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Note

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

Ralf Lichtinghagen *

Department of Clinical Chemistry, Medizinische Hochschule Hannover, Germany

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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.

* Department of Clinical Chemistry, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625, Hannover, Germany. Tel.: +49 511 532 3940; fax: +49 511 532 5671.
E-mail address: Lichtinghagen.ralf@mh-hannover.de.

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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

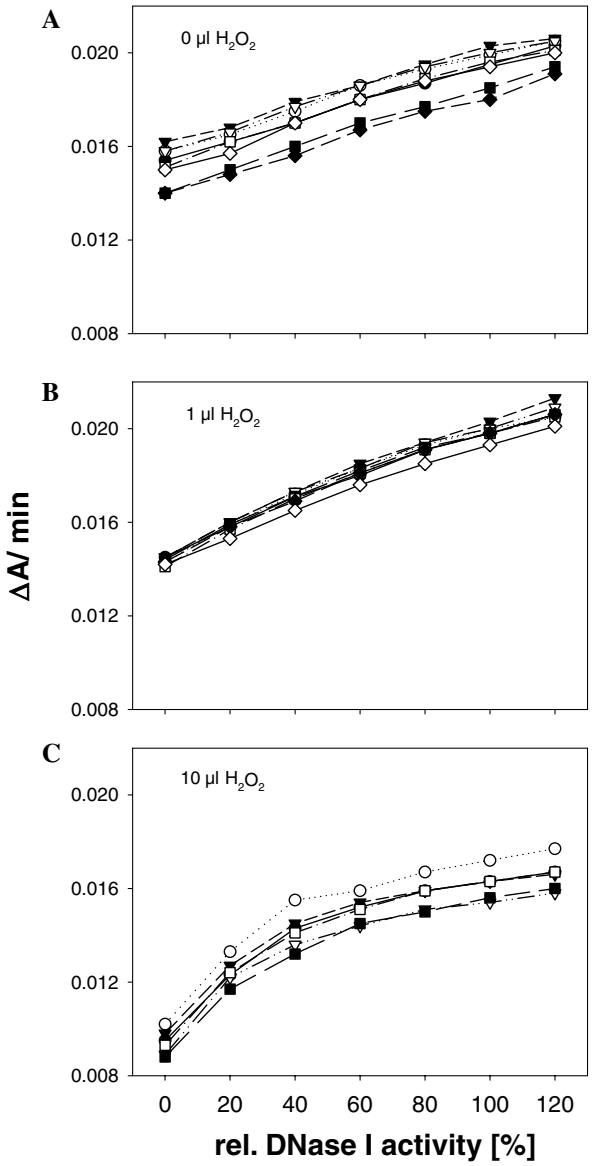

Fig. 1. Kinetic determination of DNase I activity with and without an overnight preincubation step of the substrate solution with hydrogen peroxide. Shown are results derived from three different protocols: without a preincubation step (A), preincubation of the colorimetric substrate solution with 1 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).

protocols ((A–C), see Fig. 1) using 0, 1, and 10 μl hydrogen peroxide in the colorimetric substrate solution. Fig. 1 shows variable spreading patterns in an interassay comparison with an advantage for protocol (B).

Within-assay and between-assay precision data as shown in Fig. 2 approve the highest validity for protocol (B). Coefficients of variation (CV) for between-assay precision were between 3% and 5% for three different control levels 70%, 85%, and 100% relative enzyme activity (see Fig. 2D). The critical difference between two values is defined by the validity of the assay in the corresponding range as shown by the validity data of the measured controls. It can be estimated by about 3× SD between these values [8]. We could demonstrate 3× SD intervals of less than 15%. Therefore we conclude that the introduced kinetic method can differentiate between 100% and 85% (i.e., a 15% loss) of enzyme activity.

For a placebo-controlled double-blind investigator initiated trial the original ampoules had to be transferred into glass vials identical to placebo. Concerns about loss of dornase alpha activity due to contact with glass made it necessary to test for stability. After transferring

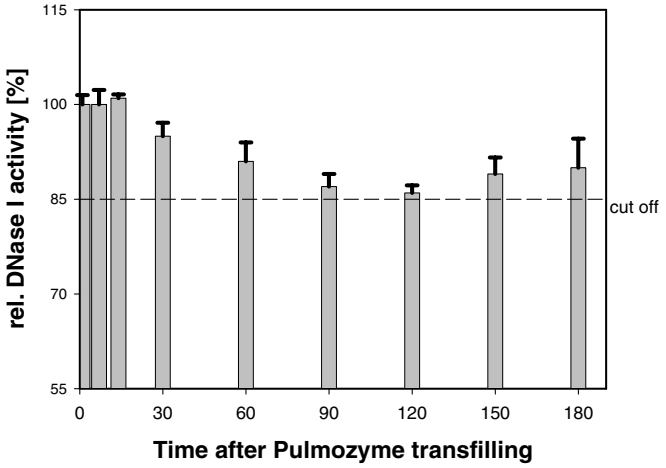

Fig. 3. Stability of Pulmozyme® after transferring into glass vials. Pulmozyme® was transferred into glass vials under sterile conditions and stored over a period of 180 days at 4 °C. In this interval enzyme activities from three different vials were assayed at predetermined times (day 1, 7, 14, 30, 60, 90, 120, 150, 180). Shown are mean values and SD of a 1:400 Pulmozyme® dilution (2500 U/L equivalent to 100%). A cut-off value of 85% remaining enzyme activity was defined as a minimum quality criterion.

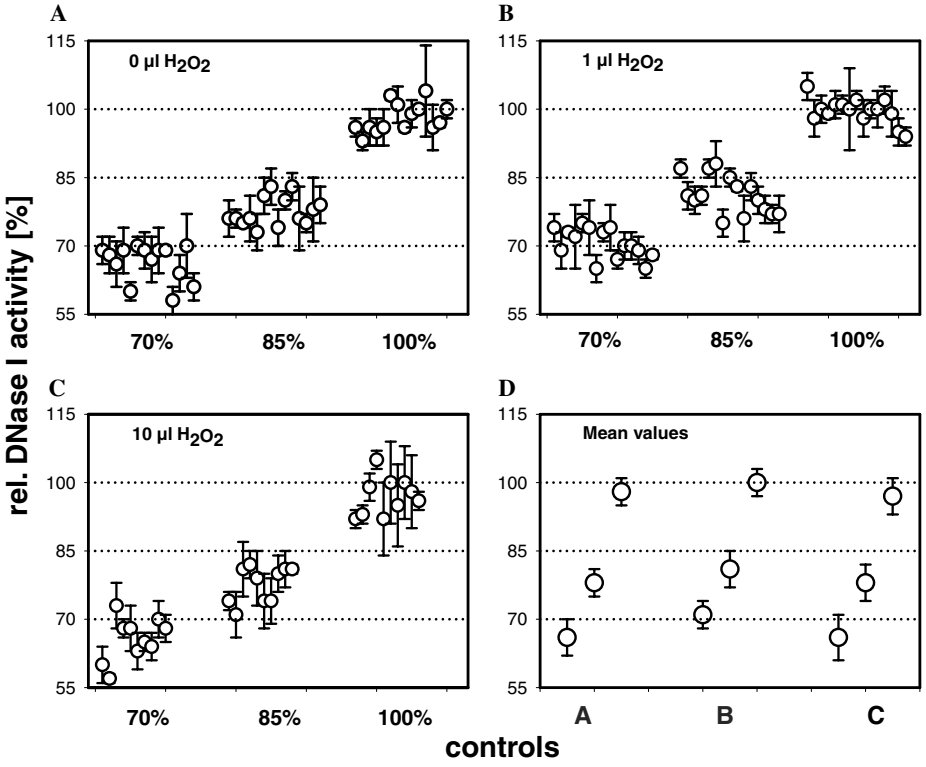

Fig. 2. Within-assay and between-assay variation of the kinetic determination of human DNase I activity with and without hydrogen peroxide preincubation of the substrate. A 100% control (2500 U/L) was obtained from a 1:400 dilution of Pulmozyme® in buffer C. From this dilution the 80% and 75% control solutions were generated. The validity of the kinetic assay was evaluated under different conditions: no preincubation of the substrate solution, preincubation of the colorimetric substrate solution with 1 μl H_2O_2, preincubation of the substrate solution with 10 μl H_2O_2. Between-assay variation was determined on 15 different days (see A and B) and 10 different days (see under C). Measurements were performed in quadruplicate. Mean values (%) and SD for between-assay precision are shown under (D). Best results were found for the assay conditions B (1 μl H_2O_2) with control values of $70 \pm 3\%$, $81 \pm 4\%$, and $100 \pm 3\%$, respectively.

dornase alpha from the original polyethylene vials into glass vials the enzyme activity was measured every 30 days for 180 days in total and compared with the activity in the original ampoules. The minimum quality criterion was a residual enzyme activity of 85% (cut-off value) over a period of 180 days. Fig. 3 shows the result of these measurements, and reveals a stable reduction of the enzyme activity of about 10% from day 30 to the end of the period.

Quality control of Pulmozyme® stability may be one possible application of the current method, which in our hands shows better validity than the non-kinetic procedure. However, the method of Sinicropi et al. may be more applicable to measurement of clinical samples that exhibit a wider range of concentrations [7]. Other applications for the kinetic method may also be suitable for pharmacological testing. A determination of *dornase alpha* activity should be done prior to treatment with combinations of nebulizer solutions, e.g. Pulmozyme® in combination with different antibiotics or antiasthmatic agents (e.g. salbutamol). First results show an incompatibility of Pulmozyme® and the antioxidant sodium metabisulfite used as an excipient in some liquid pharmaceutical forms of tobramycin (*Gernebcin®*, *TOBRA-cell®*) [9]. Furthermore this method may be useful in evaluating new inhaler systems (e-flow), as the nebulization process can be accompanied by a tremendous loss of *dornase alpha* activity due to excessive energy transfer, as observed with ultrasonic devices [10].

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