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

Nywana Sizemore,[1] Richard C. Dudeck,[2] Charles M. Barksdale,[2] Gerald D. Nordblom,[2] Williams T. Mueller,[3] Patrick McConnell,[3] D. Scott Wright,[2] Antonia Guglietta,[4] and Be-Sheng Kuo[2,5]

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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 (TGF_α, TGF_β, PDGF, and FGF) or lymphokines ($IL_{1\beta}$ and TNF_α). These validated methods have been routinely applied to assay of 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.

[1] Department of Oncology, Vanderbilt University, Nashville, Tennessee 37232.

[2] Departments of Pharmacokinetics and Drug Metabolism, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105.

[3] Department of Biotechnology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105.

[4] Department of Immunopathology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105.

[5] To whom correspondence should be addressed.

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 (TGF_α), can bind to the EGF receptor (EGFR) with similar affinity as EGF (5). The wide distribution of EGF and it's 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 IgG_{1k}) 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 H_2O_2 Microwell Peroxidase Substrate System was purchased from Kirkegaard & Perry

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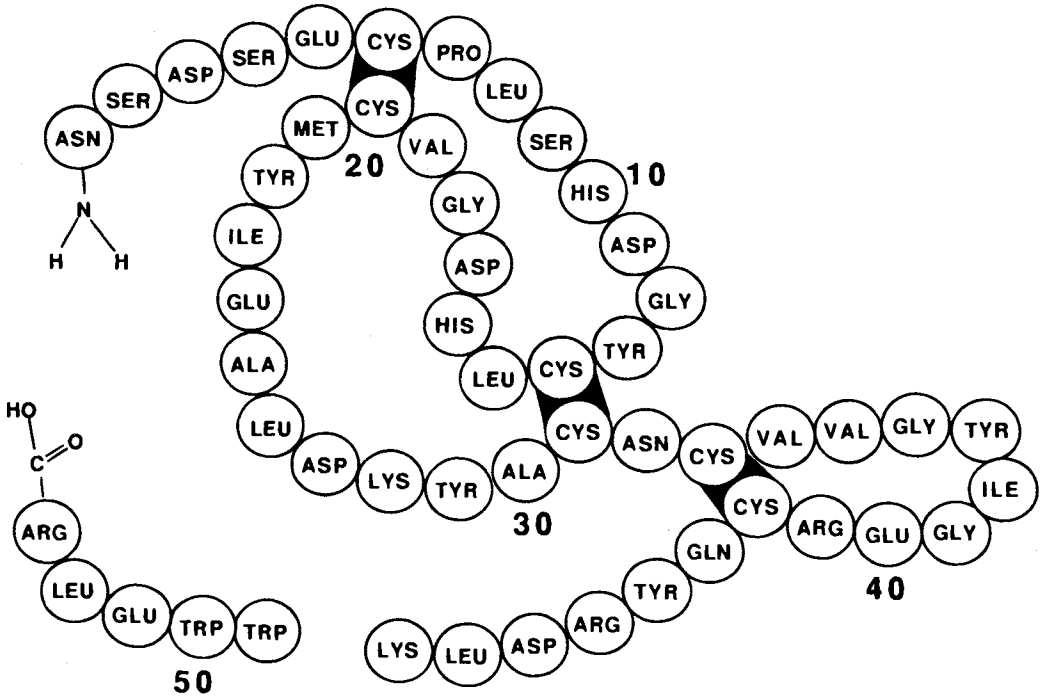

Fig. 1. Chemical Structure of hEGF1–48 and hEGF1-53.

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., Worchester, 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 anitserum had a working titer of 1:12,000 and a ED_{90} 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 glutadehyde. The molar ratio of hEGF/BSA was 7.5. The resulting mixture was passed through a S-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 ED_{90} 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 dialyzed 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 trytophan 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

concentrations (low, medium, high, and dilution) (50, 100, 200, and 1000 pg/ml for hEGF1-48; and 200, 500, 750, and 1000 pg/ml for hEGF1-53). The 1000 pg/ml QC for both assays was used as a dilution QC and was diluted at the time of the assay. The QC of 1000 pg/ml was diluted to 100 pg/ml in the hEGF1-48 assay and to 200 pg/ml in the hEGF1-53 assay. All standard stock solutions and quality control solutions were stored at −70°C.

Enzyme Immunoassay Procedures

Microtiter plates were coated with 100 μl per well of mouse anti-human EGF1-53 type H at 1 μg/ml in 0.01 M sodium bicarbonate/0.1% thimerosal, pH 9.6, and incubated overnight at 4°C. The plates were washed 3 times with 300 μl/well of plate wash buffer (0.02 M Tris containing 0.15 M NaCl, 0.1% Tween 20, and 0.1% thimerosal, pH 7.4). The remaining protein binding sites on the plates were blocked by incubating for 1 hr at 37°C with 300 μl/well blocking buffer (0.02 M Tris containing 2% BSA, 2% mouse plasma, 0.15 M NaCl, and 0.1% thimerosal, pH 7.4). The wells were washed as above followed by the addition of 200 μl of the standards, quality controls, or unknown samples. The plate was incubated at room temperature for 2 hr. After washing 3 times, 0.8 μg/well of diluted polyclonal rabbit anti-hEGF1-48A antibody for the hEGF1-48 assay or 0.4 μg/well of diluted polyclonal rabbit anti-hEGF1-48B antibody for the hEGF1-53 assay was added. The plates were incubated at room temperature for 1 hr. After washing 3 times, 0.1 μg/well of goat anti-rabbit IgG (H&L) horseradish peroxidase-conjugate in antibody diluent was added and incubated at room temperature for 1 hr. These antibodies were diluted in 0.02 M Tris containing 0.5% BSA, 1% mouse plasma, 0.15 M NaCl, and 0.1% thimerosal, pH 7.4. After washing 3 times, 200 μl of a mixture of TMB peroxidase substrate:H_2O_2 (1:1) was added and incubated for 15 min at room temperature. Finally, 50 μl of 2 M sulfuric acid stop solution was added to each well. The plates were then read at 450 nm, and data analysis was completed using validated EIA Smart data reduction and quality control software. The weighted log of the absorbance (ABS) was plotted against log hEGF concentration, and a straight line was computer-fitted by least squares regression analysis. Quality control and unknown sample concentrations were computed by interpolation from the standard curve.

Validation Requirements

The requirements for validation included a 3-day pre-study and a sample analysis validation. Three separate weighings of hEGFs were used to prepare three separate standard stock solutions and fourth separate weighing was used to prepare the quality control samples. Over the 3-day validation period, two standard curves were run for each of the three standard stock solutions. For each of the six assays in the 3-day pre-study validation, a standard curve and a set of quality control samples (consisting of the low, medium, and high QCs analyzed as unknowns) were run. Each standard curve consisted of a minimum of five standard concentrations prepared from the standard stock solution being analyzed. This 3-day pre-study validation produced a total of six standard calibration curves and n = 6 for each quality control concentration. In a single seventh run, ten replicates of the low, medium, high, and dilution QCs were analyzed to evaluate intra-assay variability. All standards and quality controls were run in duplicate. The acceptance criteria was that the percent relative standard deviation (%RSD) and percent relative error (%RE) of the back calculated values of each calibration standard concentration and quality control concentration must be ≤15% RSD and within ±15% RE. The sample analysis validation consisted of running a standard calibration curve consisting of five standard concentrations plus at least one set of quality control samples with the set of unknown samples to be assayed during each run. All standards, quality controls, and unknowns were run in duplicate. The acceptance criteria was that the quality control values must fall within either ± two standard deviations or ±10% of the established mean from the pre-study validation, whichever range is greater. If in any run the quality controls did not fall in this range, the run was considered to have failed and samples were reanalyzed.

Evaluation of Cross-reactivity

In the hEGF1-48 assay, hEGF1-53 and hEGF1-47 were tested at 100, 200, and 500 pg/ml. In the hEGF1-53 assay, hEGF1-48 and hEGF1-47 were tested at 500 pg/ml, 1000 pg/ml, and 10 ng/ml. All other peptides and proteins tested were tested at concentrations of 1, 10, and 100 ng/ml in both ELISA systems. The percent cross reactivity was determined by dividing the observed value obtained by ELISA by the actual concentrations tested.

Application to Pharmacokinetic Studies in Rats

The utility of the two immunoassay methods was demonstrated in two separate pharmacokinetic studies in fasted rats (250–350 mg/kg) receiving a 100 μg/kg intravenous bolus dose of either hEGF1-53 or hEGF1-48. Doses were administered via the jugular cannulae, followed by a 1 ml rinse of 0.9% sterile sodium chloride. Blood samples (200 μl) were drawn into tubes containing EDTA as the anticoagulant from the same cannula at pre-dose and at 1, 2, 3, 4, 6, 8, 10, 15, 30, 45, 60, 90, and 120 min following dosing. Plasma was separated and stored frozen at −70°C until analyzed with the ELISA methods described above. These unknown samples were used either undiluted or diluted with control rat plasma to produce levels of analyte which fell into the calibration range for each ELISA assay method. Pharmacokinetic parameters characterizing the plasma disposition profiles were calculated with the following equations: $t1/2 = 0.693/\lambda z$, where t1/2 is the apparent elimination half life and λz is the terminal elimination rate constant determined by nonlinear regression; MRT is the mean residence time calculated by moment analysis; CL_{tot} = Dose/$AUC_{0-\infty}$, where Cl_{tot} is the total plasma clearance of the drug and $AUC_{0-\infty}$ is the area under the plasma concentration-time curve from zero to infinite time; and $V_{ss} = CL_{tot} \cdot MRT$, where V_{ss} is the estimated steady-state volume of distribution of the peptide. All data are presented as mean ±SD.

Application to Measurement of Endogenous EGF Levels

Plasma and urine samples were obtained from apparently normal male and female human volunteers and other animal species. Plasma samples were used undiluted and urine samples were diluted 1:100 with control rat plasma. Rat plasma was

used as an assay diluent because the basal immunoreactive EGF level in rat plasma is negligible under the experimental conditions. Also the use of a non-reactive rat plasma reduces potential error associated with the use of artifical matrices. The equivalent immunoreactive concentrations (ng eq/ml) of human and non-human EGFs were determined using the ELISAs detailed above.

RESULTS

Linearity and Sensitivity of Calibration Standard Curves

In the 3-day pre-study validation process, the pooled results from a total of six assays showed a linear, weighted log-log calibration curve for the hEGF1-48 assay in the concentration range of 7.8 to 250 pg/ml, with a mean slope of 0.986 ± 0.092 and a correlation coefficient ranging from 0.993 to 0.999. The between-assay precision, expressed as percent relative standard deviation (%RSD) of back-calculated calibration standards, ranged from 5.5% to 9.6%. The between-assay accuracy, expressed as percent relative error (%RE) of back-calculated calibration standards, ranged from −11.0% to 13.9%. The limit of quantitation (sensitivity) was 7.8 pg/ml for the hEGF1-48 ELISA using 200 μl/well plasma. For the hEGF1-53 assay, a linear, weighted log-log calibration curve was obtained in the concentration range of 62.5 to 1000 pg/ml, with a mean slope of 1.106 ± 0.118 and a correlation coefficient ranging from 0.995 to 0.999. The between-assay precision ranged from 5.7% to 13.0% RSD. The between-assay accuracy ranged from −11.2% to 4.3% RE. The limit of quantitation (sensitivity) was 62.5 pg/ml for the hEGF1-53 ELISA using 200 μl/well plasma. These results indicate that the day-to-day hEGF1-48 and hEGF1-53 standard curves were stable with picogram sensitivity.

Precision and Accuracy of Assays

In the same 3-day pre-study validation process, the precision and accuracy of the ELISA methods were evaluated by assaying quality control samples (QC). Validation for both hEGF1-48 and hEGF1-53 ELISAs was evident by both the excellent intra- and inter-assay precision and accuracy obtained for the quality control samples (Table 1). The intra-assay precision observed on one occasion with 10 replicates per QC (50, 100, and 200 pg/ml) ranged from 2.3 to 8.8% RSD and the intra-assay accuracy ranged from −9.8 to 8.0% RE for the hEGF1-48 assay. The inter-assay precision on six separate occasions for the low, medium, and high concentrations (200, 500, and 750 pg/ml) ranged from 7.6 to 14.8% RSD for the hEGF1-48 assay and the inter-assay accuracy ranged from −9.7 to 8.8% RE. For the hEGF1-53 ELISA, the intra-assay precision observed with 10 replicates per QC (200, 500, and 750 pg/ml) ranged from 4.1 to 10.0% RSD and the intra-assay accuracy ranged from −2.6 to 8.5% RE. The inter-assay precision on six separate occasions for the low, medium, and high QCs (200, 500, and 750 pg/ml) ranged from 6.6 to 10.0% RSD for the hEGF1-53 assay and the inter-assay accuracy ranged from −5.7 to −1.1% RE. These results indicate that both ELISA systems were highly reproducible. For both hEGF1-48 and hEGF1-53 assays, dilution of the 1000 pg/ml QC samples with control rat plasma also yielded excellent precision and accuracy (Table 1).

Table 1. Intra- and Inter-Assay Precision (%RSD) and Accuracy (%RE) of Quality Control Samples of hEGF1-48 and hEGF1-53

	Intra-assay (n = 10)[a]			Inter-assay (n = 6)[a]		
Predicted	Observed	%RSD	%RE	Observed	%RSD	%RE
hEGF1-48						
50 pg/ml	45.1	8.8	−9.8	45.2	14.8	−9.7
100 pg/ml	93.3	2.3	−6.7	109	12.2	8.8
200 pg/ml	185	2.7	−7.4	192	7.6	−3.9
1000 pg/ml[b]	1080	8.0	8.0			
hEGF1-53						
200 pg/ml	203	7.1	1.5	198	10.0	−1.1
500 pg/ml	542	4.1	8.5	471	6.6	−5.7
750 pg/ml	730	8.2	−2.6	722	8.0	−3.7
1000 pg/ml[b]	1030	10.0	3.0			

[a] Each replicate was the mean of two duplicate wells.
[b] QC samples diluted to 100 pg/ml for hEGF1-48 and to 200 pg/ml for hEGF1-53.

Stability of Quality Controls

In the hEGF1-48 assay, the values for the QCs following the freeze-thaw cycles were 56.9, 119, 175, and 1060 pg/ml compared with validated values of 45.2, 109, 192, and 1080 pg/ml, respectively. In the hEGF1-53 assay, the values for the QCs following the freeze-thaw cycles were 177, 518, 707, and 985 pg/ml compared with validated values of 198, 471, 722, and 1030 pg/ml, respectively. Because the results were all within the acceptable QC ranges of ±2 SD of the mean, this indicates that samples would be stable through three freeze-thaw cycles. In addition, no significant changes in immunoreactive concentrations of hEGF1-48 or hEGF1-53 were observed after storage at −70°C for a period of up to six months.

Cross-reactivity

Cross-reactivity of the two ELISAs with the hEGF1-47 fragment and other possible cross-reactants was tested (human TGF_{α} and TGF_{β}, mouse EGF, human TNF_{α}, mouse $IL_{1\beta}$, PDGF ββ chain, and basic FGF). In the hEGF1-48 assay at concentrations of 100, 200, and 500 pg/ml, hEGF1-53 cross-reactivity ranged from 21.6–31.2% and hEGF1-47 cross-reactivity ranged from 9.6–14.5%. In the hEGF1-53 assay at concentrations of 500 pg/ml, 1000 pg/ml and 10 ng/ml, hEGF1-48 cross-reactivity ranged from 82.4–92.3% and hEGF1-47 cross-reactivity ranged from 18.2–34.2%. The other cross-reactants were tested at a concentration of 1, 10, and 100 ng/ml in both the hEGF1-48 and the hEGF1-53 assay. These possible cross-reactants including mouse EGF (mEGF1-53) at the concentration tested produced responses that were less than that of the lowest standard for each assay.

Application to Pharmacokinetic Study

The practicality of the present two ELISA methods were tested by quantitating the immunoreactive hEGF levels in rat plasma following an intravenous bolus dose of 100 μg/kg hEGF1-48 or hEGF1-53. Immunoreactive responses in the rat plasma samples collected prior to dosing of either peptide were below the detection limit. The kinetic profiles of the two pep-

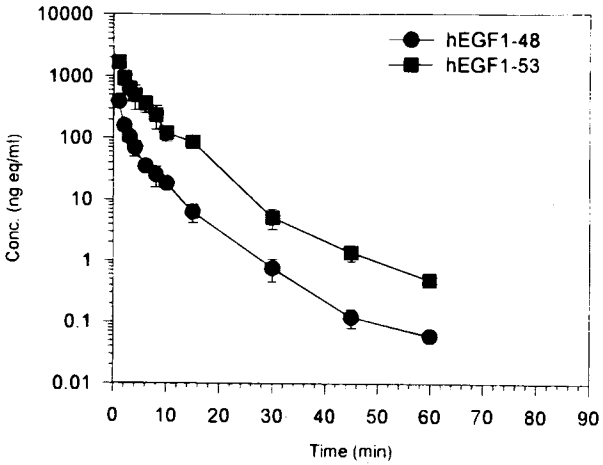

Fig. 2. Comparative Plasma Profiles of hEGF1-48 and hEGF1-53 Following a 100 μg/kg IV Bolus Dose in Male Wistar Rats.

tides were different (Figure 2). The hEGF1-48 was cleared at a CL_{tot} of 57.2 ± 2.1 ml/min/kg with a terminal elimination half life (t1/2) of 5.6 ± 1.4 min, while hEGF1-53 was cleared at a 5-fold slower rate of 11.2 ± 2.7 ml/min/kg with a terminal half life of 13.2 ± 5.0 min (Table 2). These results suggest that deletion of the C-terminal pentapeptide of hEGF1-53 had a dramatic effect on the clearance of the peptide at a 100 μg/kg dose.

Application to Analysis of Endogenous EGFs

The present two methods were adapted to measure the immunoreactive EGF levels (ng eq/ml) in urine and plasma of several different animal species (Table 3). The average levels of immunoreactive EGF in human urine and plasma detected by the hEGF1-53 assay were 51.7 ± 4.8 and 0.12 ± 0.04 ng eq/ml, respectively. Detectable levels of hEGF1-53 equivalents were found in the urine of the five non-human species tested, although the highest average level found in rabbit urine was only one-fifth the amount found in human urine. Only dog and monkey plasma contained detectable levels of hEGF1-53 equivalents. The average levels of immunoreactive EGF in human urine and plasma detected by the hEGF1-48 assay were 11.8 ± 1.7 and 0.03 ± 0.01 ng eq/ml, respectively. Very little, if any, immunoreactive EGF was found in the urine and plasma of the other 5 species tested. The urine sample values are actual and are not normalized for creatinine content.

Table 2. Pharmacokinetic Parameters of hEGF1-48 and hEGF1-53 in Fasted Male Wistar Rats Following an IV Bolus Dose of 100 μg/kg hEGF1-48 or hEGF1-53 Mean (±SD), n = 4

Kinetic Parameters	hEGF1-48	hEGF1-53
t1/2 (min)	5.6 (1.4)	13.2 (5.0)
$AUC_{0-\infty}$ (ng·min/ml)	1570 (220)	7727 (1587)
CL_{tot} (ml/min/kg)	57.2 (2.1)	11.2 (2.7)
MRT (min)	3.3 (0.4)	4.9 (0.4)
V_{ss} (ml/kg)	188 (25.6)	47.6 (8.0)

Table 3. Concentrations (Nanogram Equivalents per ml) of hEGF1-48 and hEGF1-53 Found in Urine and Plasma

Sample	hEGF1-48 (ng eq/ml)	hEGF1-53 (ng eq/ml)
hUrine (n = 6)	8.6–14.3	44.9–57.8
hPlasma (n = 8)	0.01–0.07	0.07–0.17
mUrine (n = 3)	BLQ	0.00–10.3
mPlasma (n = 3)	BLQ	BLQ
rUrine (n = 3)	BLQ	0.00–6.70
rPlasma (n = 3)	BLQ	BLQ
dUrine (n = 3)	BLQ	0.00–6.70
dPlasma (n = 3)	0.00–0.01	0.09–0.20
rbUrine (n = 3)	BLQ	6.60–9.80
rbPlasma (n = 3)	BLQ	BLQ
cmUrine (n = 3)	BLQ	0.00–9.70
cmPlasma (n = 3)	0.00–0.01	0.00–0.09

Note: BLQ: below limit of quantitation; h: human; m: mouse; r: rat; d: dog; rb: rabbit; and cm: cynomolgus monkey.

DISCUSSION

The present investigation describes the development and validation of two separate sensitive ELISA methods for quantitation of hEGF1-48 and hEGF1-53 in rat plasma. The applications of these methods to pharmacokinetic study and measurement of endogenous immunoreactive EGF responses in the biological fluids of several different animal species are also demonstrated. The readily available mouse monoclonal anti-hEGF1-53 antibody (UBI catalogue #05-109 type H), used in both assays as the primary antibody efficiently captures both hEGF1-48 and hEGF1-53 spiked in rat plasma. The in-house polyclonal rabbit anti-hEGF1-48 antibody (anti-hEGF1-48A) used to detect the hEGF1-48 cross-reacted weakly with hEGF1-53, necessitating the development of a separate but similar assay for hEGF1-53. A commercially available polyclonal goat anti-hEGF1-53 antibody in combination with the mouse monoclonal did not work in the hEGF1-53 assay system, as the background reaction with control rat plasma was too high. However, a second, different in-house rabbit anti-hEGF1-48 antibody (anti-hEGF1-48B) in combination with the mouse monoclonal antibody produced an excellent ELISA assay for hEGF1-53.

While the hEGF1-53 ELISA assay does detect hEGF1-48 adequately, the sensitivity of this assay was six fold lower than that of the preferred original hEGF1-48 ELISA assay. A goat anti-rabbit peroxidase conjugated antibody is used for spectrophotometric detection, rendering both assays very sensitive. Both assays are reliable, inexpensive and highly sensitive with a limit of quantitation of 7.8 pg/ml for hEGF1-48 and 62.5 pg/ml for hEGF1-53. Sample analysis can be completed in one working day thus allowing fast sample through put. Compared to a commercially available hEGF assay kit costing approximately four dollars per well, the low cost of our present ELISA methods (4 cents/well) makes it affordable to analyze large numbers of plasma samples for pharmaceutical and diagnostic research.

Maintaining consistency in the composition of plasma matrix between standards, quality controls, and unknown samples to be analyzed is important for accurate quantitation. Rat

Table 4. The Effect of Artificial Matrix on Determination of hEGF1-48 and hEGF1-53 Quality Control Concentrations

	hEGF1-48			hEGF1-53		
	Predicted (pg/ml)	Observed (pg/ml)	%RE	Predicted (pg/ml)	Observed (pg/ml)	%RE
Ratio (PBS-Gel/Plasma)						
No (0%/100%)	125	129	2	500	498	0.5
Low (25%/75%)	125	114	9	500	504	1
Medium (50%/50%)	125	158	27	500	574	15
High (75%/25%)	125	217	73	500	615	23

plasma from more than six separate sources were found not to interfere with either assay system, therefore the dilution of unknown samples with control rat plasma is a valid process. In addition, the use of control rat plasma, which had a basal immunoreactive response below the limit of quantitation (Table 3), as a diluent in our system for the preparation of standards, quality controls and unknown samples is crucial. In Table 4, the standard curve was produced by dilution with control rat plasma, while the quality control samples (125 pg/ml for the hEGF1-48 assay and 500 pg/ml for the hEGF1-53 assay) were prepared with increasing amounts of an artificial matrix PBS/Gel (0.01 M PBS containing 0.1% gelatin from porcine skin and 0.1% thimerosal). It was discovered that use of artificial matrices for sample preparation or dilution leads to errors in the determination of hEGF concentrations, depending on the percentage of plasma present in the samples. Many ELISA assay methods reported previously (16–21) were not subjected to this type of validation criteria. The mechanism of the dilution effect associated with the artificial matrix is unknown.

The assays for hEGF1-48 and hEGF1-53 showed some cross-reactivity with each other, but less so for hEGF1-47. No cross-reactivity was observed with other proteins even at very high concentrations of 100 ng/ml in either the hEGF1-48 or the hEGF1-53 assay. It is interesting to note that mouse EGF (mEGF) does not cross-react with either assay, even though it is 70% homologous to hEGF1-53 (3). Human transforming growth factor alpha ($hTGF_\alpha$) also does not cross-react with either assay, even though it binds to the same receptor as EGF (5).

The present ELISAs were applied to a plasma pharmacokinetic study in rats. Based on the immunoreactive EGF levels, it is evident that hEGF1-48 (CL_{tot}: 57.2 ml/min/kg) was cleared approximately five-fold faster than hEGF1-53 (CL_{tot}: 11.2 ml/min/kg) (Table 2). The plasma concentration-time curves clearly reflect the difference (Figure 2). The difference in clearance of hEGF1-48 and hEGF1-53 in rats found in the present study substantiates the results of our previous investigation using a commercially available kit for disposition analysis of these two peptides in rats (24). Appropriate dilutions of the wide-ranged hEGF plasma concentrations (spanning 5–6 log cycles) with control rat plasma were performed. For example, plasma samples from the hEGF1-48 and hEGF1-53 study at 1 min postdose had to be diluted 1000- and 3000-fold, respectively, while only a 2-fold dilution was used for time points after 45 min.

These validated EIA assays for hEGF1-48 and hEGF1-53 were then used to measure the relative immunoreactive levels of endogenous EGF equivalents present in urine and plasma samples from different animal species (Table 3). Basal levels of immunoreactive hEGF are much higher in human urine (45–58 ng eq/ml) than in human plasma (less than 0.2 ng eq/ml). The levels found in human urine and plasma were similar to those previously reported (22–25), indicating the efficacy of the two assays for use in clinical application. Plasma hEGF is mainly associated with platelets and liberated into plasma during blood clotting (26). It is known that the predominant species of plasma hEGF (urogastrone) are of high molecular weights (27,28), while those in the human urine are known to be mainly hEGF1-53 and its proteolytic products hEGF1-52, hEGF1-51, and hEGF1-50 (29,30). Detectable EGF levels were found in the urine of five non-human species tested and in plasma of dog and cynomolgus monkey by the hEGF1-53 assay, but not by the hEGF1-48 assay. The fact that no immunoreactive EGF could be detected in the plasma of mice, rabbits, and rats under the present assay systems suggest these species may be suitable animal models for pharmacokinetic study for hEGF1-53 or its derivatives.

In conclusion, two rapid, sensitive, and highly reproducible ELISA methods for hEGF1-48 and hEGF1-53 were developed and validated. These ELISAs were used to investigate EGF disposition kinetics and to assay endogenous EGF levels in the biological fluids of several different animal species, including humans. Both ELISAs will be invaluable tools for evaluating the roles of hEGF in human physiology and disease.

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