Arch Pharm Res Vol 24, No 6, 601-606, 2001

Archives of Pharmacal Research
http://apr.psk.or.kr

Determination of Recombinant Human Epidermal Growth Factor (rhEGF) in a Pharmaceutical Preparation by Capillary Electrophoresis

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(Received August 21, 2001)

A simple assay method of recombinant human epidermal growth factor (rhEGF) in a pharmaceutical preparation was studied and validated by capillary electrophoresis (CE) using micellar electrokinetic chromatography (MEKC) techniques. Factors affecting the migration behavior and separation performances of the peptide; type of buffer, pH, buffer concentration, and concentration of sodium dodecyl sulfates (SDS) were investigated to optimize the analytical performance. CE was performed using running buffer, 50.0 mM borate (pH 8.5) containing 12.5 mM SDS at 20 kV of the applied voltage. Calibration curves for the rhEGF showed good linearity ($r>0.999$) over the wide dynamic range from 1.25 to 100 μg/ml. Sample analysis was performed by using standard addition method to eliminate the matrix effects of dosage vehicle. This method is assumed to be useful for quality control (QC) of various forms of pharmaceutical products of the peptide.

Key words: Capillary electrophoresis, Recombinant human epidermal growth factor (rhEGF), Validation, Pharmaceutical preparation, Standard addition

INTRODUCTION

Human epidermal growth factor (hEGF), a single chain polypeptide containing 53 amino acid residues with three disulfide bonds (MW 6045), is detected in a variety of tissues and body fluids (Cohen, 1962; Starkey et al., 1975; Hirata & Orth, 1979 a,b; Yip *et al.*, 1986). hEGF possess a broad spectrum of biological activities including stimulation of cell proliferation in a variety of cell types and inhibition of gastric acid secretion (Carpenter *et al.*, 1979; Stoscheck *et al.*, 1986; Moonlenaar *et al.*, 1986). Exogenously administrated rhEGF has long been investigated for their potential activity to expedite wound healing process (Gregory & Morris, 1986; Schultz *et al.*, 1987; Chvapil *et al.*, 1988; Brown *et al.*, 1989; Okumura *et al.*, 1990; Schultz *et al.*, 1991).

Recombinant human epidermal growth factor (rhEGF1-53) was formulated such as ophthalmic and topical preparations for faster and better healing of wounds and burns (Arturson, 1984; Brown *et al.*, 1986; Singh *et al.*, 1987). Recently, a pharmaceutical preparation of rhEGF was developed for treatment of diabetic foot ulcers by a company (Daewoong Pharmaceutical Co., LTD, Korea) and licenced preparations are expected to come to market.

Capillary electrophoresis (CE) has become an useful separation technique in a wide variety of applications ranging from small inorganic ions to large biopolymers, especially to the analysis of peptides and proteins. An attractive approach to apply CE is straightforward: proteins and peptides often yield inefficient and broad peaks with HPLC; CE, on the other hand, could potentially serve as a complementary technique to HPLC with specific advantages of speed, resolution, and selectivity. This report first demonstrated the CE determination of rhEGF in a pharmaceutical preparation, and the assay method was validated.

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MATERIALS AND METHODS

Materials

Highly purified recombinant human EGF (rhEGF, more than 99% purity) prepared by means of recombinant DNA technology and expressed in *Escheria coli.* was pro-

vided by the Daewoong Pharm. Co. LTD., (Seoul, South Korea). Boric acid, sodium dodecyl sulfate (SDS), and benzyl alcohol were purchased from Sigma (St Louis, MO, USA). All other reagents were of analytical grade and used as received.

rhEGF preparation of spray solution (label claimed EGF 60,000 IU/ml) was used as analytical samples for assay. A standard formulation of rhEGF (50 μg/ml) was prepared using the formulation vehicles to validate the accuracy.

Standard stock solution (1.0 mg/ml) of rhEGF was prepared in a Millipore Milli-Q water and stored at -20°C for 3 months. Working standards of rhEGF (1.25-100 μg/ml) were prepared daily from stock solution with running buffer before each experiment. Analyte solutions were diluted with the same diluent and stored below 4°C. These peptide solutions were made in polypropylene or silanized glassware to prevent adsorption to glass surfaces. Benzyl alcohol, as a neutral marker in micellar electrokinetic chromatography (MEKC) mode and an internal standard, was spiked into the analyte solutions at the concentration of 0.002%.

Capillary electrophoresis

CE experiments were performed using a Spectra PHORESIS® 100 CE system (Thermo Separation Products, CA, USA) which equipped with a dc power supply capable of delivering up to 30 kV and on-column UV absorbance detection at 214 nm (Linear Model 200, NV, USA). A chromato-intergrator (Hitachi model D-2500, Tokyo, JAPAN) was used to record electropherograms for the quantitation work by peak area measurements. An uncoated fused-silica capillary tubing (Supelco model Celect-UVT 50) with an effective length 50 cm (total length of 75 cm) and 50 μm i.d. (360 μm o.d.) was used as a separation capillary column. Before each experiment, the capillary was rinsed with 0.1M NaOH for 3 min, deionized water for 3 min, and running buffer for 3 min by the built-in vaccum system. During the standard analysis, capillary was filled with 50 mM borate buffer containing 12.5 mM SDS (pH 8.5). Sample solution was introduced into the capillary from the anode side by applying vacuum for 3 seconds (which equivalent to 30 nl injection volume).

Assay procedure

Standard solution of rhEGF was spiked into four aliquots (each, 100 μl) of sample solution. The solutions were further made to 1.0 ml with running buffer to make the final concentration of spiked rhEGF 0-, 5-, 10-, and 15 μg/ml, respectively. The sample solutions with and without spiked rhEGF were analyzed successively on CE. Analytical results were obtained and estimated with standard addition method using an uncertainty program (a PUMA® program; KRISS, Daejon, Korea).

RESULTS AND DISCUSSION

In CE analysis of larger peptides and proteins, as compared with small peptides, factors that may affect mobility are hydrophobicity, primary sequence, conformational differences, and the chirality of amino acids aside from the mass-to-charge ratio. Therefore, the conditions affecting the migration behavior of rhEGF (pI 4.6) including type of buffer, electrolyte pH, applied voltage, concentration of both the running buffer and SDS were investigated and optimized.

Optimization of MEKC separation

The effect of pH. CE analysis of protein or peptide is preferably carried out by adjusting the pH of running buffer in the range of 8 to 11 (Lauer *et al.*, 1986) where both the uncoated silica surface and the peptides are negatively charged and electrostatic repulsion decreases adsorption phenomena. In CE, coulometric forces between sample ions and oppositely charged wall ions can slow down the separation. Higher pH of the running buffer increases the separation speed by increasing the mobility of buffer ions and the electrosomotic flow rate. Theoretically, resolution is improved when the electroosmotic flow (EOF) is suppressed and balanced against the electrophoretic migration.

Variation of the migration times of rhEGF was examined over the electrolyte pH range of 7.5 to 9.0 and applied voltages between 7.5 and 20 kV in 100 mM borate buffer without micelles (Fig. 1). The migration time slightly increased with the increase of pH, which is contrary to that would be expected on the basis of EOF increase. This means that the more negative charge over the peptide at more alkaline solution cause subsequent increase of electrophoretic flow, which rule over the apparent mobility whereas

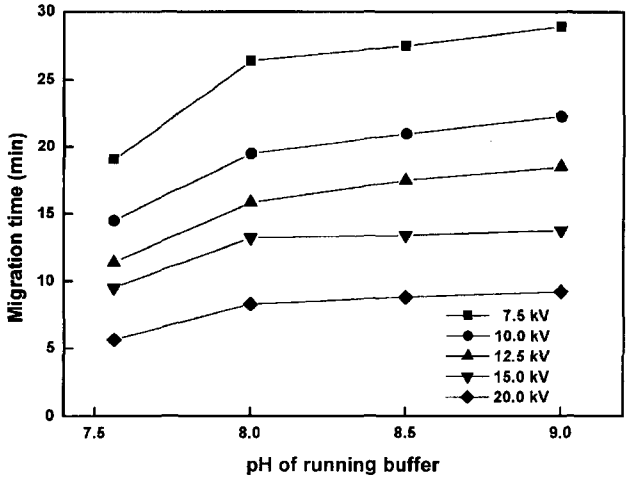

Fig. 1. Plots of the migration time of rhEGF vs. pH and applied voltages.

the pH increase might induce higher electroosmotic flow.

The capillary zone electrophoresis (CZE) at pH lower than 7 led to undesirable deformed and/or broad peak shapes with increased background currents. At pH higher than 9, stronger retaining of rhEGF resulted in too much elongation of migration time with high current values. The peptide is unstable at higher pH which might cause deamidation or peptide bond scission. The pH 8.5 was chosen as optimum pH for this work. The higher applied voltage resulted in the increase of the electroosmotic mobility and the shorter migration time was observed as expected.

The effect of running buffer. The running buffer selection depends on the solubility and charge of the sample components and is extremely important to the success of any CE separation. Buffer ion mobility affects the amount of current and subsequent heating in the capillary. Borate buffer (pH range 7. 5 to 10), in particular, is a popular buffer in CE for a wide variety of applications since this buffer has low inherent conductivity. Often, the conductivities associated with phosphate, citrate, and acetate buffers are relatively high, necessitating the selection of smaller i.d. capillaries or the adjustment of electric field strength conditions.

Similar experiments were performed using frequently used buffers in CE, tetraborate, phosphate, tricine, and Tris-HCl buffer. Tricin and Tris-HCl buffer system showed undesirable shoulder and thus broadening of the peak shape. Tetraborate and phosphate buffers produced high background currents. Accordingly, borate buffer was chosen for this work because it afforded higher sensitivity and lower baseline noise for rhEGF than other buffers studied.

The effect of buffer concentration on migration time was examined from 50 to 200 mM borate buffer at pH 8.5 as shown in Fig. 2. Higher ionic strength of running buffer decreases the effective charge, thus lowering electroosmotic mobility with subsequent elongation of the migration time. But higher buffer concentration gave concurrent increase of background currents.

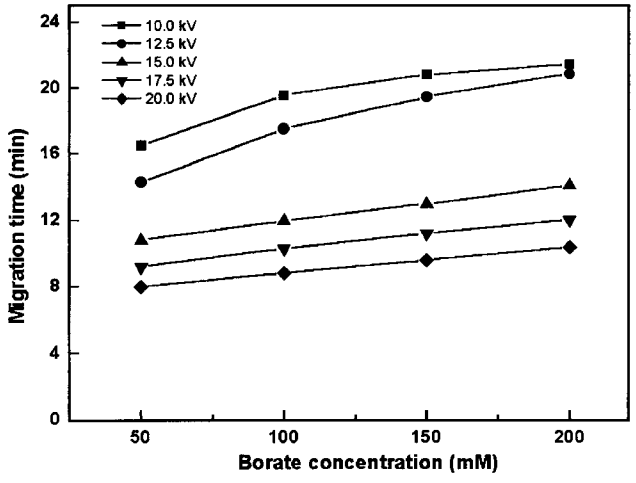

Fig. 2. Effects of buffer concentration on the migration time of rhEGF at pH 8.5.

The effect of SDS. Purity checks of peptide products are needed in biopharmaceutical industry for process control and quality control (QC) of bulk peptides and final peptide products. The reported degradation pathways of rhEGF were oxidation at the methionine residue (Rao *et al.*, 1986), deamidation at the asparagine residue (Ferraiolo & Benet, 1985), and succinimide formation at the aspartic acid (Konturek *et al.*, 1981). Deamidation of asparagine and glutamine residue is common. Physical instability of rhEGF can be from polymerization of monomer into dimer and trimer by disulfide exchange that may change biological activity of immunological properties.

The high resolution of CE is essential for monitoring the stability of products during storage. CE is suitable for this purpose because peaks appearing pure after HPLC analysis are often resolved in multiple peaks with a CE check because peptides similar in hydrophobicity in reversed-phase HPLC can be further resolved based on differences in charge-to-mass ratio. For this work, CZE technique

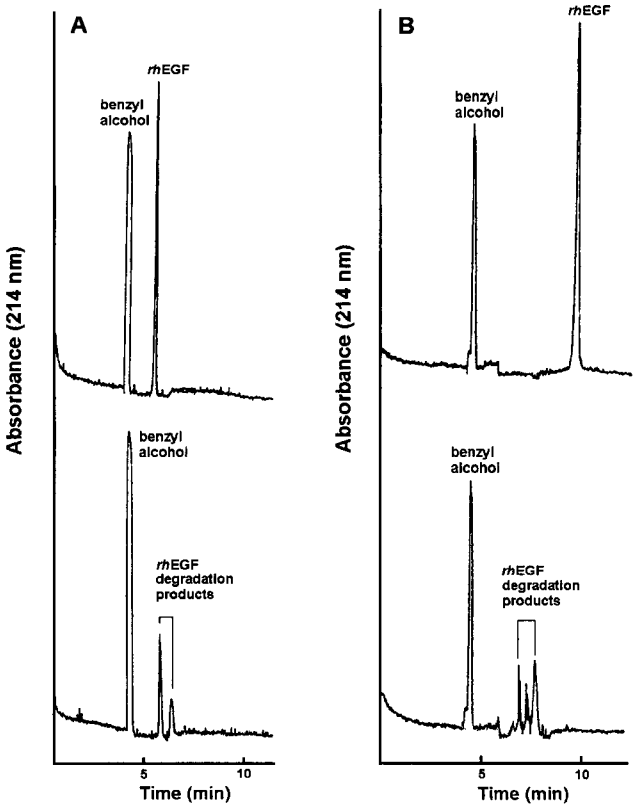

Fig. 3. Comparison of separations of rhEGF and its degradation products (A) without 12.5 mM SDS (B) with 12.5 mM SDS to the buffer, 50 mM sodium borate (pH 8.5) was used.

couldn't fully resolve native rhEGF from its closely related variants or degradation products as shown in Fig. 3A.

When anionic surfactant SDS micelles were added to the electrolyte, the migration time of the analyte became longer and the native form of rhEGF could be separated from its degradation products (Fig. 3B). The above findings suggested that the anionic SDS micelles are adsorbed to the peptide surface together via hydrophobic interaction and subsequently more negatively charged form of the peptide move more slowly toward the cathode whereas electroosmotic velocity from anode to cathode is nearly constant.

The effect of SDS concentration on the migration time was examined using a 50 mM borate buffer containing 12.5-100.0 mM SDS at pH 8.5 (Fig. 4). The migration time greatly increased up to 12.5 mM SDS, then slightly increased between the concentration range of 12.5-100 mM. This means that more negative charge of the peptide induce hydrophobic interaction with or adsorption of the SDS anion to the peptide surface. In all likelihood, SDS must not only induce negative charges on peptide molecules but also partially acts as MEKC separation mode, although extent of the contribution of MEKC modes to the migration behavior of peptides is not quantitatively estimated in the present study.

Assay of rhEGF in formulation

Pharmaceutical or diagnostic products typically are not consisted of pure drug substance, but rather of formulated mixtures. For this type of assay the complexity and/or variability of the sample matrix also plays an important role with regard to quantitative precision and accuracy of the results.

When the method was applied to quantify rhEGF present in a prepared dosage form (spray solution), the migration time of rhEGF was reduced about 2 min (8 min vs 10 min) in MEKC mode with 12.5 mM of SDS due to matrix effects of the formulation vehicle (Fig. 5A). When SDS concentration was increased the migration of rhEGF became longer again. A similar migration time of standard rhEGF could be obtained using a running buffer containing 50 mM SDS (Fig. 5B). Poloxamer 407 (MW 9800~14600 range) which is an emulsifying ingredient in the formulation vehicle greatly influenced migration time. We considered that the block copolymer may interfere with the adsorption of the anionic micelle to the peptide surface since increased micellar concentration caused subsequent delayed migration time of rhEGF again.

Assay of rhEGF from a commercial formulation, spray solution for diabetic ulcer, was performed using standard addition method to correct for matrix effects. The assay result was 54.3 ± 4.1 (mean ± S.D.) μg/ml (n=3).

Method validation

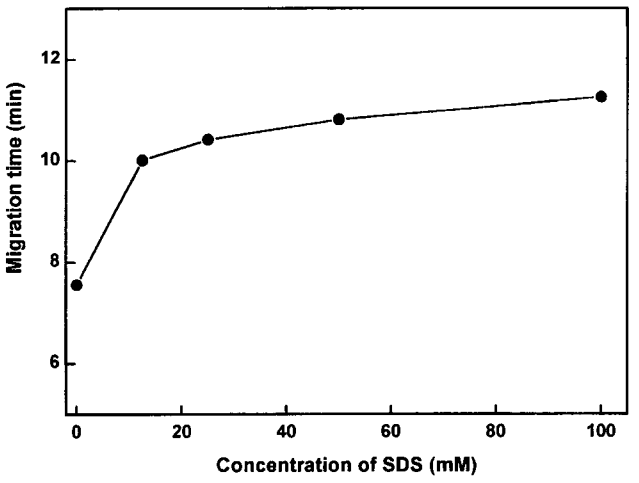

Fig. 4. Effect of SDS concentration on migration time of the rhEGF in 50 mM borate buffer at pH 8.5.

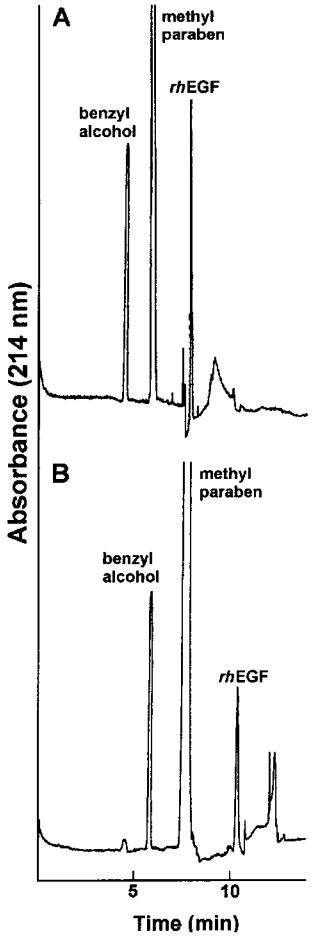

Fig. 5. Electropherograms of a pharmaceutical formulation (A) with 12.5 mM SDS; (B) with 50 mM SDS in 50 mM borate buffer (pH 8.5) with benzyl alcohol as a neutral marker, each respectively.

As required by the demands of good laboratory practice, as well as regulatory agencies, analytical procedures are typically validated for accuracy, precision, specificity, detection limit, linearity, and dynamic range. This is particularly important in pharmaceutical biotechnology when the method is to be used for process or quality control purposes. The optimized CE method was validated by assessment of linearity, limit of quantitation, method precision and accuracy.

Using benzyl alcohol as an internal standard, the calibration curve for the determination of rhEGF was linear over the concentration range from 1.25 to 100 μg/ml with correlation coefficient greater than 0.999. The limit of detection, defined as the concentration of rhEGF resulting in a signal-to-noise ratio of 3, was 0.5 μg/ml. The intra- and inter-day precision and accuracy of the assay are shown in Table I. The concentrations of rhEGF were found to deviate within a narrow range, from -1.4 to 2.8% of the theoretical concentrations in standard formulations. The relative standard deviation was less than 2.3%, which suggests that the assay is both precise and accurate.

CONCLUSION

Optimum condition for the CE determination of rhEGF in a pharmaceutical formulation applying MEKC technique was found from observing several factors affecting migration behaviors of the analyte in various parameters. The optimum CE run for resolving rhEGF, degradation products and formulation vehicles simultaneously was performed with 50 mM borate buffer (pH 8.5) containing 12.5 mM SDS at an applied voltage of 20.0 kV. The CE analysis afforded rapid and reproducible results for the determination of rhEGF over a wide range of concentration. The quantitation of rhEGF in a pharmaceutical preparation such as solution preparation was possible using standard addition method without pre-concentration or derivatization. The method could be used for the quality control of EGF products during manufacturing process, and especially in screening of contaminants and stability studies.

Table I. Reproducibility of rhEGF in EGF standard formulation by standard addition method

Spiked conc. (μg/ml)	Determined conc. (μg/ml)	Recovery (%)	C.V. (%)	Number
Intra-day				
2.50	2.52	101.1	1.7	3
5.00	4.96	99.2	1.3	3
10.00	10.2	100.2	0.3	3
Inter-day				
2.50	2.57	102.8	2.3	5
5.00	4.93	98.6	1.3	5
10.00	10.04	100.4	0.4	5

ACKNOWLEDGEMENTS

We greatly acknowledge the financial support provided by the Korea Research Foundation (99-005-F00033). Also, we deeply appreciate the Daewoong Pharm. Co. in Seoul for kind providing rhEGF and pharmaceutical formulations.

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