Development and Validation of Two Solid-Phase Enzyme Immunoassays (ELISA) for Quantitation of Human Epidermal Growth Factors (hEGFs)

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Purpose. The purpose of the present investigation was to develop and validate two separate enzyme-linked immunosorbent assays (ELISA) for quantitation of exogenous human epidermal growth factor (hEGF1-53) and its truncated fragment (hEGF1-48) in rat plasma.

Methods. The present assay systems were based on the sandwiching of the antigen between a monoclonal mouse anti-hEGF1-53 antibody, pre-coated on a 96-well polystyrene plate, and a polyclonal rabbit anti-hEGF1-48 antibody, which is then detected with a peroxidase-labeled goat anti-rabbit antibody.

Results. The calibration curves for hEGF1-48 and hEGF1-53 in plasma were validated over a concentration range of 7.8–250 and 62.5–1000 pg/ml, respectively. Determined from replicate assays of hEGF1-48 quality control samples, the intra-assay precision and accuracy were ≤8.8% RSD and within ±9.8%; and the inter-assay precision and accuracy were ≤14.8% RSD and within ±9.7% RE, respectively. Determined from replicate assays of hEGF1-53 quality control samples, the intra-assay precision and accuracy were ≤10.0% RSD and within ±8.5%; and the inter-assay precision and accuracy were ≤10.0% RSD and within ±5.7% RE, respectively. The limit of quantitation of the hEGF1-48 and hEGF1-53 assay using 200 µl plasma per well is 7.8 and 62.5 pg/ml, respectively. These two ELISA methods are specific to hEGFs and do not cross-react with mouse EGF or other growth factors (TGFα, TGFβ, PDGF, and FGF) or lymphokines (IL-1 and TNFα). These validated methods have been routinely applied to assay plasma samples from various pharmacokinetic studies in rats receiving intravenous hEGFs. Both assay methods were also adapted to assay endogenous hEGFs in biological fluids of different animal species.

Conclusions. Two sensitive ELISA methods have been validated for quantitation of hEGF1-53 and hEGF1-48 in rat plasma. Their utility has been demonstrated in the application of assaying immunoreactive concentrations of exogenous and endogenous epidermal growth factors.

KEY WORDS: ELISA; epidermal growth factors; hEGFs; method development; and assay validation.

INTRODUCTION

Mouse epidermal growth factor (mEGF) was first purified from adult male mouse submaxillary gland in 1972 (1). Human EGF (hEGF) was later purified from urine in 1975 by Cohen and Carpenter (2). The naturally occurring EGF both in mice and human has 53 amino acids with a molecular weight of about 6 kD and 37 of the 53 amino acids in the two peptides are identical (3). EGF can invoke a variety of responses in vitro and in vivo including stimulating cell proliferation and inhibiting gastric acid secretion (4). It is also known that other proteins, such as transforming growth factor alpha (TGFα), can bind to the EGF receptor (EGFR) with similar affinity as EGF (5). The wide distribution of EGF and its receptor (10) indicate an important role for this peptide in human health and disease.

Since EGF is a potent mitogen, its applications for wound healing are likely to be important in the future. Clinical applications include using EGF for topical wound healing (7). Since exogenous EGF can stimulate the growth of gastrointestinal epithelium (8,9), EGF could possibly be used as a therapeutic agent for acceleration of gastrointestinal epithelial regeneration. Recombinant human epidermal growth factor (hEGF1-53) and its C-terminal truncated fragment (hEGF1-48), shown in figure 1, have been under evaluation for treatment of gastrointestinal, hepatic and kidney lesions (8–13). If hEGFs were to be used as a therapeutic, development of simple methods for determination of exogenous growth factors in plasma would be important.

Many studies have focused on hepatic binding, intracellular transport and lysosomal degradation of EGF (14,15), but relatively few studies on plasma disposition have been conducted (16,24). Many ELISA methods for hEGF1-53 with similar sensitivities have been reported (16–21). However, from an industrial perspective, these assays were not properly validated using quality control samples, according to good laboratory practice (GLP). No immunoassay method has been reported for the hEGF1-48 peptide. This report describes the development and validation of two sensitive ELISAs to measure the immunoreactive concentrations of hEGF1-48 and hEGF1-53 in rat plasma. These new methods were validated according to the requirements of pharmaceutical industry and have been routinely applied to pharmacokinetic studies in rats. These immunoassay methods may also be adapted to assay EGFs in biological fluids of other species.

MATERIALS AND METHODS

Materials

Human epidermal growth factor (hEGF1-53; MW 5935) (Figure 1), obtained from PreproTech Inc. (Rocky Hill, NJ), was produced by means of recombinant DNA technology and expression in Escherichia coli. Human EGF1-48 (hEGF1-48; MW 5447) was obtained by cleavage of the C-terminal pentapeptide from hEGF1-53 with trypsin treatment. Mouse anti-hEGF1-53 type H (monoclonal IgG1) used as the primary antibody in ELISA was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Goat anti-rabbit IgG (H&L) peroxidase conjugate was from Rockland Inc. (Gilbertsville, PA), TMB (3,3',5',5'-tetramethylbenzidine) and H2O2 Microwell Peroxidase Substrate System was purchased from Kirkegaard & Perry

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Laboratories Inc. (Gaithersburg, MD). Control rat plasma, containing EDTA as an anti-coagulant, was from Pel-Freeze (Rogers, AR). Bovine serum albumin and porcine skin gelatin were purchased from Sigma Chemical Co. (St. Louis, MO). Immulon 4 microtiter plates were purchased from Baxter (McGaw Park, IL). Plates were washed using the Milenia Microwash 4 (Diagnostic Products, Los Angeles, CA). Absorbance reading of microtiter plates was accomplished on the Cayman Chemical Autoreader (Cayman Chemical Co., Ann Arbor, MI).

**Preparation of Rabbit Anti-hEGF IgG Antibodies**

Polyclonal rabbit anti-hEGF antibody used as the secondary antibody in hEGF1-48 assay system was raised in New Zealand rabbits. They were immunized on the intradermal sites of their back first with 200 μg of hEGF1-48 and 200 μg of Adjuvax (Alpha-Beta Technology, Inc., Worcester, MA) in an emulsion produced from normal saline and Freund’s complete adjuvant, followed by two biweekly booster immunizations with Freund’s incomplete adjuvant containing 200 μg hEGF1-48 and 200 μg Adjuvax. Bleedings and subsequent immunizations were performed biweekly until the antisera had a working titer of 1:12,000 and a ED₅₀ sensitivity of 7.8 pg/ml. The polyclonal antibody used in the hEGF1-53 assay system was raised in rabbits in a manner similar to that for the hEGF1-48 assay, but the immunogen was a bovine serum albumin (BSA)-hEGF1-48 conjugate. The conjugate was prepared by overnight incubation of a 1-ml mixture containing 1.6 mg hEGF1-48, 2.6 mg BSA, and 30 μl glutaraldehyde. The molar ratio of hEGF/BSA was 7.5. The resulting mixture was passed through a 5-25G Sephadex column and washed with 50 mM ammonium bicarbonate. The two eluent fractions containing highest protein content, monitored by the UV absorbance at 280 nm, were pooled as immunogen. The antiserum used for the hEGF1-53 ELISA assay had a working titer of 1:9,000 and a ED₅₀ sensitivity of 62.5 pg/ml. The IgG fractions from the working antisera were diluted 1:1 with phosphate-saline (PBS, pH 7.4) and purified on a protein A-sepharose column. Elution of the IgG was accomplished by adding 0.1M glycine, pH 3.0, to the column and collecting the peak fractions. The IgG solutions were then diazoyed extensively against PBS.

**Preparation of Standard and Quality Control Plasma Samples**

The lyophilized hEGFs were dissolved in 20 mM sodium phosphate buffer containing 0.01% Tween 80, pH 6.0, at approximately 0.5 mg/ml for spectrophotometric determination of actual concentrations at 276 nm using an extinction coefficient (EC) of 1.22 for hEGF1-48 and 2.94 for hEGF1-53. The EC for hEGF1-48, which was in terms of ml/mg using 1 cm path length, was estimated by the ratio of optical density at that wavelength to different protein concentrations prepared, where the actual amount of each protein at each concentration was determined by quantitative amino acid analysis. The EC of hEGF1-53 was calculated from the EC of tyrosine and tryptophan using the theoretical tyrosine and tryptophan content in the hEGF1-53 molecule and the experimentally determined molecular weight of the hEGF1-53 molecule. Determination of protein content in the stock solution is essential for preparation of accurate concentrations of plasma standards, quality controls, and dosing solutions. Plasma hEGF standards were prepared fresh for each analytical run by diluting the stock solutions with control rat plasma to yield final concentrations of 7.8, 15.6, 31.2, 62.5, 125, and 250 pg/ml for hEGF1-48 and concentrations of 62.5, 125, 250, 500, and 1000 pg/ml for hEGF1-53. Quality control samples (QC) for assay validation were prepared by diluting the hEGFs with control rat plasma to four different