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Competitive immunoassay for recombinant hirudin using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A competitive immunoassay based on capillary electrophoresis (CE) with laser-induced fluorescence (LIF) has been developed for the determination of recombinant hirudin (r-hirudin) in biological mixtures. Hirudin, a thrombin inhibitor, is a polypeptide of 65 amino acids. To check purity levels and perform pharmacokinetic studies of (r-hirudin), specific and reproducible analysis methods are demanded. The work involved the development of separation conditions allowing for routine analysis of plasma samples. In this study, r-hirudin was labeled with fluorescein isothiocyanate (FITC), and FITC-labeled r-hirudin was purified using high-performance liquid chromatography. The purified product was then mixed with the sample followed with the addition of anti-hirudin antibody. Free, antibody-bound, and tagged r-hirudin could be separated within 5 min by CE analysis using uncoated fused-silica capillary with high reproducibility. The developed method can be used to determine r-hirudin with good precision and a detection limit lower than 20 n*M*. This result demonstrates the feasibility of the CE–LIF immunoassay method for the determination of r-hirudin in plasma samples.

Keywords: Immunoassays; Hirudin; Polypeptides

1. Introduction

Hirudin is a thrombin-specific inhibitor first isolated from the medicinal leech, *Hirudo medicinale*, which has a molecular mass of 6797 and is composed of 65 amino acids with an isoelectric point (p*I*) of about 4.3 [1,2]. Recombinant DNA technology has made available sufficient amounts of recombinant (r-) hirudin. Today, r-hirudin is being studied as a potential anticoagulant and antithrombotic agent through clinical trials [3]. Therefore, to check purity levels and perform pharmacokinetic studies of r-hirudin, specific and reproducible analysis methods are demanded. Usually, the amount of r-hirudin is quantified by immunoassay [4–6] using an antibody against r-hirudin. Immunoassay is an analytical method that uses antibodies or antibody-related reagents for the determination of sample components [7]. The selective nature of antibody binding allows these reagents to be employed in the development of methods that are highly specific and that can often be used directly on even complex biological matrices such as blood, plasma, or urine. By combining the selectivity of antibody–analyte interactions with the vast array of antibodies that can be produced in nature and the availability of numerous readily detectable labels (e.g., radioisotopes or enzymes), such as radioimmunoassays (RIAs) and enzyme-linked immunosorbent assay (ELISA), immunoassays can be designed for a wide variety of analytes

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while also providing low limits of detection. These characteristics, along with the relatively low cost generally associated with these methods, have continued to make immunoassays a popular method in many clinical and pharmaceutical applications. Nevertheless, there are several disadvantages because most conventional immunoassays are carried out manually with multiple incubation, washing and rising steps that may take hours to complete.

Capillary electrophoresis (CE) has proven to be a powerful separation tool and is therefore being used as a useful separation method for rapid and efficient immunoassays [8,9]. Also, CE, combined with appropriate labeling techniques and laser-induced fluorescence (LIF) detection, can provide high sensitivity. Therefore, immunoassay based on CE–LIF can perform rapid separations with high detection sensitivity, and is capable of determining multiple analytes simultaneously. In addition, only a minute amount of sample ranging from 10 to 20 μl is required. Immunoassay based on CE–LIF has also been applied to some drugs [9] and biological hormones [10], such as insulin and human growth hormone [11].

Immunoassay based on CE–LIF can be performed in a noncompetitive or a competitive format [12]. In a competitive assay, a fluorescently labeled antigen competes with antigen for binding to a limited amount of antibody. CE–LIF separation of the mixture produces two distinct fluorescent peaks corresponding to each labeled antigen and labeled antigen–antibody complex, the intensities of which are related to the original concentration of unlabeled antigen. 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, r-hirudin has a low molecular mass (6900) and its binding to the much heavier antibody (150 000) 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 CE. Therefore, in this study, immunoassay based on CE–LIF was performed in a competitive format.

The focus of this work is to develop a technique that combines the selectivity of immunoassay, the separation of CE, and the sensitivity of LIF for the detection of r-hirudin in plasma. A competitive assay in which a tracer of fluorescently labeled r-hirudin is used to monitor the r-hirudin will be demonstrated in this paper. The CE separation of the labeled tracer from antibody complexes eliminates the washing steps common to ELISA, thus reducing the labor and time necessary for the assay.

2. Experimental

2.1. Apparatus

All experiments were performed on an automated A P/ACE 5000 CE system (Beckman Instrument, Fullerton, CA, USA) fitted with an LIF detector. The 488 nm line of a 5 mW argon ion laser detector was utilised as the excitation source of the LIF detector, and the emitted fluorescence was collected at 520 nm. An untreated fused-silica capillary column of 37 cm (effective length 30 cm)×50 μm I.D. was from Beckman Instrument. Sample injections were performed under pressure for 5.0 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). The applied voltage was 24 kV and the current was 82 μA. Data were analyzed using Beckman System Gold software, version 8.1.

2.2. Chemicals and reagents

Fluorescein isothiocyanate (FITC), sodium tetraborate, boric acid, trifluoroacetic acid (TFA) and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). Polyclonal anti-hirudin (sheep IgG) was obtained from Cedarlane Labs. (Hornby, Canada). Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Recombinant hirudin was kindly provided by the Doping Control Center (Seoul, South Korea). All solutions were prepared with deionised water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

r-Hirudin was labeled with FITC using the Fluoro Tag FITC conjugation Kit from Sigma. The labeled peptide was purified with column chromatography (Sephadex G-25M), and the elution buffer was 10 m*M* sodium phosphate-buffered saline (PBS; 10 m*M* sodium phosphate, 138 m*M* NaCl, 27 m*M* KCl, pH 7.4). The purified fraction was stored at 4°C until use.

2.3. Purification of FITC-labeled r-hirudin

The appropriate FITC–r-hirudin peaks were then purified by reversed-phase high-performance liquid chromatography (HPLC), using a Vydac C_{18} column [13]. The LC mobile phase composition was a mixture of 0.1% TFA in acetonitrile and 0.1% TFA in water and peaks were monitored by UV absorbance at 220 nm. Purified FITC–r-hirudin fractions were lyophilised, and reconstituted with PBS buffer, pH 7.4. The FITC–r-hirudin samples were then divided into 5-μl portions and kept frozen until use.

2.4. Immunoassay protocol

FITC–r-hirudin, r-hirudin, and anti-hirudin solutions were diluted to the appropriate concentrations with 10 m*M* PBS, pH 7.4. A 15-μl volume each of FITC–r-hirudin and anti-hirudin solution were mixed in a 0.5-ml microcentrifuge tube and incubated at room temperature for 20 min before injection for CE–LIF assay of r-hirudin. To perform competitive assay, 15 μl of a fixed amount of FITC–r-hirudin was mixed with 15 μl of varying amounts of r-hirudin. To each was added 15 μl of fixed amount of polyclonal antibody. These mixtures were allowed to incubate at room temperature for 20 min and were analyzed by CE.

2.5. Electrophoretic conditions

Electrophoresis was performed at 25°C using 7 m*M* sodium borate and 15 m*M* sodium phosphate (pH 9.1) containing 50 m*M* SDS to reduce analyte–wall interaction. The samples were pressure injected for 5 s and a 37 cm (effective length 30 cm)×50 μm I.D. untreated fused-silica capillary column was used. To obtain the reproducible results, the capillary was rinsed with water (10 min), 25 m*M* phosphate buffer, pH 7.0, containing 60 m*M* SDS (5 min), and running buffer (10 min). Capillary column washing by SDS is known to be more effective than NaOH or HCl in removing protein from the capillary column inner walls [14]. The applied voltage was 24 kV and the current was 82 μA.

3. Results and discussion

3.1. Labeling and purification of r-hirudin

The technique of using CE to study competitive immunoassays has been applied to various peptides, and its sensitivity was greatly improved with the use of a fluorescence labeled tracer. It is very important to use a pure tracer in order to obtain a sensitive immunoassay because an impure tracer is prone to increase nonspecific binding, which results in an elevated detection limit [9,15,16]. However, there is a difficulty in making peptides and proteins with multiple primary amino groups (lysines) into a homogeneous labeled antigen. FITC labeling of r-hirudin which had four lysine residues in addition to the N-terminal valine residue resulted in multiple fluorescent products as shown in Fig. 1A. Therefore,

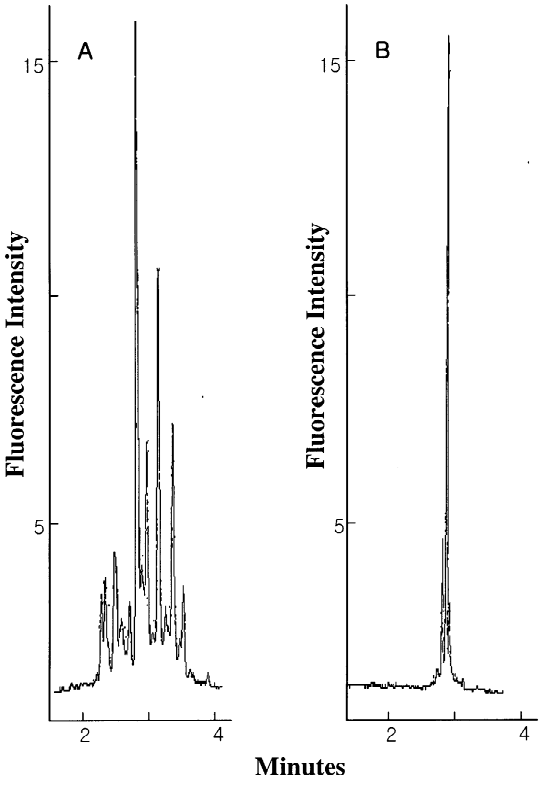

Fig. 1. CE monitoring of FITC–r-hirudin: (A) unpurified FITC-labeled r-hirudin; (B) purified FITC-labeled r-hirudin. 37 cm×50 μm I.D. untreated fused-silica capillary, 7 m*M* borate and 15 m*M* phosphate buffer (pH 9.1) containing 50 m*M* SDS, 24 kV, LIF detection (excitation, 488 nm/emission, 520 nm).

in order to obtain a sufficiently homogeneous labeled antigen, we purified labeled antigen by reversed-phase HPLC. The purity of FITC–r-hirudin was checked by CE–LIF and we conformed a sufficiently homogeneous FITC–r-hirudin peak in Fig. 1B.

3.2. Optimisation of the immunoassay based on CE–LIF detection

In an immunoassay based on the CE–LIF system, analysis time is important because the complex is not stable within the long separation time [10]. Therefore, during the course of the slower run, the complex dissociates, giving no peak or poorly formed peaks for the complex. The separation time is shorter when using a more narrow capillary with higher electric field strength, causing the dissociation of the complex to be lowered during the course of the run. Therefore, the separation was performed in a 50 μm I.D. capillary to avoid heating problems associated with high strength in a 75 μm I.D. capillary. The application of CE to biofluid samples is more difficult due to the adsorption of sample components to the capillary wall, which affect the reproducibility of the assay [17]. In order to overcome such problems, we conducted studies using micellar electrokinetic chromatography method with SDS. Therefore, CE was performed with 7 m*M* sodium borate and 15 m*M* sodium phosphate buffer (pH 9.1) containing 50 m*M* SDS in an uncoated capillary (37 cm×50 μm I.D.) at a voltage of 24 kV. In our immunoassay based on the CE–LIF system, 15 μl of $17.8 \cdot 10^{-7}$ *M* FITC–r-hirudin were added to $6.7 \cdot 10^{-7}$ *M* of anti-hirudin antibody and incubated at room temperature for 10 min before injection for CE–LIF analysis. Under the above conditions, with LIF detection, the free FITC–r-hirudin peak and immunocomplex containing FITC–r-hirudin peak were detected (Fig. 2). The electropherogram of FITC–r-hirudin alone in Fig. 2A showed a single peak, confirming that FITC–r-hirudin was homogeneous. Addition of anti-hirudin antibody resulted in the decrease of the FITC–r-hirudin peak and the formation of the immunocomplex peak in Fig. 2B. These two peaks were well separated within 3.5 min. The asymmetric peak that corresponded to immunocomplex is likely due to the binding of the antibody

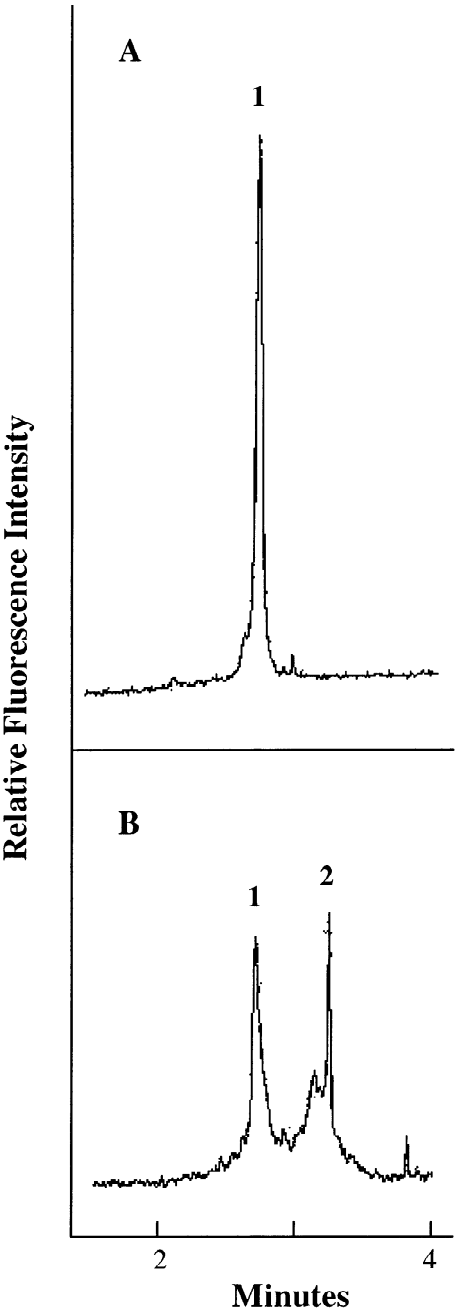

Fig. 2. CE–LIF electropherograms of (A) $17.8 \cdot 10^{-7}$ *M* FITC-labeled r-hirudin; (B) mixture of $17.8 \cdot 10^{-7}$ *M* FITC-labeled r-hirudin and $6.7 \cdot 10^{-7}$ *M* polyclonal anti-hirudin antibody. Peaks: 1=free FITC-labeled r-hirudin; 2=immunocomplex. CE conditions as in Fig. 1.

to one or two FITC–r-hirudins because of the microheterogeneity of antibody [11].

Before a successful competitive immunoassay based on CE–LIF can be accomplished, the concentration of labeled antigen and antibody must be determined. It has also been shown that the best detection limit of competitive assays is achieved with a minimal amount of fluorescent tracer that competes for the limiting amounts of antibody [18]. To balance these two criteria, titrations of the antibody and the tracer were performed to assess the optimal concentrations of these two reagents.

The titration of the anti-hirudin antibody was done using a fixed concentration of $8.9 \cdot 10^{-7}$ *M* dilution of the original FITC–r-hirudin. The dilution was chosen as it was the lowest concentration level to give a signal that could be easily distinguished from the background. Fig. 3 shows the titration curve plotted from solutions containing a fixed amount of FITC-labeled r-hirudin and varied amounts of antibody. With increasing amount of antibody in sample mixture, unbounded free FITC–r-hirudin peak height decreases gradually, which is accompanied by an increase of the immunocomplex peak. The free FITC–r-hirudin peak height did not show a clear decrease at anti-hirudin antibody concentrations higher than $6.7 \cdot 10^{-7}$ *M*. Assuming the plateau region reflects almost 100% binding of the FITC–r-hirudin, antibody concentration of $6.7 \cdot 10^{-7}$ *M* would be reasonable starting concentrations for the assay. Starting with an antibody concentration of $6.7 \cdot 10^{-7}$ *M*, a titration of the FITC–r-hirudin was carried out.

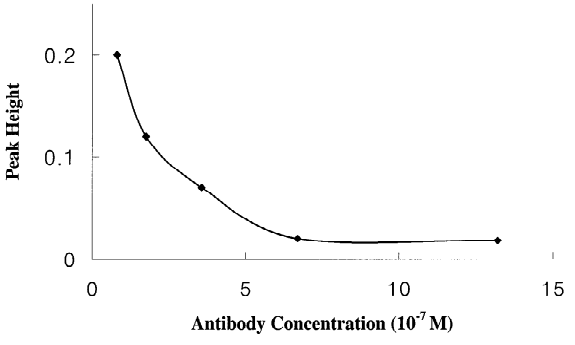

Fig. 3. Titration curve of the peak heights of free FITC–r-hirudin verse anti-hirudin antibody concentration. Increasing amounts of anti-hirudin antibody were added to an aliquot of $8.9 \cdot 10^{-7}$ *M* FITC-labeled r-hirudin. CE conditions as in Fig. 1.

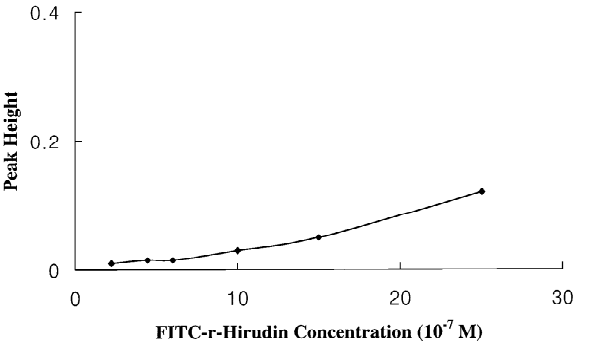

Fig. 4. Titration curve of the peak heights of unbounded FITC–r-hirudin versus FITC-labeled r-hirudin. Increasing amounts of FITC-labeled r-hirudin were added to an aliquot of $6.7 \cdot 10^{-7}$ *M* anti-hirudin antibody. CE conditions as in Fig. 1.

Fig. 4 shows that the titration curve of the peak height of free FITC–r-hirudin was according to the increase FITC–r-hirudin concentration. As shown in Fig. 4, the peak heights of free FITC–r-hirudin with FITC–r-hirudin concentration increase were nearly zero without significant change at concentration lower than $5.0 \cdot 10^{-7}$ *M*. However, when the concentration was higher than $5.0 \cdot 10^{-7}$ *M*, the peak height of free FITC–r-hirudin increased with the increase of FITC–r-hirudin concentration. The deviation of the curve from a straight line parallel to the *x*-axis at around $5.0 \cdot 10^{-7}$ *M* is due to the increase in free FITC–r-hirudin by the completion of binding of FITC–r-hirudin and anti-hirudin antibody.

FITC–r-hirudin and anti-hirudin antibody were incubated for 10 min in the above experiments. In order to determine the effect of incubation time in this system, FITC–r-hirudin and anti-hirudin antibody solution were incubated at room temperature for 0, 10, 20, 40 and 60 min before injection into the capillary. Fig. 5 shows a plot of resulting in free FITC–r-hirudin peak height as a function of incubation time. As the reaction of antibody with FITC-labeled r-hirudin proceeds, the increase of immunocomplex peaks and consequently, free FITC–r-hirudin decreases with incubation time. After 20 min of incubation time the free FITC–r-hirudin shows no outstanding decrease, indicating equilibration of the reaction and that antibodies can no longer attach after that incubation time.

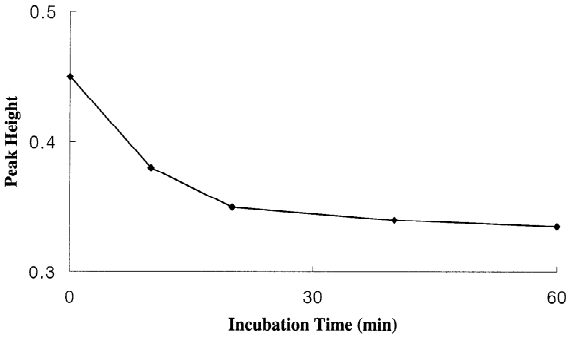

Fig. 5. Immunocomplex formation as a function of the incubation time. Components were mixed as shown in Fig. 2B and analyzed by CE immediately after incubation at room temperature for the periods time of indicated. The points display the peak height of free FITC–r-hirudin. CE conditions as in Fig. 1.

3.3. Competitive immunoassay based on CE–LIF for r-hirudin

Since r-hirudin has a low molecular mass (6900) and its binding to the much heavier antibody (150 000) did not lead to any observable mobility difference between free antibody and complexed antibody, immunoassay based on CE–LIF was performed in a competitive manner using FITC–r-hirudin as tracer. For the assay, $17.8 \cdot 10^{-7}$ *M* of FITC–r-hirudin and $6.7 \cdot 10^{-7}$ *M* of anti-hirudin antibody were used as the starting reagents for the assay. Although the lower limit of detection would be located at FITC–r-hirudin concentration of $5 \cdot 10^{-7}$ *M*, a better working range would lie where the concentration of FITC–r-hirudin is higher than $5 \cdot 10^{-7}$ *M*. Fig. 6 shows electropherograms obtained when the competitive immunoassay based on CE was performed for the determination of r-hirudin in blank plasma and plasma spiked with r-hirudin at concentrations of 100 and 400 ng/ml. At an applied field strength of 648 V/cm, the free labeled antigen was well separated from the complex in a run of 3.5 min. Both free and bound labeled antigen follow the behavior expected of competitive assays; as increase in the amount of r-hirudin led to a decrease in signal for the complex and an increase in signal for the free labeled antigen. No clean-up of the plasma samples prior to the CE runs was required, and the plasma was injected without further dilution. As can be seen,

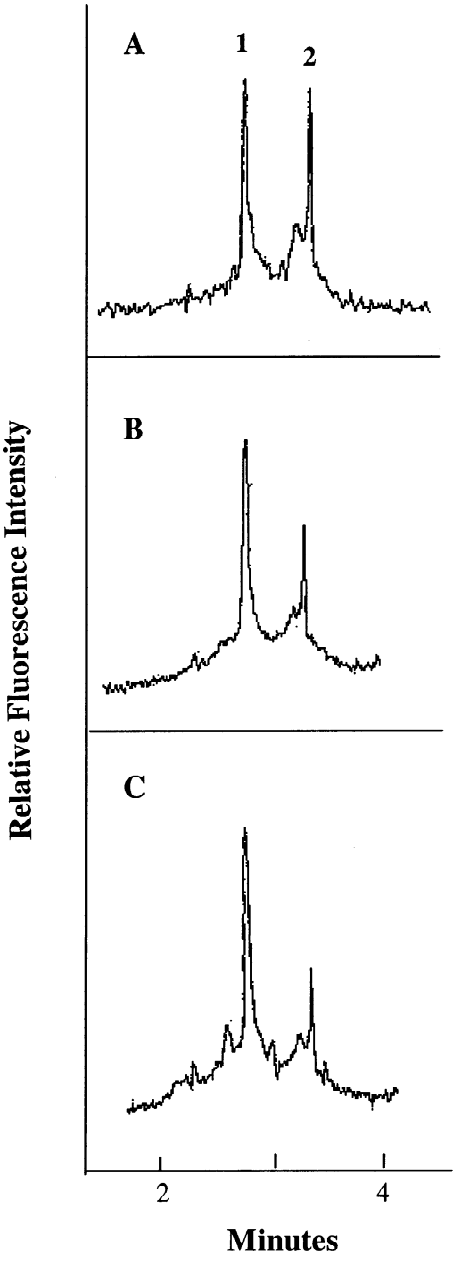

Fig. 6. Competitive immunoassay based on CE–LIF of r-hirudin in undiluted plasma. Each sample contained $6.7 \cdot 10^{-7}$ *M* of anti-hirudin antibody and $17.8 \cdot 10^{-7}$ *M* of FITC-labeled r-hirudin. (A) r-Hirudin 0 ng/ml; (B) r-hirudin 100 ng/ml; (C) r-hirudin 400 ng/ml. Peaks: 1=free FITC-labeled r-hirudin; 2=immunocomplex. CE conditions as in Fig. 1.

the high salt and protein content of plasma did not have an adverse effect on separations. A calibration curve was generated by competitive immunoassay plasma containing increasing concentration of r-hirudin. We constructed the calibration curve for r-hirudin by plotting the peak heights of free FITC–r-hirudin against the r-hirudin concentrations within a 14.2–114.4 n*M* range of r-hirudin without an internal standard (Fig. 7). The calibration curve shows the sigmoidal shape typical of concentration response curves of competitive immunoassay. Although competitive assay inherently yields nonlinear calibration plots, it is possible to fit our data to linear models at the 10–80 n*M* concentration range. The linear approximation was feasible because of the relatively small concentration range that was used.

The reproducibility of migration time and peak height and detection limit were tested. The relative standard deviations (RSDs) for plasma samples with 100 ng/ml and 500 ng/ml concentrations of r-hirudin were studied. Migration time RSDs for 100 ng/ml and 500 ng/ml were 0.1% and 0.2%, respectively. Peak height RSDs for the two points were 5.6% and 4.0%, respectively. Therefore the reproducibility can be considered to be satisfactory for quantitative and qualitative analysis.

The limit of detection (LOD) for r-hirudin was calculated from electropherograms obtained after sample preparation of plasma spiked with r-hirudin. The limit of detection of this method was found to be 14.2 n*M* for r-hirudin.

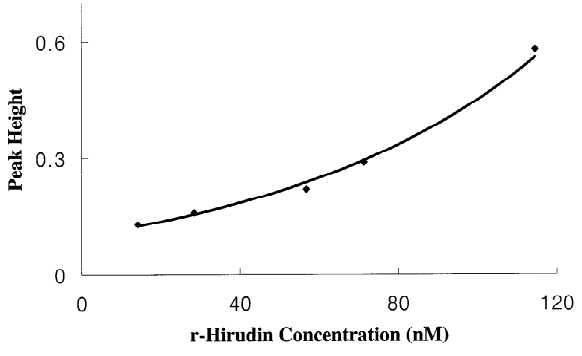

Fig. 7. Calibration curve for r-hirudin in plasma measured by competitive immunoassay based on CE–LIF. The presented data is based upon peak heights of free FITC–r-hirudin.

4. Conclusion

In the present work, we developed a method for the determination of r-hirudin in plasma using a competitive immunoassay based on CE–LIF. CE–LIF was performed in an uncoated fused-silica capillary (37 cm×50 μm I.D.) with 7 m*M* borate and 15 m*M* phosphate buffer (pH 9.1) with 50 m*M* SDS. The adsorption of proteins to the capillary walls is prevented and the immunocomplex is stable in presence of SDS [19]. The assay requires very little sample preparation of 10 μl, minimal incubation time of 20 min, and short analysis time of 3.5 min. The results indicate that immunoassay based on CE–LIF allow for rapid and quantitative determination of analytes in complex sample matrices such as plasma. Therefore, we believe that this assay method could be applied to quantify r-hirudin in biofluids for pharmacokinetic studies.

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References

[1] F. Markwardt, Haemostasis 21 (1991) 11.
[2] A. Bossi, P.G. Righetti, C. Visco, U. Breme, M. Mauriello, B. Valsasina, G. Orsini, E. Wenisch, Electrophoresis 17 (1996) 932.
[3] V. Steiner, R. Knecht, O. Börnsen, E. Gassmann, S.R. Stone, F. Raschdorf, J.-M. Schlaeppi, R. maschler, Biochemistry 31 (1992) 2294.
[4] S. Spinner, F. Scheffauer, R. Maschler, G. Stöffler, Thromb. Res. 51 (1988) 617.
[5] L. Iyer, J. Shavit, M. Koza, R. Calabria, S. Moran, J. Fareed, Thromb. Res. 69 (1993) 59.
[6] L. Iyer, M. Adam, J. Amiral, J. Fareed, E. Bermes Jr., Semin. Thromb. Hemost. 21 (1995) 184.
[7] D.S. Hage, Anal. Chem. 65 (1993) 420R.
[8] J.J. Bao, J. Chromatogr. B 699 (1997) 463.
[9] D. Schmalzing, W. Nashabeh, M. Fuchs, Clin. Chem. 41 (1995) 1403.
[10] N.M. Schultz, R.T. Kennedy, Anal. Chem. 65 (1993) 3161.
[11] K. Shimura, B.L. Karger, Anal. Chem. 66 (1994) 9.
[12] D. Schmalzing, S. Buonocore, C. Piggee, Electrophoresis 21 (2000) 3919.

[13] N.G. Hentz, J.M. Richardson, J.R. Sportsman, J. Daijo, G.S. Sittampalam, Anal. Chem. 69 (1997) 4994.
[14] B. Verzola, C. Gelfi, P.G. Righetti, J. Chromatogr. A 868 (2000) 85.
[15] F.-T.A. Chen, R.A. Evangelista, Clin. Chem. 40 (1994) 1819.
[16] T.J. Pritchett, R.A. Evangelista, F.-T.A. Chen, J. Cap. Electrophoresis 2 (1995) 145.
[17] W. Thormann, S. Molteni, J. Caslavska, A. Schmutz, Electrophoresis 15 (1994) 3.
[18] J.P. Ou, Q.G. Wang, T.M. Cheung, S.T.H. Chan, W.S.B. Yeung, J. Chromatogr. B 727 (1999) 63.
[19] V.M. Okun, B. Ronacher, D. Blaas, E. Kenndler, Anal. Chem. 72 (2000) 4634.