Pergamon

0731-7085(94)00095-6

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 11, pp. 1363–1367, 1994

0731-7085/94 $7.00 + 0.00

Development and validation of capillary electrophoresis assay for Ribonuclease A

RANJANI NELLORE and HITESH R. BHAGAT*†

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 N. Pine Street, Baltimore, MD 21201, USA
†Oravax, Inc., 230 Albany Street, Cambridge, MA 02139, USA

Abstract: A quick and reproducible capillary electrophoresis assay was developed and validated for bovine pancreatic Ribonuclease A (Rnase A). Effects of separation buffer pH and concentration as well as separation power were evaluated on Rnase A samples in phosphate buffered saline in the 5–100 μg ml^{-1} concentration range. The separation variables yielded significant influence on peak elution, with phosphate buffer (pH 2.5; 0.2 M) at separation power of 2 W providing an optimum peak at about 4 min. The peak sharpness and detection limit was further improved by a base/sample/acid injection technique. The optimized method showed good linearity with inter- and intraday RSD of peak area in the 2–5% range. Heat, as well as guainidine hydrochloride denatured samples, showed loss of enzymatic activity, changes in tyrosine fluorescence and abnormal electropherograms. The assay was also compared to a bicinchoninic acid assay that is routinely used for protein quantitation, and found to have greater sensitivity and reproducibility.

Keywords: *Capillary electrophoresis; sample stacking; Ribonuclease A; fluorescence; enzyme activity.*

Introduction

High-performance capillary electrophoresis (HPCE) is a powerful analytical tool for the rapid separation and quantitation of charged molecules, and has proven to be particularly useful for the analysis of proteins and peptides [1, 2]. Ribonuclease A (Rnase A) has been extensively studied for protein folding kinetics and freeze drying stability [3–6]. It is currently being used in our laboratory as a model protein for controlled delivery using biodegradable polymeric microspheres. A quick and reproducible assay technique was desired for this protein.

Materials and Methods

Chemicals and reagents

Ribonuclease A (Type XII-A) and its substrate, ribonucleic acid were purchased from The Sigma Chemical Co. (St Louis, MO, USA). Sodium phosphate, sodium hydroxide and other buffer ingredients were of analytical grade and were obtained from The Sigma Chemical Co. Sterile water for irrigation was used for preparation of all solutions.

Capillary electrophoresis

A PACE 2100 capillary electrophoresis unit (Beckman Instruments Inc., CA, USA) equipped with a fixed wavelength UV detector was used. The unit is controlled using System Gold software (Beckman Instruments Inc.). The separations were performed at constant power using a 27 cm long fused silica capillary with an internal diameter of 75 μm. The temperature during operation was maintained at 20 ± 0.1°C. Samples were pressure injected for 3 s at 85 psig and detected at 200 nm. The fused silica capillaries were washed with sodium hydroxide (0.1 N), water, and running buffer for 5 min each prior to the runs. The separation buffer was degassed and filtered before use and the buffer vials were changed after every 12–15 analysis (triplicate injections of four to five samples). The analysis conditions investigated for method optimization were pH, buffer concentration and separation power. The pHs investigated were 2.5 (phosphate) 8.0 and 9.0 (borate). The buffers were investigated at 0.1, 0.15 and 0.2 M concentration, at separation power of 0.5, 1.0, 1.5 and 2.0 W. The sample was prepared fresh in phosphate buffer (pH 7.4; 0.1 M) with sodium chloride (34 mM).

*Author to whom correspondence should be addressed.

After optimization of the assay conditions, standard curves were generated in the concentration range of 5–100 μg ml^{-1} on three different days for interday variability and three runs were performed during a day for intraday variability. Peak area was plotted against the concentration. Reproducibility of the assay was determined by calculating % RSD of peak area.

Activity assay

The enzymatic activity of Rnase A was determined by the method of Kunitz [7]. The hydrolysis of the substrate, ribonucleic acid is accompanied by a decrease in absorbance at 300 nm, the rate of decrease being directly proportional to the enzymatic activity of Rnase A. The absorbance measurements were carried out in 1-cm light path quartz cuvettes using a Shimadzu UV160U spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) with a temperature controlled cuvette holder maintained at 25 ± 0.5°C.

Fluorescence measurements

The tyrosine fluorescence of Rnase A was measured using a Perkin–Elmer LS 50 Luminescence spectrometer (Perkin–Elmer Limited, Buckinghamshire, UK). The emission scans were obtained by excitation at the isosbestic point of 268 nm. Protein samples were prepared in phosphate buffer (pH 7.4; 0.1 M) with sodium chloride (34 mM).

BCA assay

Protein quantitation was also conducted by the BCA assay (Pierce, Rockford, IL, USA). The assay involves reaction between cuprous ion, bicinchoninic acid and the protein, leading to the formation of a purple reaction product which exhibits a strong absorbance at 562 nm. The absorbance measurements were made on the Shimadzu spectrophotometer using bovine serum albumin as standard. Reproducibility of analysis was monitored in the range of 10–200 μg ml^{-1}.

Results and Discussion

The surface of the fused silica capillaries that are used in HPCE have free silanol groups that have a negative charge at a pH greater than 3. During separations, proteins tend to get adsorbed on these surfaces resulting in poor resolution and peak broadening. However, these interactions can be minimized by performing the separations at low pH where the surface silanols are protonated and the protein is also positively charged and also, at pH higher than the isoelectric point of protein [8]. At low pH, the electroosmotic flow is also reduced and in some cases may lead to undesirably long retention times [9]. In the case of Rnase A, better sensitivity and reproducibility were obtained in phosphate buffer (pH 2.5) with a retention time of about 4 min.

Effect of buffer concentration

The effect of phosphate buffer concentration at pH 2.5 is shown in Fig. 1. At lower buffer concentration Rnase A migrated faster. This is postulated to be due to decreased resistance to the movement of ions, leading to increased ionic mobility of the protein [10].

Effect of separation power

Figure 2 shows the effect of separation power on peak retention in phosphate buffer (pH 2.5; 0.1 M). Increasing the separation power from 1.0 to 2.0 W leads to reduction in elution time from about 5.5 to 3.5 min. In general, increasing the voltage across the capillary results in faster migration of analyte but there is also a simultaneous increase in the current which may lead to increased heating of the sample, resulting in poor separation. Since the heating is directly proportional to the power applied, there is an optimum amount of power that can be applied to the capillary. For our system, we have found it to be about 2 W. Application of greater separation power frequently results in abortion of analysis due to development of microbubbles in the capillary (presumably due to overheating).

Effect of injection conditions

Simple pressure injection of sample plug occasionally lead to broad peaks with shoulder or tailing, specially at low concentrations. A base/sample/acid plug injection method was used to obtain sharper sample bands. When a plug of NaOH (0.1 M) was injected prior to the sample and a plug of phosphoric acid (0.1 M) was injected after the sample, sharper peaks and increased sensitivity were observed (Fig. 3) in phosphate separation buffer (pH 2.5; 0.2 M). This phenomenon can be attributed to the fact that the voltage applied during electrophoresis causes the base and acid plug to move toward each other, pushing the sample

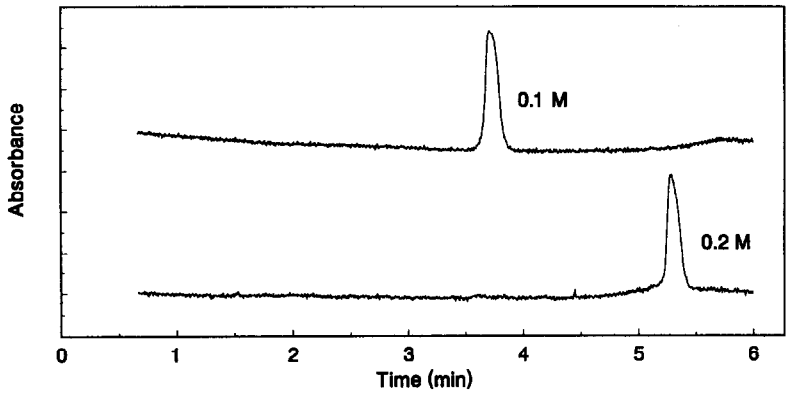

Figure 1
Effect of separation buffer concentration (phosphate, pH 2.5) on Rnase A peak retention.

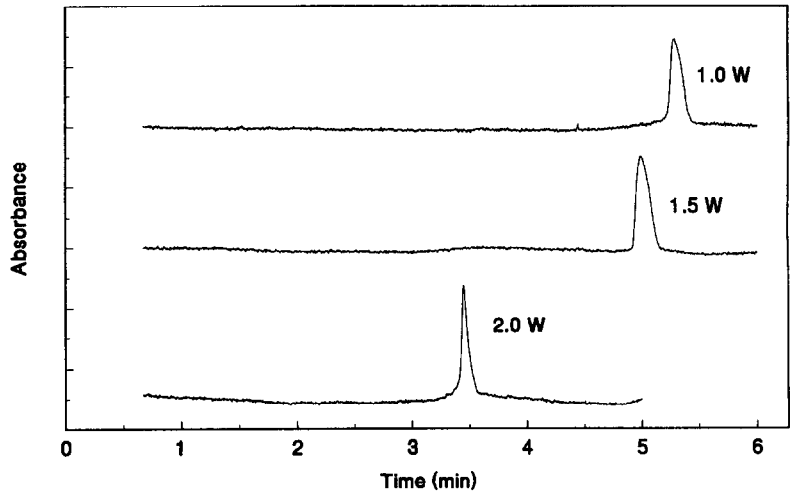

Figure 2
Effect of separation power on Rnase A peak retention in phosphate separation buffer (pH 2.5; 0.1 M).

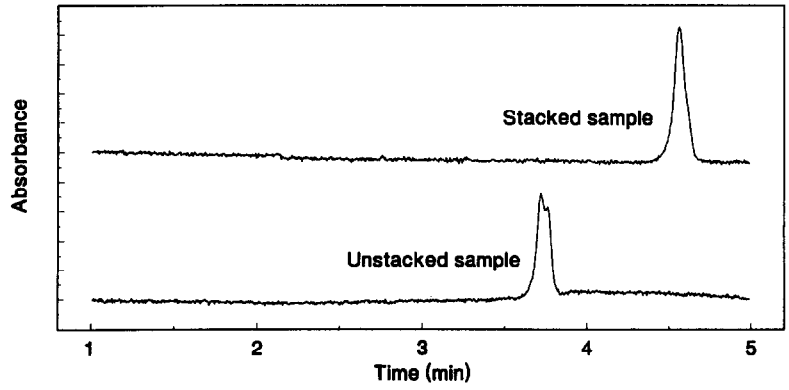

Figure 3
Effect of sample stacking on Rnase A peak resolution in phosphate separation buffer (pH 2.5; 0.2 M).

plug together. Neutralization of the two results in formation of water, leading to a region of low resistance and, therefore, high field strength. This in turn causes the sample to accelerate forward and become focused on the column [11].

Validation of optimized method

The assay technique was validated using phosphate buffer (pH 2.5; 0.2 M) at a separation power of 2 W. Under these conditions, the elution time was about 4.5 min. Regression of concentration vs peak area provided good correlation. The assay was fairly reproducible, with RSD of peak area in the range of 2.5–5.1%.

Capillary electrophoresis of denatured Rnase A

Heat and guanidine denatured samples were injected at the same concentration as native Rnase A. The heat denatured protein had the same retention time as the native protein but the peak appeared to be much smaller and broader. This could be due to aggregation of the denatured protein resulting in non-uniform sample injection. The guanidine denatured Rnase A could not be detected due to high background absorbance of the denaturant.

Tyrosine fluorescence

Figure 4 shows the emission spectra for the native as well as denatured Rnase A, following excitation at the isosbestic point at 268 nm.

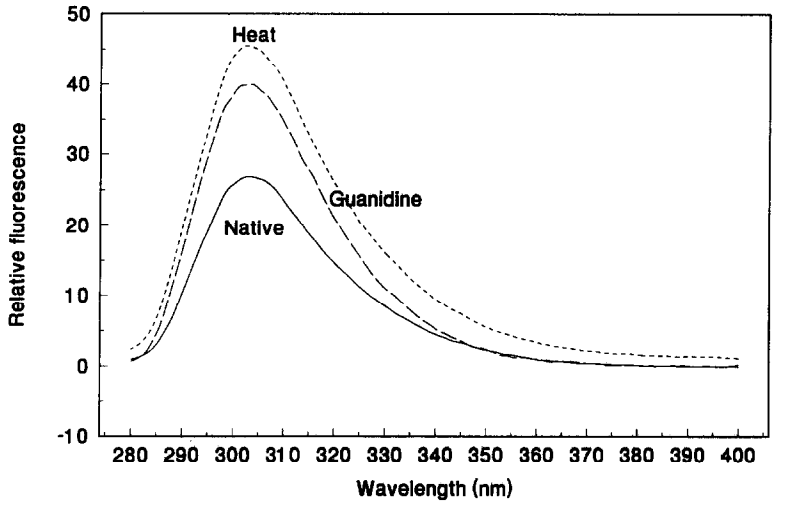

Figure 4
Tyrosine fluorescence spectra for native and denatured Rnase A in phosphate buffer (pH 7.4; 0.1 M) with sodium chloride (34 mM).

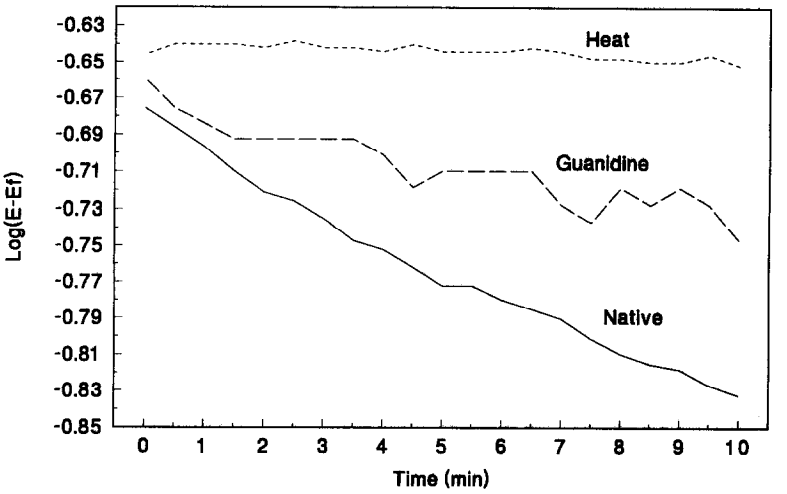

Figure 5
Kinetics of enzymatic hydrolysis of ribonucleic acid by native an denatured Rnase A. E is absorbance at time t and E_f is absorbance at infinity.

Tyrosine fluorescence intensity is increased for the denatured protein with maximum fluorescence obtained for the heat denatured sample. This implies a significant change in the environment of the tyrosine residues of the heat denatured Rnase A [12].

Activity assay

In Fig. 5, a linear decline in absorbance is seen for the native Rnase A as enzymatic hydrolysis of ribonucleic acid occurs. The slope of the line (log $(E - E_f)$ vs t), where E is absorbance at time t and E_f is absorbance at infinity) was decreased for the guanidine denatured protein with almost no decrease in absorbance seen for the heat denatured Rnase A. Thus, heat denaturation results in almost complete loss of activity of Rnase A as a result of the structural changes observed in the fluorescence spectrum.

BCA assay

The validated BCA assay for Rnase A had inter- and intraday RSD in the range of 6.9–12.5%. While this method is widely used for routine analysis of protein content, it was found to have higher variability than the HPCE assay. Furthermore, the method is less sensitive and requires much large quantity of sample (500 μl) compared to HPCE (10 μl). Also, BCA would not be applicable for individual protein analysis in a matrix containing two or more proteins.

References

[1] P. Grossman, J. Colburn, H. Lauer, R. Nielsen, R. Riggin, G. Sittampalam and E. Rickard, *Anal. Chem.* **61**, 1186–1194 (1989).
[2] Chen, T. Fu, *J. Chromatogr.* **559**, 445–453 (1991).
[3] M. Lyles and H. Gilbert, *Biochemistry* **30**, 619–625 (1991).
[4] M. Townsend, P. Byron and P. DeLuca, *Pharm. Res.* **7**, 1086–1091 (1990).
[5] M. Townsend and P.DeLuca, *J. Pharm. Sci.* **79**, 1083–1086 (1990).
[6] M. Townsend and P. DeLuca, *J. Pharm. Sci.* **80**, 63–66 (1991).
[7] M. Kunitz, *J. Bio. Che.* **164**, 563 (1946).
[8] S. Rabel and J. Stobaugh, *Pharm. Rres.* **10**, 171–186 (1993).
[9] R. McCormick, *Anal. Chem.* **60**, 2322–2328 (1988).
[10] P. Pande, R. Nellore and H. Bhagat, *Anal. Biochem.* **204**, 103–106 (1992).
[11] C. Schwer and F. Lottspeich, *J. Chrom.* **623**, 345–355 (1992).
[12] J. Lakowicz, in *Principles of Fluorescence Spectroscopy*, pp. 341–379. Plenum Press, New York (1983).

[Received for review 28 March 1994; revised manuscript received 5 July 1994]