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Determination of Recombinant Human Epidermal Growth Factor (rhEGF) in a Pharmaceutical Formulation by High Performance Liquid Chromatography with Electrochemical Detection

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A novel HPLC method with electrochemical detection has been developed for the determination of recombinant human epidermal growth factor (rhEGF) in pharmaceutical products. rhEGF was separated from other components in formulation on a reversed-phase C18 column with 24% acetonitrile in 0.1 M phosphate buffer (pH 4.75). The optimum electrochemical oxidation of EGF was obtained at 0.85 V vs. Ag/AgCl in a glassy carbon working electrode due to electroactive tyrosine, tryptophan, methionine, and arginine residues. The quantitation range was from 1.0 to 200 ng of rhEGF with the linear correlation coefficient greater than 0.999. The method was successfully applied for the quantitation of rhEGF in a pharmaceutical preparation.

Key words: HPLC, Electrochemical detection, Recombinant human epidermal growth factor

INTRODUCTION

Human epidermal growth factor (hEGF) is a mitogenic 53 amino acid polypeptide which are present in a variety of tissues and body fluids of mammalian species (Cohen, 1962; Starkey *et al.*, 1975; Hirata & Orth, 1979a,b; Yip *et al.*, 1986). EGF is one of the growth factors which has been investigated for their potential to expedite the wound healing process (Gregory & Morris, 1986; Schultz *et al.*, 1987; Chvapil *et al.*, 1988; Brown *et al.*, 1989; Schultz *et al.*, 1991). Recently, a pharmaceutical formulation that allows administration of rhEGF as a solution form to diabetic foot ulcers was developed and is expected to come to market in the near future.

HPLC has the advantages of precision and accuracy and unlike bioanalytical procedures, can differentiate between intact form of EGF and its degradation products. In connection with pharmaceutical studies of EGF, the reversed-phase HPLC (RPHPLC) has widely been used for the assay of hEGF in a variety of matrices (Hirata & Orth, 1979b; Dibias & Rhodes, 1991; Senderoff *et al.*, 1994; Son & Kwon, 1995). However, the HPLC-UV method lacks the sensitivity required at levels of a few micrograms per quality control. It is, therefore, necessary to develop a more specific and sensitive method to determine hEGF in various forms of pharmaceuticals including cosmetic products.

Electrochemical detection has proved to be a reliable, sensitive and specific method for the determination of peptides containing tyrosine, tryptophan or arginine in biological samples (Bennett *et al.*, 1981; Dawson *et al.*, 1985; Sagara *et al.*, 1988; Ding *et al.*, 1990; Meriluoto *et al.*, 1998). hEGF contains 11 electrochemically active amino acids as underivatized, 5 tyrosine, 2 trypthophan, 3 arginine, and 1-methionine residues. This paper describes an HPLC assay method with electrochemical detection of rhEGF in a pharmaceutical preparation using standard addition method.

MATERIALS AND METHODS

Materials

Recombinant human EGF (rhEGF, more than 99% purity) prepared by genetic engineering was provided by the Daewoong Pharm. Co. (Seoul, Korea). HPLC-grade acetonitrile was purchased from Burdick and Jackson (Muskegon,

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MI, USA). All other chemicals were of analytical grade, and were purchased from Sigma (St. Louis, MO, USA). An EGF sample, spray solution for the treatment of diabetic foot ulcer (label claimed 60,000 IU/ml) was used to validate sample quantitation. Standard formulation of rhEGF (50 μg/ml) was made using formulation matrices to validate accuracy (recovery) of the method. rhEGF stock solution (1.0 mg/ml) was prepared in deionized water and stored at -20°C for 3 months. Working standard solutions of rhEGF (0.05-10 μg/ml) were freshly prepared in mobile phase before each experiment. Sample solution was diluted using the same diluent and stored below 4°C. All other peptide solutions, whether reference standards or analytes, were made in polypropylene or silanized glassware to prevent adsorption to glass surfaces.

Chromatography

HPLC was performed with a Jasco series chromatograph (Tokyo, Japan) which comprised of PU 980 dual pumps, UV-975 UV detector and 807-IT integrator used together with HP3396A (Hewlett Packard, PA, USA) Integrator or y-t recorder (Gasukuro Kogyo 72R, Tokyo, Japan). Electrochemical detection was performed with an amperometric detector (Metrohm 656; wall-jet type, cell volume < 1 μl) with a glassy carbon working electrode under the control of potentiostat (Metrohm 641 VA detector, Herisau, Switzerland). The electrochemical cell temperature was maintained constant using a thermostatic jacket with water circulator (Techne, C-400, England). The HPLC column temperature controlled with a column oven (Eldex CH-50, USA) was maintained at 40°C during HPLC analysis and the HPLC effluent was dual-monitored using UV- and ECD detector. Solvent was degassed with high purity nitrogen (99.999%, RIGAS Co., Daejon, Korea).

Compounds were separated on a Jupiter, 5-μm particle (300Å) C18 column (250 × 4.6 mm i.d., Phenomenex, CA, USA). The mobile phase was 24:76 (v/v) acetonitirle-0.1 M sodium phosphate buffer (pH 4.75). Flow rate was 1.0 ml/min and the injection volume was 20 μl. The working electrode was operated at 0.85 V vs. Ag/AgCl (3 M KCl) reference electrode. UV detection at 214 nm was also performed to confirm the identity of the electrochemically detected peaks.

Glassy carbon working electrode was polished with 0.3 μm alumina powder slurry on polishing cloth at the beginning of a days use. The electrode can be slowly passivated after running overnight under these conditions. Therefore it was polished everyday before running. Measurement at high concentration of rhEGF (10 μg/ml) needs more frequent polishing.

Hydrodynamic voltammetry

A hydrodynamic voltammogram was constructed by performing replicate analyses of rhEGF standard (1.0 μg/ml) at different electrode potentials starting from 0.7 V to 1.0 V (vs. Ag/AgCl). The peak currents at each potential were calculated from peak height values and were plotted against the potential.

Assay of EGF formulations

Certain amounts (~100 μl) of analyte samples were diluted 10-fold using mobile phase (diluent). From this solution, four aliquots (each, 100 μl) were pipetted into eppendorf tubes; the solution were further made to 1.0 ml in order to make the final concentration of spiked rhEGF 0-, 0.5-, 1.0, and 2.0 μg/ml, respectively. The analyte solutions were injected to HPLC and the analytical results were calculated using a PUMA® program (KRISS, Daejon, Korea).

RESULTS AND DISCUSSION

Chromatography

Reversed-phase HPLC is a versatile analytical tool in pharmaceutical studies of EGF. HPLC is capable of resolving parent and possible modified or fragmented forms of EGF found in biological samples. HPLC-ECD method has an advantage such as higher sensitivity than conventional HPLC-UV detection method. Adjustments of mobile phase pH, buffer type, buffer strength and the concentration of organic modifier play an important role in the electrochemical detection of peptides (Meriluoto *et al.*, 1998).

A phosphate-based mobile phase was chosen for this work because it resulted in a better peak shape and a lower detection limit for rhEGF than other commonly used buffers, such as acetate-, borate-, and carbonate based mobile phases (data, not shown). pH is an important factor in maintaining optimum sensitivity because the peak oxidation potential is reduced at higher pH. As shown in Fig. 1, the retention time of rhEGF decreases when the pH of mobile phase increases from pH 3.5 to 6.0. As the non-ionic state of hEGF is retained longer by the nonpolar stationary phase, the ionic fraction of the acidic peptide hEGF increases as the pH increases from 3.5 up to 6.0 under investigation. As the pH of the mobile phase increased, the oxidation current of the peptide increased but reached a maximum value around the isoelectric pH (pH 4.6) (Fig. 1).

The concentration effects of phosphate buffer (pH 4.75) on the peak current and capacity factor are shown in Fig. 2. Ionic strength of the mobile phase at 0.10 M gave maximum sensitivity while capacity factor decreased almost linearly with the buffer concentration.

The elution of a peptide from a reversed-phase column is extremely sensitive to the smallest variations in the concentration of the organic modifier in mobile phase (Oliver,

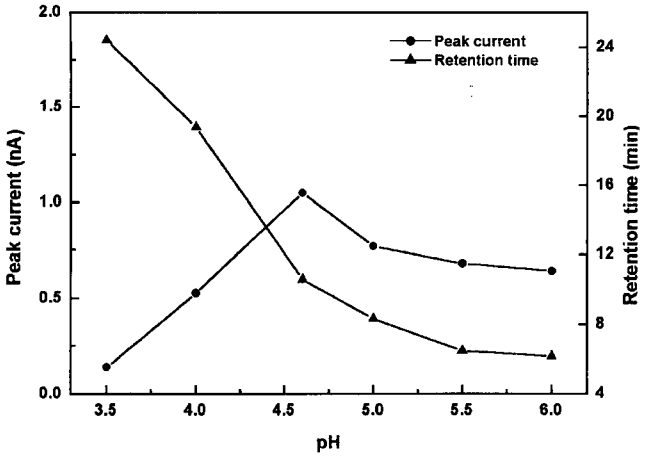

Fig. 1. Effects of pH on the retention time and peak current of rhEGF. Column, Jupiter C18 (250 × 4.6 mm i.d., 5-μm, 300Å); mobile phase, 24:76 (v/v) acetonitrile-0.1 M sodium phosphate buffer; flow-rate, 1.0 ml/min; electrochemical detection, 0.85 V; sample volume, 20 μl.

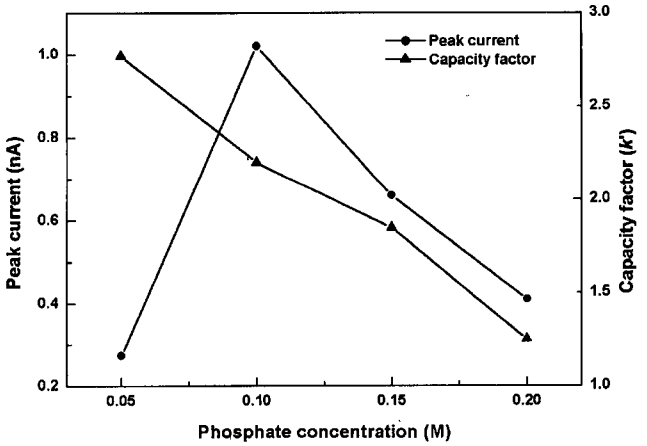

Fig. 2. Effects of ionic strength of mobile phase on peak currents and capacity factors of rhEGF. Column, Jupiter C18 (250 × 4.6 mm i.d., 5-μm, 300Å); mobile phase, 24:76 (v/v) acetonitrile-sodium phosphate buffer (pH 4.75); flow-rate, 1.0 ml/min; electrochemical detection, 0.85 V; sample volume, 20 μl.

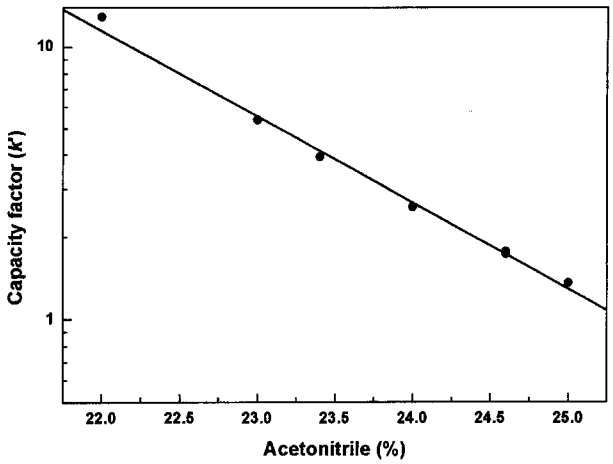

Fig. 3. Effect of the concentration of acetonitrile on the capacity factor of rhEGF. Column, Jupiter C18 (250 × 4.6 mm i.d., 5-μm, 300Å); mobile phase, 24:76 (v/v) acetonitrile-0.1 M sodium phosphate buffer (pH 4.75); flow-rate, 1.0 ml/min; electrochemical detection, 0.85 V; sample volume, 20 μl. The fitted line was log y=8.017-0.316× (r=- 0.997).

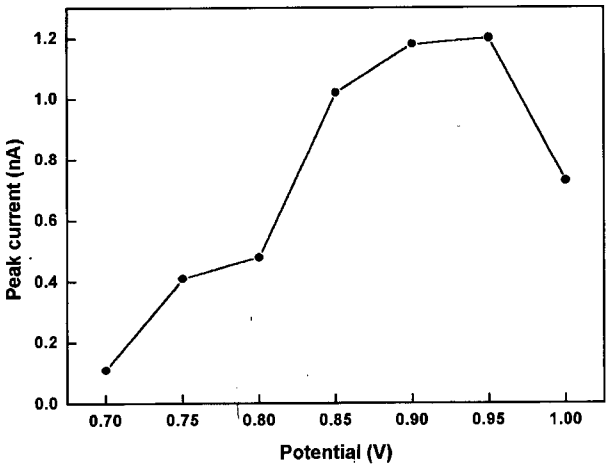

Fig. 4. Hydrodynamic voltammogram of rhEGF. Chromatographic conditions, other than oxidation potential, were as for Fig. 1.

1989). The effect of acetonitrile concentration on the capacity factor of rhEGF is presented in Fig. 3. The capacity factor of rhEGF decreased in a log-linear manner as the percentage of acetonitrile increased.

A hydrodynamic voltammogram was obtained to determine the optimum oxidation potential of rhEGF (Fig. 4). Oxidation of rhEGF at 0.95 V gave the maximum signal. But more than 40% of the signal was lost at higher potentials (≥ 1.0 V) because of the increased baseline noise and electrode passivation over time (18). In this paper, we chose 0.85 V as the polarization potential which showed better sensitivity but lower background currents.

Increase in column temperature from 30°C to 40°C led to increase in retention, but separation selectivity of rhEGF from methyl paraben increased from 1.3 to 2.0. Stepwise elevation of the detector cell temperature from 25°C, 30°C to 40°C showed the relevant increase of peak currents but with less precision of the current values (R.S.D., 0.63%, 3.19%, and 5.54%, each respectively).

Degradation peaks caused by frequent frozen and thaw cycles of the peptide did no interfere with determination of EGF. The native form of rhEGF could be well separated from these degradation products which possibly can be formed from long-term storage. Three degradation peaks eluted faster than the rhEGF peak (retention time, less than 6 min).

Oxidative electrochemistry proved to be a reliable and

sensitive method for the determination of rhEGF, which contains electrochemically active tyrosine, tryphtophan, methionine, and arginine as constituent amino acids. Typical electrochemical detection chromatograms obtained from a pharmaceutical preparation containing rhEGF are shown in Fig. 5.

Method validation

The calibration curve for the determination of rhEGF with electrochemical detection was linear over the concentration range 0.05 to 10 μg/ml (1.0 ng-200 ng rhEGF) with correlation coefficient greater than 0.999. The limit of quantitation (LOQ), defined as the concentration of rhEGF resulting in a signal-to-noise ratio of 5 was 1.0 ng per injection, which is more sensitive than conventional UV detection (20 ng). This electrochemical detection method have advantage that no derivatization is necessary, and can be applied to the quality control of EGF preparations. 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 2.4 to 0.6% of the theoretical concentrations in standard formulations. The relative standard deviation was less than 2.7 %, which suggested that the assay is both precise and accurate.

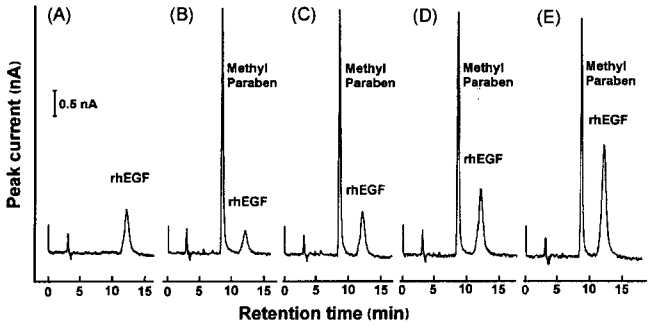

Fig. 5. Chromatograms of rhEGF with electrochemical detection. (A) EGF standard (1.0 μg/ml), (B) sample formulation (diluted 100-fold with mobile phase), (C) spiked sample with EGF 0.5 μg/ml, (D) spiked sample with EGF 1.0 μg/ml, and (E) spiked sample with EGF 2.0 μg/ml.

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				
0.500	0.488	97.6	1.3	3
1.000	0.992	99.2	0.9	3
2.000	2.012	100.6	0.7	3
Inter-day				
0.500	0.502	100.4	2.7	3
1.000	1.002	100.2	2.3	3
2.000	2.003	100.2	0.8	3

Table II. Assay results of EGF formulation by RPHPLC with electrochemical detection (mean ± S.D.)

Formulation[a]	Content (μg/ml)	Found (μg/ml)	n
A	50	56.2 ± 2.5	5
B	50	49.0 ± 0.6	5

A, an EGF formulation; B, a reference standard formulation.
[a]The formulation matrices contain poloxamer 407 and methyl paraben.

Assay of rhEGF in formulations

The method was used to quantify rhEGF in a spray solution for the treatment of diabetic foot ulcer. Fig. 5 and Table II show the chromatograms and assay results for a sample and standard formulation of rhEGF. Chromatograms of rhEGF formulation and the spiked samples are no different, which indicate that other ingredients in the formulations have no effect on the determination of rhEGF by this electrochemical detection system.

CONCLUSIONS

RPHPLC with the electrochemical detection proved to be a reliable, simple and sensitive method for the determination of rhEGF. The pH, buffer type and strength, organic modifier concentration of the mobile phase, and the working potential were optimized to obtain maximum sensitivity. The quantification of rhEGF in formulations such as spray was possible without concentration and derivatization. We are currently investigating the applicability of this detection system to the determination of rhEGF levels in biological fluids. In conclusion, this method would be useful for the quality control of rhEGF pharmaceutical products.

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