Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, No. 7, pp. 597–601, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00

Short Communication

Determination of subtilisin proteolytic activity by flow-injection analysis with fluorescence detection

C. HAYES POWELL* and R. BRETT NELSON

Allergan Optical, A Division of Allergan Inc., 2525 Dupont Drive, Irvine, CA 92715, USA

Keywords: *Flow-injection analysis (FIA); fluorescence detection; subtilisin; proteolytic enzyme; hydrophilic contact lenses; enzymatic cleaners.*

Introduction

Proteolytic enzymes are used to hydrolyse protein deposits that form on hydrophilic contact lenses during the course of normal wear [1]. Ultrazyme™ Enzymatic Cleaner (Allergan, Inc., Irvine, CA) contains a proteolytic enzyme and can be dissolved in 3% hydrogen peroxide (a contact lens disinfectant) to effect simultaneous disinfection and enzymatic cleaning [2]. The active ingredient in Ultrazyme tablets is subtilisin A (SUB). This communication describes an activity assay for SUB that was developed for product development and quality control purposes.

Methods for determining proteolytic enzymes include the use of artificial substrates [3, 4] and the derivatization of proteolytic products with fluorescent labels [5, 6]. The activity of SUB has been determined by employing chromophorically modified protein substrates to improve assay sensitivity [7]. In addition, Remazobrilliant Blue-hide powder has been used as a substrate for an automated method for the analysis of proteolytic enzymes [8]. The method reported here was designed to be used primarily for the rapid determination of SUB enzymatic activity in Ultrazyme tablets and was based on dialytic separation of low molecular weight proteolysis products from casein and the native fluorescence of those reaction products. Because of the relatively high content of tyrosine and (especially) tryptophan in casein and the high fluorescence quantum yield of tryptophan residues, the following flow-injection analysis (FIA) method represents a sensitive, direct route for determining proteolytic activity without the use of chromophorically altered or labelled substrates.

Experimental

Apparatus

The basic FIA system consisted of a Quik-Chem automated ion analyser (Lachat Instruments) and a McPherson model FL750BX fluorescence spectrometer with a deuterium source and modified inlet and outlet tubings (0.8 mm i.d.) connected to the 24-μl flow cell. The wavelength of the grating excitation monochromator was set to 280 nm, while emission was continuously monitored at all wavelengths >320 nm by employing a cut-off filter. The H-type membrane (Technicon, see Fig. 1) measured 3 × 25 cm. The sampling rate was set to 2 min (30 s for injector loop filling), and the pump speed was set to give a total flow rate of about 7 ml min^{-1} from the acceptor and reaction streams. The reaction stream passed through the upper chamber of the dialysis block, while the acceptor stream flowed through the lower chamber. After each run, all reagent lines were flushed with 0.05 M NaOH

* Author to whom correspondence should be addressed.

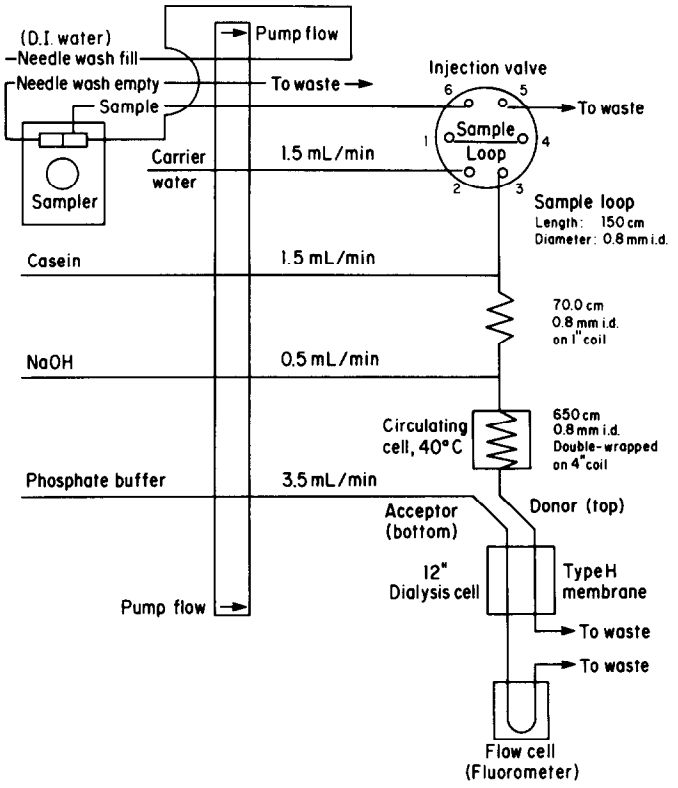

Figure 1
Flow diagram of the FIA system.

for 1 min to clean the system; then, all lines were flushed with water for a minimum of 2 min.

Chemicals and reagents

The standard SUB was a certified reference standard (NOVO) of ~1.5 Anson units per gram. The *N*-acetyl-L-cysteine, sodium carbonate, tartaric acid, and sorbitol were equivalent to the grades used in Ultrazyme. The sodium phosphate, casein (Hammerstein type), and sodium hydroxide were all reagent grade.

Standard preparation

An amount of the SUB standard equivalent to 1.5 Anson units was weighed into a 100-ml volumetric flask, dissolved in and diluted to volume with a blank base and mixed well. The blank base consisted of appropriate portions of the tablet excipients (acetylcysteine, sodium carbonate, sorbitol, polyethylene glycol 3350, and tartaric acid), equivalent to that contained in 100 tablets, dissolved in 1.0 l of distilled, deionized water. Working standards of 75 and 100% of the nominal concentration of SUB in the Ultrazyme tablet were then prepared by accurately pipetting 30.0 and 40.0 ml of stock solution into 50-ml volumetric flasks and diluting the solutions to volume with the blank base.

Sample preparation

One Ultrazyme tablet was placed in a 10-ml volumetric flask and allowed to dissolve in 5 ml of distilled, deionized water. The solution was then diluted to volume with water and mixed well. In a typical run, at least 10 samples are prepared.

Procedure

All reagent lines were placed in water, the pump started, and the fluorescence spectrophotometer and/or UV detector turned on. Next, the water bath was turned on and set to 60°C (since the water was circulated externally to the reaction coil, the actual temperature of the coil was about 40°C). After 15 min, the pump tube lines were placed in the proper reagents (casein, buffer, sodium hydroxide and water), the sampling probe was placed in the first standard, and the analogue-to-digital con-

verter was started. Duplicate or triplicate injections were made from each solution, with standard sets bracketing after every 9 or 10 injections of samples.

Results and Discussion

Test solutions were reacted with casein within the FIA system to form fluorescent peptides and amino acids, particularly tyrosine- and tryptophan-containing residues. The residues were separated via dialysis from unreacted casein and swept into the acceptor stream and to the fluorescence detector flow cell (Fig. 1). Quantitation was based on a comparison of the response of unknown solutions with standard SUB solutions of known activity run under identical conditions. A negative y-intercept was observed when analysis was performed in the linear concentration range of the S-shaped response curve; thus, a two-point standard curve was used routinely to correct for the non-zero intercept.

Because the reaction solution was found to be pH- and temperature-dependent, these parameters were optimized by adjusting the concentration of the sodium hydroxide reagent and the temperature of the water bath to yield maximum response. The phosphate buffer pH, when maintained at a value of >7.0, had little effect on the overall system response.

FIA, rather than segmented flow, technology was used because of the ability of the former to completely resolve the baseline with each peak, resulting in better accuracy with regard to long-term baseline drift.

Figure 2 shows a typical trace of the FIA fluorescence output. After data acquisition into a single file (up to 5 h run time on the HP Laboratory Automation System), the assay calculations were computed automatically with a BASIC linear regression program and a method representing optimized parameters for peak height determination.

Linearity of response

A standard response curve (Fig. 3) was generated to determine the linearity of the method from 40 to 200% (4.8×10^{-4} to 2.4×10^{-3} Anson units/ml) of the nominal activity of SUB in an Ultrazyme tablet. The y-intercept (y_0) over y at 100% label claim (y_0/y_{100}) was usually negative and was found to depend on

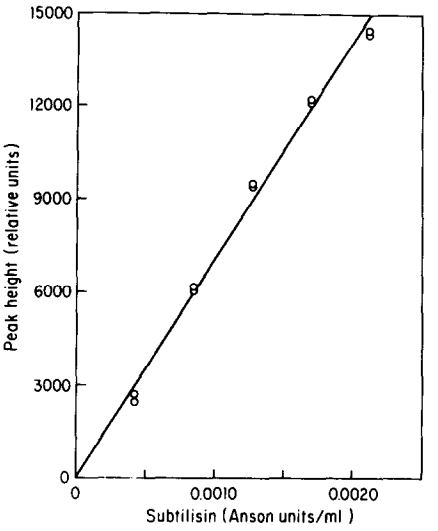

Figure 3
Standard response curve used to determine linearity of method.

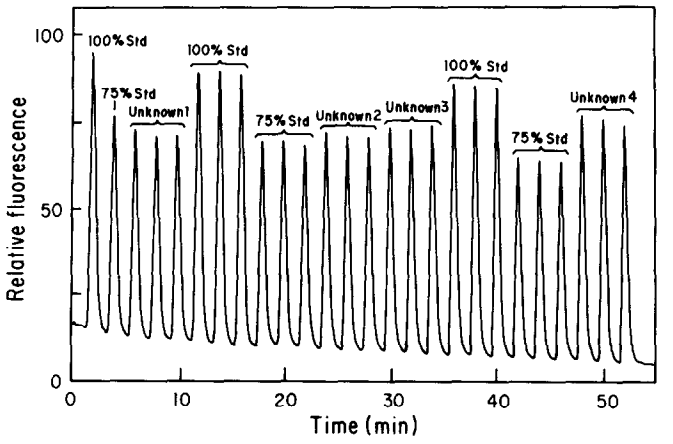

Figure 2
Results of SUB assay using fluorescence spectrometry and a flow injection analyser.

the membrane, with more efficient membranes giving a smaller negative y-intercept and a larger dynamic linear range. The correlation coefficient was 0.996 over the full linear test range. The slight S-shape of the curve resulted from the dynamics of passage of the peptides and amino acids through the membrane (saturation effect) and from the fact that a threshold concentration of the enzyme is required for observation.

Because the slope decreases at high enzyme concentration (>150% of nominal), care was taken to ensure accuracy of the regression data analysis by working within the area of rectilinear response. A two-point calibration with linear interpolation of results was used to correct for the typically non-zero (negative) y-intercept.

Potential interference from tablet excipients

The effects of varying excipient concentrations on assay values were found to be negligible when using fluorescence as the means of detection. Excipients considered to have the potential for affecting the assay were sodium carbonate, tartaric acid, and acetylcysteine which contributed to the pH and ionic strength of the samples for assay. In addition, acetylcysteine, because it exhibited absorption at 280 nm (a significant drawback to using UV spectroscopy as a detection method), had the potential for quenching fluorescence (at high concentrations) or enhancing fluorescence (at low concentrations). Varying the concentration of acetylcysteine from 50 to 150% of the nominal value in a tablet, however, resulted in only a 5.5% increase the SUB assay value. Similar changes in sodium carbonate and tartaric acid resulted in an increase of measured SUB of 7.6 and 0.2%, respectively (Table 1). The effect of varying the non-absorbing (at 280 nm) components (all except acetylcysteine) was the same for UV and fluorescence detection. However, because acetylcysteine accounted for up to 50% of the total response by UV detection, the effect of varying this component from 50 to 150% of the nominal concentration was an assayed result 75 to 125% of the actual value. Assuming no fluorescence quenching, the fluorescence results in Table 1 represent ranges in actual proteolytic activity produced by varying the excipient levels. This information was useful for optimization of the formulation as well as for analytical qualification. Thus, in terms of specificity and ruggedness, fluorescence was preferred to UV absorption.

Potential interference from degradation products

In terms of stability, UV and fluorescence

Table 1
Effect of excipients on subtilisin assay in Ultrazyme tablets using fluorescence detection

Concentration of added excipient*	Recovery† (%)	Difference (%)
Acetylcysteine		
50	97.7	5.5
150	103.2	
Sodium carbonate		
50	94.5	7.6
150	102.1	
Tartaric acid		
50	103.7	0.2
150	103.9	

*Expressed as percentage of theoretical concentration.
†Median value of three samplings.

Table 2
Comparison of fluorescence and UV detection in the FIA analysis of stability samples of Ultrazyme tablets

Lot no.	Storage conditions	Activity (Anson units/tablet) Fluorescence	UV	Difference (%)
6915A		0.00898	0.00906	+0.9
6915B		0.00893	0.00898	+0.6
6933-45A	120 days at 45°C	0.00951	0.00959	+0.8
6933-45B	120 days at 45°C	0.01002	0.01007	+0.5
6933-45C	120 days at 45°C	0.00978	0.00989	+1.1
6933-5A	120 days at 5°C	0.00999	0.00992	−0.7
6933-25/85C	120 days at 25°C/85% RH	0.01036	0.01011	−2.4
6933-37/85C	120 days at 37°C/85% RH	0.00984	0.00966	−1.8
	Mean	0.009676	0.009660	−0.2
6933-50C	120 days at 50°C	0.00968	0.01146	+18.4
6933-65C	120 days at 65°C	0.00496	0.01081	+117.9

detection gave equivalent results for samples stored below 45°C for up to 120 days. Increasing the storage temperature, however, increased the difference between the results obtained by the two methods (Table 2). The likely explanation for this is that UV absorbing substances which interfered with the detection were produced at high temperatures by decomposition of tablet excipients, specifically, acetylcysteine. This observation together with the fact that fluorescence detection was more sensitive further supported the conclusion that fluorescence was the detection method of choice for the FIA determination of enzymatic activity of subtilisin. Another, but much less likely, explanation for the apparent difference in high-temperature stabilities as measured by the two methods is that the functionality of the enzyme responsible for hydrolytic release of tryptophan units from casein becomes deactivated, whereas the functionality of the enzyme responsible for hydrolytic release of tyrosine is not affected even under extreme storage conditions.

References

[1] R.M. Kiral and J.G. Vehige, *Contact Lens Spectrum* **3(10)**, 31–42 (1988).
[2] N. Tarantino, R.C. Courtney, L.A. Lasswell, D. Kono and J. Frank, *Int. Contact Lens Clinic* **15(1)**, 25–32 (1988).
[3] K. Kuromizu, H. Higuchi, O. Abe and Y. Shimokawa, *J. Chromatogr.* **267**, 199–208 (1983).
[4] A.E. Klein, J. Freiberg, S. Same and M.A. Carroll, *J. Assoc. Offic. Anal. Chem.* **72**, 881–882 (1989).
[5] R. Garesse, J.V. Castell, C.G. Vallejo and R. Marco, *Eur. J. Biochem.* **99**, 253–259 (1979).
[6] P.T. Chien, *J. Amer. Ind. Hyg. Assoc.* **39**, 808–814 (1978).
[7] R. Chavira, T.J. Burnett and J.H. Hageman, *Anal. Biochem.* **136**, 446–450 (1984).
[8] P.B. Newman and D.J. Etherington, *Anal. Biochem.* **57**, 347–355 (1974).

[Received for review 9 February 1990; revised version received 7 May 1990; accepted 8 May 1990]