulmozyme®, 1,000,000 U/L, Hoffmann-La Roche, Basel, Switzerland) of 0, 500, 1000, 1500, 2000, 2500, and 3000 U/L, respectively. Since the assay design focussed on the determination of remaining enzyme activity, the 1:400 dilution (=2500 U/L) of *dornase alpha* was defined as 100% enzyme activity for comparison with the 1:400 diluted samples. In this context the calibration curve ranged from 0% to 120% of nominal enzyme activity. Δ*A*/min was measured at 600 nm and 37 °C. The analysis was made over 50 consecutive time intervals of 25 s each (*Cobas mira* calculation model: *logit/log4* (*Passing-Bablock*)). The first value included in the kinetic analysis was taken after 12 time intervals. All samples and controls were measured in quadruplicate.

3. Results and discussion

An automated kinetic method was developed for the quantification of human desoxynuclease (DNase I) activity. The method was adapted from an endpoint determination procedure by Sinicropi et al. [7], which employs a substrate comprised of polymerized native DNA complexed with methyl green. Hydrolysis of DNA results in production of free methyl green and a consequent decrease in the absorbance of the solution at 620 nm. In the original publication the authors reported two assay procedures (high and low range) for different DNase I concentrations in the analyzed samples. Incubation periods of 1–24 h were necessary to decolorize free methyl green in the substrate solution. The present method was developed because of the need for a simple cost-effective method for the precise determination of enzyme activity that might be reduced by less than 15%. Simple kinetic protocols including preincubation steps of freshly prepared substrate solutions with hydrogen peroxide were evaluated. We compared three

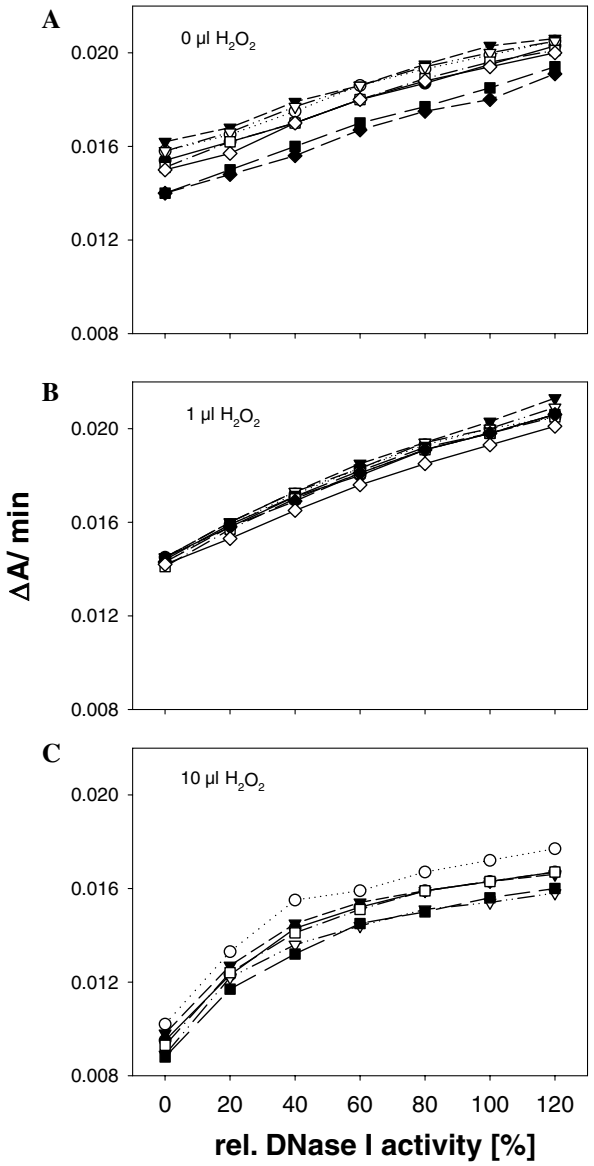

Fig. 1. Kinetic determination of DNase I activity with and without an overnight preincubation step of the substrate solution with hydrogen peroxide. Shown are results derived from three different protocols: without a preincubation step (A), preincubation of the colorimetric substrate solution with 1 μl H_2O_2 (B), and preincubation with 10 μl H_2O_2 (C), respectively. For the assay calibration Δ*A*/min values were plotted against the relative enzyme activity of the standards as described in Section 2. Different determinations on eight consecutive days predict the slightest spreading of values and the best slope for protocol (B).