Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1013 (2003) 215–220
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

# Analysis of vasopressin using capillary electrophoresis with laser-induced fluorescence detector based on competitive immunoassay

Kyoung-Youn Han, Eunmi Ban, Young Sook Yoo*

*Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, South Korea*

**Abstract**

A competitive immunoassay based on CE–LIF has been developed for the determination of vasopressin in biological mixtures. Vasopressin participates in the hormonal control of water metabolism and the constriction of arterioles in humans. Thus, detection of vasopressin is important in diagnosing pathological conditions and physiological water metabolism. The peptides were fluorescently tagged with FITC and purified by HPLC. The purified product was then mixed with the cerebrospinal fluid sample followed with the addition of anti-vasopressin antibody. It was possible to separate antibody-bound and free FITC-tagged vasopressin within 10 min by CE–LIF analysis using uncoated fused-silica capillary with high reproducibility.


*Keywords:* Immunoassays; Vasopressin; Peptides

## 1. Introduction

Vasopressin (Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Leu–Gly–NH2) is synthesized in the hypothalamus and secreted in the posterior pituitary. The major effects of this peptide are participation in the hormonal control of water metabolism and the constriction of arterioles in human [1,2]. The basic nonapeptide secreted from the posterior pituitary is arginine vasotocin (AVT) in non-mammalian vertebrates [3] and arginine vasopressin (AVP) in mammals [4]. These peptides, which differ by only one amino acid, are consistently associated with control of urine production throughout the vertebrate series. The renal action of these neurohypophysial peptides is due to the defined antidiuretic effects of AVP in the mammalian distal nephron, where AVP acts to increase apical membrane water permeability of kidney tubules. AVP's action on the water permeability of the kidney tubule is mediated by the so-called V2-type receptor, which activates adenylate cyclase and results in an increase in intracellular levels of cAMP, and ultimately, the insertion of water channels (aquaporins) in the apical membrane of the principle cells [5]. This increased water permeability permits reabsorption of nascent urine across the tubule wall and is largely responsible for AVP-induced antidiuresis in mammals. Thus, detection of vasopressin is important in diagnosis of pathological conditions due to physiological water metabolism. Peptides constitute a large and important family of hormones and neurotransmitters [6]. Quantitation of these compounds in complex biological samples for studies of intercellular communi-

*Corresponding author. Tel.: +82-2-958-5066; fax: +82-2-958-5170.
*E-mail address:* ysyoo@kist.re.kr (Y.S. Yoo).

0021-9673/03/$ – see front matter 
doi:10.1016/S0021-9673(03)01432-8

cation can be challenging due to the low concentration of peptide messengers, often 1–150 p*M*, and the presence of multiple peptides in biological samples [7]. Recombinant DNA technology has made available sufficient amounts of recombinant vasopressin [8].

Immunoassays are commonly used in clinical, pharmaceutical and chemical analyses for detection and quantification of trace analytes in biological fluids and other complex matrices [9,10]. These assays offer high selectivity for known determined analytes due to specific antibody/antigen binding. Conventional immunoassays usually use solid-phase techniques, in which case quantification is achieved by measuring either the radioactivity as in radioimmunoassay (RIA) or enzyme activity as in enzyme linked immunosorbent assay (ELISA). Recently, with the development of a radioimmunoassay, the physiological range of vasopressin has been established [11]. Radioimmunoassays (RIAs) are the method of choice for such measurements because of their specificity and good concentration detection limits; however, RIAs have some disadvantages including long analysis times, the ability to detect only one analyte per assay, and the potential for interference from cross-reactive species. For these latter problems, samples can be separated by HPLC and the fractions analyzed by RIA; however, this is a cumbersome method that results in loss of sensitivity due to the large sample volumes required. Usually, the amount of vasopressin is quantified by immunoassay using an antibody against vasopressin [12,13]. Although most immunoassays show a high selectivity and sensitivity, they are still labor intensive, requiring a number of incubating and washing steps that may take hours to complete. Recently, significant progress has been made in the development of rapid and sensitive immunoassays based on capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection [14,15]. CE–LIF immunoassay can be performed in a noncompetitive or a competitive format [16]. In a competitive CE immunoassay, a fluorescently-labeled antigen (Ag*) (tracer) competes with the antigen of interest (Ag) for binding to a limiting amount of antibody (Ab). Separation of the mixture by CE with laser-induced fluorescence detection (LIF) produces two distinct peaks corresponding to Ag* and Ag*–Ab complex, the intensities of which can be related to the original concentration of Ag [17]. CE-based immunoassays have mass sensitivity, speed, and an ease of automation that is superior to RIAs [18,19]. In addition, since a separation step is used for the isolation of bound and free tracer, multiple analytes can be detected in a single analysis. The majority of published competitive CE immunoassays have utilized monoclonal antibodies instead of polyclonal antibodies because monoclonals usually yield narrower antibody–antigen complex peaks in the electrophoresis separation [20]; however, monoclonal antibodies require extra preparation relative to polyclonal antisera and are not available for many targets. In a noncompetitive assay, the complex formed by the antigen with the labeled antibody must be separated from the excess labeled antibody. In our experiments, vasopressin has a low molecular mass (1.1 kD) and its binding to the much heavier antibody (150 kD) did not lead to any observable mobility difference between free antibody and complexed antibody. The separation of free antibody and complexed antibody was difficult in free zone capillary electrophoresis. Therefore, in this study we chose the CE-based competitive immunoassay.

The objective of this work is to develop a technique that combines the selectivity of immunoassays, the separation capability of CE, and the sensitivity of LIF for the quantitation of vasopressin to use in pharmacokinetics. A competitive immunoassay in which a tracer of fluorescence-labeled vasopressin is used to monitor the vasopressin, is demonstrated in this paper. Firstly, we show the preparation and purification of fluorescence-labeled vasopressin for CE–LIF detection and competitive immunoassay. We also present the fact that the pre-column competitive immunochemical reactions between vasopressin and antibody followed by the CE separation of fluorescence-labeled vasopressin from the antibody-bound vasopressin can be used to determine the amount of unlabeled vasopressin.

## 2. Materials and methods

### 2.1. Chemicals

Sodium tetraborate, boric acid, fluorescein iso-

thiocyanate (isomer I), vasopressin, Sephadex-25M gel column and trifluoroacetic acid were purchased from Sigma (St. Louis, MO, USA). Polyclonal anti-vasopressin (sheep IgG) was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). Acetonitrile and acetone were purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 2.2. Apparatus

Separations were carried out using a Beckman P/ACE 5000 capillary electrophoresis system (Beckman Coulter Instrument, Fullerton, CA, USA) equipped with a LIF detector (Beckman P/ACE System Laser Module 488; 488 nm excitation, 520 nm emission). Samples were injected by pressure at 0.5 p.s.i. for 5.0 s into an untreated fused-silica capillary column (27 cm×50 μm I.D.) from Polymicro technologies (Phoenix, AZ, USA) and separation at 20 °C using a running voltage of 10 kV. In between runs, pressure (20 p.s.i.) rinses with 1 *M* HCl (5 min), water (10 min), 0.5 *M* NaOH (10 min), and running buffer (10 min) were performed. Post-run analyses of data were performed using the System Gold chromatography data system, version 8.1 (Beckman). All HPLC separations were performed with a Vydac $C_{18}$ column (25 cm×2.2 mm I.D., W.R. Grace, Columbia, MD, USA). The samples were analyzed by a Waters 550 RP HPLC system with UV detector (Waters, Milford, MA, USA). A model V550, double-beam scanning spectrophotometer (Jasco, Tokyo, Japan) was used to measure absorbance of gel filtration fractions after fluorescence labeling.

### 2.3. Preparation and purification of FITC-labeled vasopressin

Reaction conditions were a modification of Cheng [21]. FITC (Molecular Probes, Eugene, OR, USA) was dissolved in acetone (1 mg/ml) and 10 μl of the FITC solution were added to a 1-ml solution containing a concentration of 2 mg/ml of vasopressin dissolved in 100 m*M* carbonate buffer, pH 9.0. Upon addition of FITC, the reaction vial was protected from light and allowed to mix for 2 h at room temperature with gentle stirring. The mixture was passed through a Sephadex 25M column pre-equilibrated with phosphate buffer saline (PBS), pH 7.4, to remove low molecular mass components and free FITC. After the appropriate fractions were collected, the samples were monitored by the absorbance at 280 nm by spectrophotometry. The appropriate FITC–vasopressin peaks were then purified by reversed-phase HPLC [22]. All HPLC separations were performed with a Vydac $C_{18}$ column. The following gradients were used on all HPLC determinations: 0–5 min (5% to 10% B), 10–35 min (10% to 60% B), and 35–40 min (100% B), where A was 0.1% trifluoroacetic acid (TFA) in water and B was 0.1% TFA in 90% acetonitrile. Peaks were monitored by UV absorbance at 214 nm. Purified FITC–vasopressin fractions from HPLC were evaporated under a gentle flow of nitrogen, and reconstituted with 500 μl of PBS buffer, pH 7.4. The FITC–vasopressin samples were then divided into 15-μl portions and kept frozen until use.

### 2.4. Formation of the vasopressin–anti-vasopressin complex

FITC–vasopressin, vasopressin, and anti-vasopressin antibody solutions were diluted to the appropriate concentrations with 100 m*M* PBS, pH 7.4. Fifteen microliters each of FITC–vasopressin and anti-vasopressin antibody solution were mixed in a 0.5-ml microcentrifuge tube and incubated at room temperature for 20 min before injection for the CE–LIF assay of vasopressin. To perform a competitive assay, 15 μl of 100 n*M* FITC–vasopressin were mixed with 15 μl of 500 and 1000 ng/ml vasopressin. To each were added 15 μl of 0.2 mg/ml polyclonal antibody. After 20 min of incubation at room temperature, the samples were analyzed by CE.

## 3. Results and discussion

### 3.1. Preparation and purification of FITC-labeled vasopressin

In the CE-based competitive immunoassay, fluorescent-labeled antigen and antibody were added

in specific amounts to the sample to be analyzed. The labeled antigen competed with the unlabeled antigen for the limited number of antibody binding sites. After equilibrium was established in the free solution, a small volume of the incubate was injected into the capillary, whereupon free and bound labeled antigen are separated by CE and quantitated by measurement of fluorescence intensity. In the CE–LIF-based immunoassay, chemical derivation is performed independently before the labeled peptide is used in the assay. While precolumn fluorescent labeling is useful in amino acid and DNA analysis, it is not always useful in peptide analysis [23]. Because most peptides and proteins have multiple reactive sites with fluorescent reagents, the labeling reaction produces a complex mixture of products. After unlabeled free FITC dye was eliminated using a Sephadex-25M gel column, the appropriate FITC–vasopressin fraction was analyzed by CE. As we expected, the result showed multiple peaks. However, in order to accomplish a successful CE–LIF competitive immunoassay, homogeneous labeled antigen (Ag*) was required. Therefore, in order to obtain a sufficiently homogeneous labeled antigen, we purified labeled antigen by reversed-phase HPLC [22]. As shown in Fig. 1A, homogeneous labeled fluorescent vasopressin was obtained.

### 3.2. CE–LIF-based immunoassay for vasopressin

For the CE–LIF-based immunoassay, 15 μl of FITC–vasopressin were added to 0.2 mg/ml of anti-vasopressin and incubated at room temperature for 10 min before injection. CE was performed with 80 m*M* borate buffer (pH 8.9) containing 19 m*M* NaCl and 50 m*M* SDS in an uncoated capillary at a voltage of 10 kV. With LIF detection, only the free FITC–vasopressin and immunocomplex containing FITC–vasopressin were revealed in the electropherograms. Fig. 2A shows the free FITC–vasopressin was well separated from the complex in a run time of 7 min. The addition of anti-vasopressin antibody to FITC–vasopressin resulted in the forma-

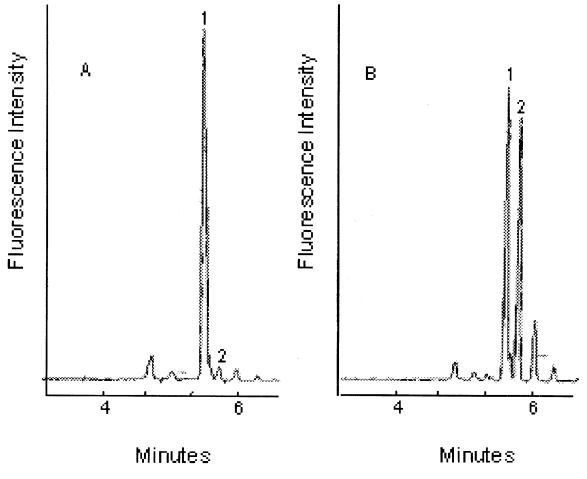


Fig. 1. Electropherograms of (A) FITC-labeled vasopressin and (B) FITC-labeled vasopressin spiked with FITC. Peaks: 1, FITC-labeled vasopressin; 2, FITC. Conditions: 27 cm×50 μm, 80 m*M* borate buffer (pH 8.9) containing 19 m*M* NaCl and 50 m*M* SDS, 10 kV, LIF detection.

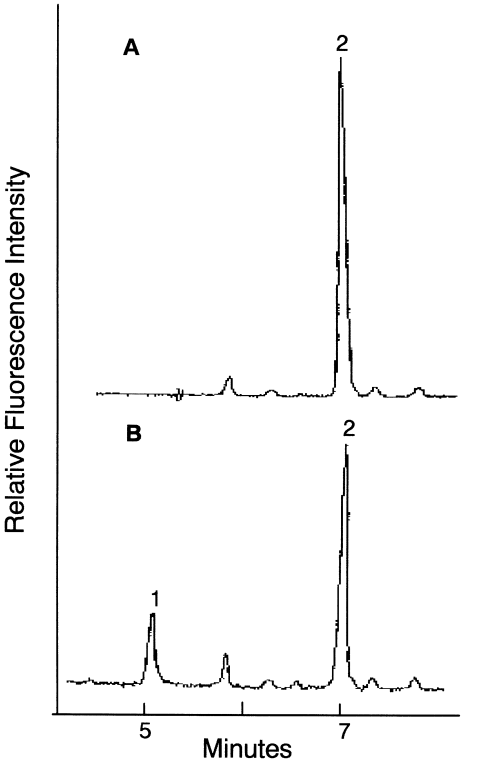


Fig. 2. CE–LIF electropherograms of (A) 100 n*M* FITC-labeled vasopressin; (B) mixture of 100 n*M* FITC-labeled vasopressin and polyclonal anti-vasopressin antibody. Peaks: 1, immunocomplex; 2, free-labeled vasopressin. CE conditions as in Fig. 1.

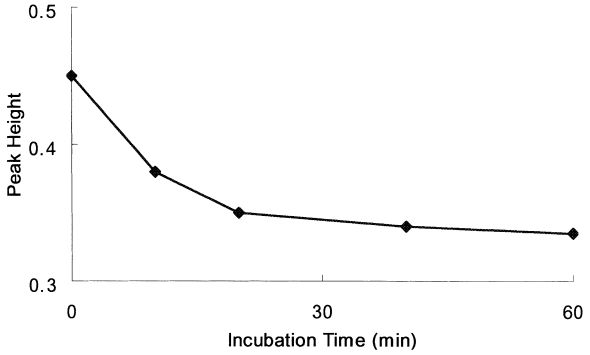


Fig. 3. Peak height of free FITC–vasopressin as a function of the incubation time. Components were mixed as in Fig. 2. CE conditions as in Fig. 1.

tion of multiple immunocomplex peaks at 5 min and a decrease in the FITC–vasopressin peaks at 7 min. Other investigators have reported that antibody–antigen binding remains unaffected by up to 75 m*M* SDS [24] even though SDS has the potential to open the tertiary structure of a protein. In our experiments, we used 50 m*M* SDS in running buffer and our results indicate that 50 m*M* SDS in the running buffer facilitates satisfactory separation of the compounds of interest.

### *3.3. Effect of incubation time on complex formation*

Vasopressin and anti-vasopressin were incubated for 10 min in the above experiments. In order to determine the effect of incubation time on CEIA in this system, vasopressin and anti-vasopressin solution were incubated at room temperature for 0, 10, 20, 40 and 60 min before injection into the capillary. As shown in Fig. 3, decrease of the free FITC–vasopressin peak can be observed with the increase of incubation time. However, there was little difference in peak height of the free FITC–vasopressin at incubation times longer than 20 min. This indicates that equilibrium is reached in about 20 min.

### *3.4. Competitive CE–LIF-based immunoassay in uncoated capillary*

Competitive immunoassay of vasopressin was performed with 100 n*M* of FITC–vasopressin and various concentrations of vasopressin competing to

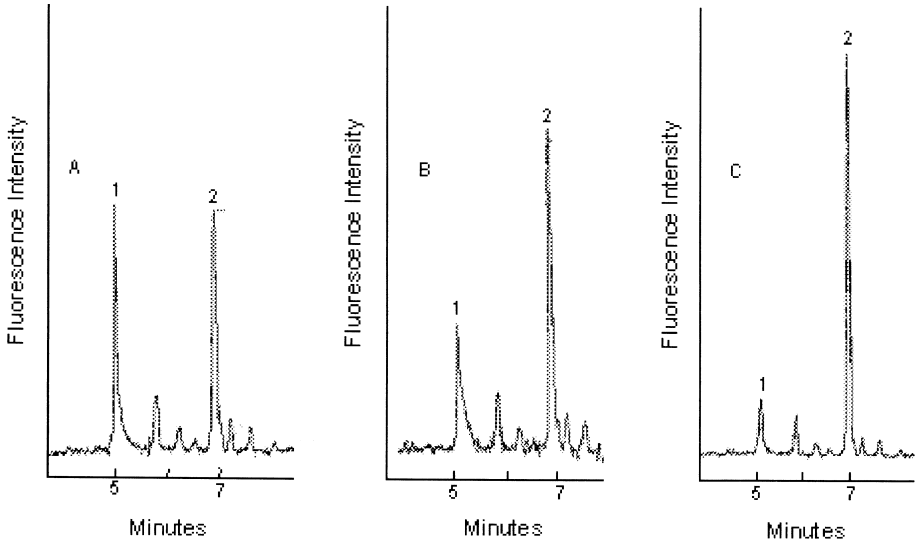


Fig. 4. Competitive immunoassay based on CE–LIF of vasopressin in buffer. Each sample contained 100 n*M* of FITC-labeled vasopressin and polyclonal anti-vasopressin antibody. (A) Vasopressin 0 ng/ml; (B) vasopressin 500 ng/ml; (C) vasopressin 1000 ng/ml. Peaks: 1, immunocomplex; 2, free-labeled vasopressin. CE conditions as in Fig. 1.

complex with 0.2 mg/ml of anti-vasopressin antibody. Both free and bound labeled antigen followed the behavior expected of competitive immunoassays. As the concentration of unlabeled vasopressin increased, the peak height and area of the immunocomplex in the electropherogram decreased while that of the free FITC–vasopressin increased (Fig. 4). Migration time reproducibility of free labeled vasopressin and immunocomplex, given in terms of percent relative standard deviation (RSD), was 4.5% and 5.1%, respectively. The results presented describe a method to label vasopressin with fluorescin and use of immunoassay method to analyze the peptides using CE–LIF. The CE–LIF-based competitive immunoassay proved to be a sensitive method detecting vasopressin in the n*M* range. Furthermore, the speed and separation power of CE has the advantages of reducing reagent consumption, simplification of assay methodology by elimination of washing steps, and potentially providing multianalyte and multilane capabilities. This assay can be easily modified for the detection of other peptides and proteins. Based on our result, we believe that CE-based immunoassay should prove to be a valuable analytical resource for bioresearch, biodiagnostics, and the biopharmaceutical industry.

## Acknowledgements

The authors wish to thank Miss Bae for her helpful comments on the manuscript. This work was supported in part by a grant from KIST (A studies on Metabolomics; 2E17500) and a grant from the Brain Science Research Group from the Ministry of Science and Technology (2N23950).

## References

[1] E.B. Verney, Proc. R. Soc. Lond. Ser. B 18 (1997) 292.
[2] E.M. Stricker, J.G. Verbalis, in: M.J. Zigmond et al. (Ed.), Fundamental Neuroscience, 1999.
[3] R. Acher, Gen. Comp. Endocrinol. 102 (1996) 157.
[4] R. Acher, J. Chauvet, Front. Neuroendocrinol. 16 (1995) 237.
[5] S. Nielson, P. Agre, Kidney Int. 48 (1995) 1057.
[6] P.C. Emson, Prog. Neurobiol. 13 (1979) 61.
[7] T.E. Robinson, J.B. JusticeJr. (Eds.), Microdialysis and the Neurosciences, Techniques in the Behavioral and Neural Sciences, Vol. 7, Elsevier, Amsterdam, 1991.
[8] H. Gainer, R.L. Fields, S.B. House, Exp. Neurol. 171 (2001) 190.
[9] D.E. Rebeski, E.M. Winger, Y.-K. Shin, M.L. Lelenta, M.M. Robinson, R. Varecka, J.R. Crowther, J. Immunol. Methods 226 (1999) 85.
[10] J.R. Crowther, ELISA; Theory and Practice, Humana Press, New Jersey, 1995.
[11] I. Heuser, G. Bissette, M. Dettling, U. Schweiger, U. Gotthardt, J. Schmider, C.-H. Lammers, C.B. Nemeroff, F. Holsboer, Depress. Anxiety 8 (1998) 71.
[12] C.A. Ecelbarger, G.-H. Kim, J.B. Wade, M.A. Knepper, Exp. Neurol. 171 (2001) 227.
[13] J.M. Warne, K.E. Harding, R.J. Balment, Comp. Biochem. Phys. Part B 132 (2002) 231.
[14] N.M. Schulz, R.T. Kennedy, Anal. Chem. 65 (1993) 3161.
[15] L. Tao, R.T. Kennedy, Electrophoresis 18 (1997) 112.
[16] I. German, R.T. Kennedy, J. Chromatogr. B 742 (2000) 353.
[17] N.M. Schultz, R.T. Kennedy, Anal. Chem. 65 (1993) 3161.
[18] R.T. Kennedy, L. Tao, N.M. Schultz, D.R. Rose, in: J. Landers (Ed.), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1997, p. 523.
[19] J.J. Bao, J. Chromatogr. B 699 (1997) 463.
[20] M.J. Schmerr, A.L. Jenny, Electrophoresis 19 (1998) 409.
[21] Y.-F. Cheng, N.J. Dovichi, Science 242 (1988) 562.
[22] N.G. Hentz, J.M. Richardson, J.R. Sportsman, J. Daijo, G.S. Sittampalam, Anal. Chem. 69 (1997) 4994.
[23] J.Y. Zhao, K.C. Waldron, J. Miller, J.Z. Zhang, H. Harke, N.J. Dovichi, J. Chromatogr. 608 (1992) 239.
[24] L. Steinman, J. Caslavska, W. Thormann, Electrophoresis 16 (1995) 1912.