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Considerations in the development of a sensitive HPLC assay for human epidermal growth factors in human plasma ☆

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Abstract

A sensitive assay was developed for human epidermal growth factors (hEGF) 1-48 (dosed), hEGF 1-53 (endogenous), without interference from potential metabolites hEGFs 1-47 or 1-46. Spiked human plasma samples were injected directly, utilizing on-line immunoaffinity HPLC (anti-hEGF) clean-up. No change in capacity was noted after 81 cycles. After release from the immunoaffinity column, the fragments were further resolved by strong cation-exchange (SCX) via a column switching valve. Method development also required interfacing immunoaffinity, ion-exchange, and detection components. Immunoassays on collected fractions yielded a detection limit of 1 $\mu g\,ml^{-1}$, although a detection limit of 75 $pg\,ml^{-1}$ appears feasible.

Keywords: Tandem immunoaffinity and SCX HPLC; Trace quantitation; Direct plasma injection; Epidermal growth factor

1. Introduction

Human epidermal growth factor (hEGF 1-53, Fig. 1) is a 53 amino acid protein implicated as having an important role in cell division and tissue growth [1]. hEGF 1-48, an analog of hEGF 1-53 in which residues 49-53 have been removed, is under evaluation as a therapeutic agent for the treatment of necrotizing enterocolitis. Prior to a detailed study of the disposition of these growth factors, an assay for dosed hEGF 1-48, endogenous hEGF 1-53, and expected metabolites of hEGF 1-48 was required. Cross-reactivity of these fragments precluded use of a direct immunoassay for each component in plasma. Therefore, a method affording separation and quantification of fragments was needed; compatibility with automation was considered advantageous.

A major objective in the sample clean-up from plasma was isolation of hEGF fragments from other matrix proteins. In-line HPLC immunoaffinity chromatography (IAC), prepared from polyclonal antibody IgG directed against hEGF 1-48, allowed retention of only hEGF related fragments from direct injection of human plasma. Use of low pressure IAC for preparative isolation of EGF from human urine or mouse submaxillary gland has been reported [2]. Its analytical use in tandem with additional HPLC is novel.

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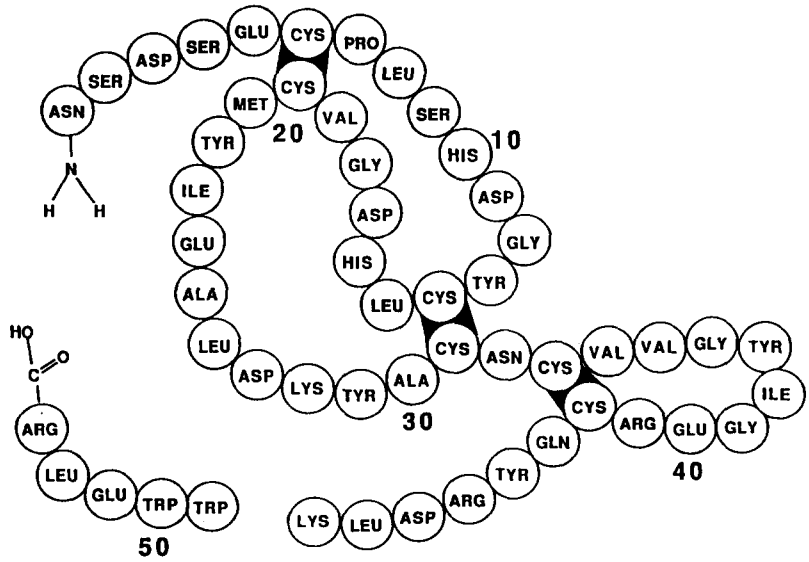

Fig. 1. Amino acid sequence and disulfide pairings for hEGF [1].

Subsequent release of the purified analytes was then required. It was important to retain the immunological activity of the IAC antibodies after elution to allow repeated use of the immunoaffinity (IA) column. In addition, it was important to retain the immunoreactivity of the hEGF analytes after elution, to afford their detection via an immunoassay, if necessary.

Because the hEGF analytes eluted from the IA column without resolution, additional chromatography was required. In addition to hEGFs 1-53 and 1-48, potential carboxypeptidase cleavage products hEGFs 1-47, and 1-46 were also of interest. Carboxypeptidase activity on hEGF 1-53 in urine has been reported [3]. Based upon the detection of minor amounts of the methionine^{21} S-oxide analog of murine EGF 1-53 in EGF from murine submaxillary gland preparations [4], an oxidation product of hEGF 1-48, hEGF 1-48 methionine^{21} S-oxide, was also expected.

A reverse-phase HPLC (C18; acetonitrile (0.1% TFA) gradient) assay, using absorbance detection (220 nm), has been reported [5] for hEGFs 1-53, 1-48, 1-47, and 1-46. However, resolution of 1-48 (dosed, active) and 1-46 (inactive in bioassays) was inadequate for detection using an immunoassay. Greater than baseline resolution was necessary for an immunoassay, as fraction collection of peaks not resolved completely would produce cross-contamination between analytes, and result in erroneous data.

Detecting the resolved analytes also posed a challenge. Owing to their potency, low plasma concentrations of dosed or endogenous analytes were anticipated (detection limits of about 50 pg ml^{-1}, 10 femtomoles ml^{-1}). Consequently, an ultrasensitive immunoassay, LC/MS/MS, or derivatization/laser-induced fluorescence (LIF) would be required.

2. Experimental

2.1. Chemicals and reagents

Reagent grade potassium chloride and monobasic potassium phosphate, phosphoric acid (85%) and trifluoroacetic acid (98.5%) were from EM Industries, Inc., Gibbstown, NJ, USA. Sodium azide (practical) was from Eastman Organic Chemicals, Rochester, NY, USA. Sodium chloride (0.9%, USP) was from Baxter Healthcare Corp., Deerfield, IL, USA. Acetonitrile and methanol (HPLC grade), and sodium cyanide (reagent grade) were from Mallinckrodt Chemical, Inc., Paris, KY, USA. Water was purified using a Milli-Q system (Millipore Corp., Milford, MA, USA). Glycine (99%), carboxypeptidase B solution (type I-DFP), and thermolysin were from Sigma, St. Louis, MO, USA. Hydrogen peroxide (USP, 3%) was from Parke-Davis, Warner-Lambert Co., Morris Plains, NJ, USA. Naphthalene-2,3-dicarboxaldehyde was purchased from Oread Laboratories, Lawrence, KS, USA. Human plasma (0.04% EDTA) was from Inter-

state Blood Bank, Inc., Memphis, TN, USA. hEGFs 1-48 and 1-53 were supplied by Parke-Davis Pharmaceutical Research, Warner-Lambert Co., Ann Arbor, MI, USA.

Adjuvax was purchased from Alpha-Beta Technology, Inc., Worchester, MA, USA. Freund's complete/incomplete adjuvant was from ICN ImmunoBiological, Irvine, CA, USA. Bovine serum albumin (BSA), 2-aminoethanol, cyanogen bromide Sepharose-4B, porcine gelatin, sodium bicarbonate (ACS reagent), and trizma were from Sigma, St. Louis, MO, USA. Recombinant Protein-A on Sepharose-4B was from Repligen, Cambridge, MA, USA. Goat anti-rabbit IgG/horseradish peroxidase was from Rockland, Gilbertsville, PA, USA. 3,3′,5,5′-Tetramethylbenzidine was from Kirkegaard and Perry Laboratories, Gaiphersburg, MD, USA.

2.2. General apparatus

The SCX column was a 4.6 × 20 mm polysulfoethyl aspartamide guard column (The Nest Group, Inc., Southborough, MA, USA). Sample injection utilized an ISS-200 LC sample processor (Perkin-Elmer Corp., Norwalk, CT, USA) fitted to pump # 1 (see below). For UV detection (220 nm), an SP8480 XR was used with a ChromJet integrator (Spectra-Physics, San Jose, CA, USA).

Electrospray ionization mass spectrometry (EIMS) was performed using a Fisons (Manchester, UK) VG Biotech Trio-2000 quadrupole mass spectrometer with an m/z range of 3000. Spectra were obtained using an ESI voltage of 3 kV, and a cone voltage of 60 V. The source temperature was maintained at 65 °C. The injection solvent was methanol–water–acetic acid (49.5:49.5:1, v/v/v).

For LC/MS, a Michrom UMA HPLC (Michrom BioResources, Inc., Pleasanton, CA, USA) fitted with a Reliasil C18 microbore column (0.5 × 150 mm, 300 Å) was used in-line with a Finnigan MAT TSQ70S mass spectrometer (San Jose, CA, USA) with a Vestec electrospray interface.

2.3. Preparation of immunoaffinity column

Six New Zealand female rabbits were immunized over multiple interdermal sites of their backs with 200 μg of hEGF 1-48 and 200 μg Adjuvax in 1 ml Freund's complete adjuvant. Fourteen and 28 days later, additional immunizations in 1 ml Freund's incomplete adjuvant containing 200 μg EGF and 200 μg Adjuvax were performed. The animals were bled 10 days later. Anti-serum from one rabbit (# 250), giving the highest titer, was loaded on a protein A-Sepharose column (2 × 5.5 cm, glass) and the IgG eluted with 20 ml glycine buffer (0.1 M, pH 3.0). The eluted IgG was then dialyzed against sodium bicarbonate buffer (0.1 M, pH 8.8).

Polyclonal rabbit anti-hEGF 1-48 IgG (57 mg) in 13.6 ml of buffer A (0.1 M sodium bicarbonate, pH 8.8, containing 0.5 M sodium chloride) was added to 5.7 g of CNBr treated Sepharose 4B gel in 80 ml of 1 mM HCl, in a 25 × 220 mm glass column. The column ends were plugged, and the column was rotated gently, mixing for 2 h at room temperature. After draining the solvent, 80 ml of buffer A containing 1 M 2-aminoethanol was added to the remaining gel. After mixing for 2 h, the column was run dry and the gel washed four times with the sequence of 100 ml of buffer A, followed by 100 ml sodium acetate (0.1 M, pH 4.0, containing 0.5 M sodium chloride).

The immunoaffinity column was prepared by packing a 4 × 24 mm stainless steel HPLC guard column with the gel until the column remained filled after HPLC flushing (0.6 ml min^{-1}) with phosphate buffered saline (0.9%, pH 7.4, PBS).

2.4. Tandem immunoaffinity and SCX system

The IA and SCX columns were connected through a VICI 10-port valve (Valco Instruments Co., Inc, Houston, TX, USA) controlled through a time sequence programmed on the ISS 200 autosampler (Fig. 2 and Table 1).

Pump # 1 (for IAC) was an SP8700 pump (Spectra-Physics, San Jose, CA, USA) operating at 0.6 ml min^{-1} using mobile phases: (1A) 0.9% sodium chloride containing 10 mM potassium phosphate (pH 7.4) with 20 ppm sodium azide; (1B) 100 mM glycine (hydrochloric acid to pH 2.50)–methanol (1:1, v/v). The gradient program is described in Table 1.

Pump # 2 (for SCX) was a series 410 LC pump (Perkin-Elmer Corp., Norwalk, CT, USA) operating at 1 ml min^{-1} using mobile phases: (2A) 5 mM potassium phosphate (pH 3.0); (2B) 600 mM potassium chloride and 5 mM potassium phosphate (pH 3.0). The gradient program is described in Table 1.

Where indicated, fractions were collected by hand over a 1.5 min interval centered at the retention time of each compound.

2.5. Quantitation of hEGF reference standards

The hygroscopic nature of hEGF precluded its accurate weighing. Instead, the amount of hEGF used in reference stock solutions were determined using a standard procedure for protein chemistry. The UV absorbance of the stock solution, showing a single peak on HPLC, was related to its extinction coefficient.

The purity of hEGF 1-48 (97 + %, with no single impurity greater than 1.5%) was confirmed by: (1) quantitative amino acid analysis demonstrating the correct amino acid composition for hEGF 1-48, and (2) demonstration of a single compound by chromatography. The extinction coefficient of hEGF 1-48 was determined by relating the UV absorbance of the above sample in solution to the amount of hEGF 1-48 present (as indicated from quantitative amino acid analysis [6a] provided by the Protein Structure Research Laboratory, University of California, Davis, USA). The extinction coefficient for hEGF 1-48 was also used as an estimate for hEGF 1-47.

In contrast, the extinction coefficient used for quantitating hEGF 1-53 stock solutions was derived from theoretical calculations based on individual amino acid extinction coefficients; this approximation appeared reasonable, as comparable calculations for hEGF 1-48 were in close agreement with the observed experimental value. Absorbance values used for 1 mg ml^{-1} in water: hEGF 1-48 = 1.22 at 276 nm; hEGF 1-53 = 2.9 at 280 nm [6b].

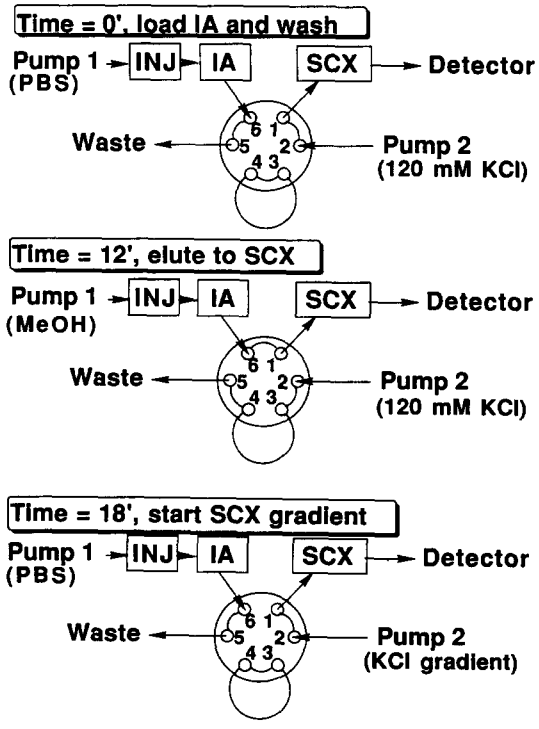

Fig. 2. Summary of some timed events and connections for the tandem IAC-SCX column switching system.

2.6. Preparation of fragment standards

Reference standards for hEGF fragments were prepared using established enzymatic or chemical treatment of hEGF 1-48. These standards were characterized by mass spectrometry and by reverse-phase HPLC retention times. Significant fragrament adherence during the purification of these small scale reactions precluded accurate determination of the yields. Therefore, yields were estimated based on HPLC product peak area, relative to that of the starting material (hEGF 1-48).

hEGF 1-47

hEGF 1-48 (0.4 ml of 0.43 mg ml^{-1} in PBS, pH 7.4) was added to 0.1 M ammonium bicarbonate (1 ml, pH 8). Following treatment [5] with 0.015 ml of carboxypeptidase B solution (type I-DFP, 1 mg per 0.3 ml), complete conversion of hEGF 1-48 to hEGF 1-47 occurred after 15 min at room temperature. Purification by preparative HPLC (Whatman 10 × 250 mm, C18) using a linear gradient (4 ml min^{-1}) from 20 to 50% acetonitrile (0.1% TFA) in water over 30 min afforded the product, eluting at 20 min. LC/MS (acetonitrile (0.1% TFA) gradient): m/z calculated 5317.0; m/z observed, 5314.2.

hEGF 1-46

hEGF 1-48 (0.43 mg) in 1.0 ml of 0.1 mM ammonium bicarbonate (pH 8) was treated with 0.025 ml of thermolysin (1 mg ml^{-1} in 20 mM potassium phosphate, pH 7.4 [6c]). The reaction was kept at room temperature until strong cation-exchange (SCX) chromatography (gradient from Table 1) indicated that the product comprised 50% of the reaction mixture. Adding additional ammonium bicarbonate buffer was sometimes necessary to increase the reaction rate, perhaps owing to a loss in buffering capacity of the reaction mixture. LC/MS (acetonitrile (0.1% TFA) gradient): m/z calculated 5203.7; m/z observed, 5199.9.

Table 1
Pump gradient and column switching valve programs for the tandem IAC-SCX system

Time (min)	Switching valve position	Mobile phase composition	
		Pump #1 (P1)	Pump #2 (P2) (25% acetonitrile and)
0–8	P1/IA to waste P2 to SCX	PBS, pH 7.4	120 mM KCl
8–8.1	P1/IA to waste P2 to SCX	MeOH/glycine (step gradient)	120 mM KCl
12	P1/IA to SCX P2 to waste	MeOH/glycine	120 mM KCl
18	P1/IA to waste P2 to SCX	MeOH/glycine	120 mM KCl
18–18.1	P1/IA to waste P2 to SCX	PBS, pH 7.4 (step gradient)	120 mM KCl
18–30	P1/IA to waste P2 to SCX	PBS, pH 7.4	120 mM KCl
19–24	P1/IA to waste P2 to SCX	PBS, pH 7.4	120–150 mM KCl (linear gradient)
24–25	P1/IA to waste P2 to SCX	PBS, pH 7.4	150–210 mM KCl (linear gradient)
25–32.5	P1/IA to waste P2 to SCX	PBS, pH 7.4	210–240 mM KCl (linear gradient)
32.5–32.6	P1/IA to waste P2 to SCX	PBS, pH 7.4	240–120 mM KCl (linear gradient)
32.6–35.6	P1/IA to waste P2 to SCX	PBS, pH 7.4	120 mM KCl

IA = immunoaffinity column; SCX = strong cation-exchange column; MeOH–glycine = MeOH–glycine (100 mM, pH 2.5) (1:1, v/v).

hEGF 1-48 (methionine^{21} S-oxide)

To an aqueous solution of hEGF 1-48 (0.2 ml of 0.1 mg ml^{-1}) in a 1.5 ml polypropylene Flex tube was added 0.08 ml of 3% hydrogen peroxide, and the mixture was shaken. After 10 min, the product (50% yield) was purified immediately by RP HPLC (retention time approximately 1 min earlier than hEGF 1-48) using a Whatman 10 × 250 mm C18 column, with a linear gradient over 30 min from 20% acetonitrile (0.1% TFA) to 50% acetontrile (0.1% TFA). m/z calculated 5461.2; m/z observed 5461.8.

2.7. Sample preparation

Plasma, treated with EDTA (0.04% final concentration), was frozen (−20 °C) prior to use. It was thawed, spiked with EGF, centrifuged (10 min at 10 000g) and then filtered (polysulfone, 0.2 μm, 13 mm syringe filter, Whatman Laboratory Division, Clifton, NJ, USA).

2.8. Enzyme linked immunosorbent assay (ELISA) for hEGFs 1-48 or 1-53

MicroTiter plates (96 well) were treated with 25 ng per well of mouse monoclonal anti-hEGF 1-48 IgG for 18 h at 4 °C. The plates were treated for 1 h at 37 °C with 2% BSA to block non-specific protein binding sites. Samples (0.2 ml) were added and incubated at room temperature for 2 h. Rabbit polyclonal anti-hEGF 1-48 (diluted 1/750, 0.2 ml) was added and incubated for 1 h at room temperature; 0.2 ml of goat anti-rabbit/horseradish peroxidase (1/4000 dilution) was added and incubated for 1 h at room temperature. 0.2 ml of 3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide [7] was the added, and the plate was shaken gently for 30 min at room temperature. Aliquots (0.05 ml) of 1 M sulfuric acid were added, and the plate was read at 450 nm using a Kmin Autoreader (Caymen Chemical, Ann Arbor, MI, USA).

3. Results and discussion

3.1. Sample clean-up

Spiked plasma samples were filtered (no adherence to the filter was detected at 500 pg ml^{-1}) and injected directly onto the IAC. hEGF analytes were retained and other plasma components were flushed to waste by washing the column with PBS (pH 7.4; 4.8 ml). The analytes were then eluted with methanol–glycine hydrochloride (100 mM, pH 2.5; 6 ml) (1:1, v/v). In addition, because no change in IAC capacity for hEGF was detected, antibody immunoreactivity was not altered irreversibly by the elution conditions. This allows repeated use of the in-line IAC and also afforded analyte detection via ELISA.

Although the hEGF analytes were isolated from other matrix components using IAC, they were eluted from the IAC without further resolution, one from the other. Therefore, additional chromatography was required.

3.2. Analyte resolution

An established RP HPLC method [5] did not demonstrate adequate resolution of hEGFs 1-48 (dosed, active) and 1-46 (inactive metabolite) in a mixture also containing hEGFs 1-53 and 1-47; an alternative method was needed. The difference in the number of positively-charged residues between hEGFs 1-53 (Arg^{53}), 1-48 (Lys^{48}), and 1-47 or 1-46 suggested use of SCX HPLC for their resolution. These four hEGF analytes in PBS were resolved with greater than base-line resolution using an SCX column with a potassium chloride gradient at pH 3 (not shown).

Although the fragments of major interest were resolved better by using SCX rather than RP-HPLC, one potential metabolite, from the oxidation of methionine^{21} in hEGF 1-48, was resolved only by using RP-HPLC. hEGF 1-48 (Met^{21} S-oxide) was prepared by careful hydrogen peroxide oxidation [8] of hEGF. It co-eluted with hEGF 1-48 using SCX analysis, whereas the two analytes were resolved (hEGF 1-48 (Met^{21} S-oxide) eluting earlier) using RP-HPLC. Owing to the low percentage of murine EGF 1-53 reported in the Met^{21} S-oxide form, the potential for interference of such oxidation metabolites was considered to be insignificant. Therefore, the tandem IAC-SCX assay was not changed to afford their separation.

It should be noted, however, that if subsequent resolution of hEGFs 1-47 and 1-46, or 1-48 (Met^{21} S-oxide) was required, the appropriate peaks could be directed from the SCX to a subsequent reverse-phase column (via a switching valve) to afford additional separations.

3.3. Interfacing IAC with SCX chromatography; UV detection

Early studies regarding IAC utilized samples with hEGF concentration high enough (< 10 μg ml^{-1}) that UV absorbance (220 nm) could be used for detection, and that non-specific protein binding was not a factor. The EGF fragments were eluted from the IA column with methanol–glycine (0.1 M, pH 2.5) (1:1, v/v). Fractions containing EGF were collected, and aliquots were than injected (200 μl) either onto the SCX system developed or onto an RP column [2]. Analyte peak shape remained sharp with SCX chromatography, whereas considerable peak broadening occurred with RP-HPLC. Apparently, the injection solvent, methanol–glycine hydrochloride, was too strong for use in RP-HPLC. Therefore, use of SCX chromatography in tandem with IAC offered an advantage (relative to RP HPLC) of tolerating larger injection volumes of the IAC elution solvents, such as those encountered using a column switch.

The IAC sample clean-up was used in tandem with the SCX column via a column switching valve interface (Fig. 2). The timings of the column switches and pump gradient programs were developed to minimize plasma interferences and maximize analyte recovery, while also affording reproducible results with a minimum run time (Table 1). The entire system was automated, with the autosampler used to control the timing of the switching valve and the initiation of individual pump gradients.

An additional goal was to minimize IAC column pressure, as its Sepharose beads were more pressure sensitive than silica supports used for HPLC columns. Although much of the early SCX method development was done with an analytical column (4.6 × 250 mm), its use in tandem with the IA column resulted in high IA column back-pressure (maximum approx. 2500 psi). However, replacement of the analytical SCX column with an SCX guard column (4.6 × 20 mm) in the tandem IAC-SCX system produced comparable analyte resolu-

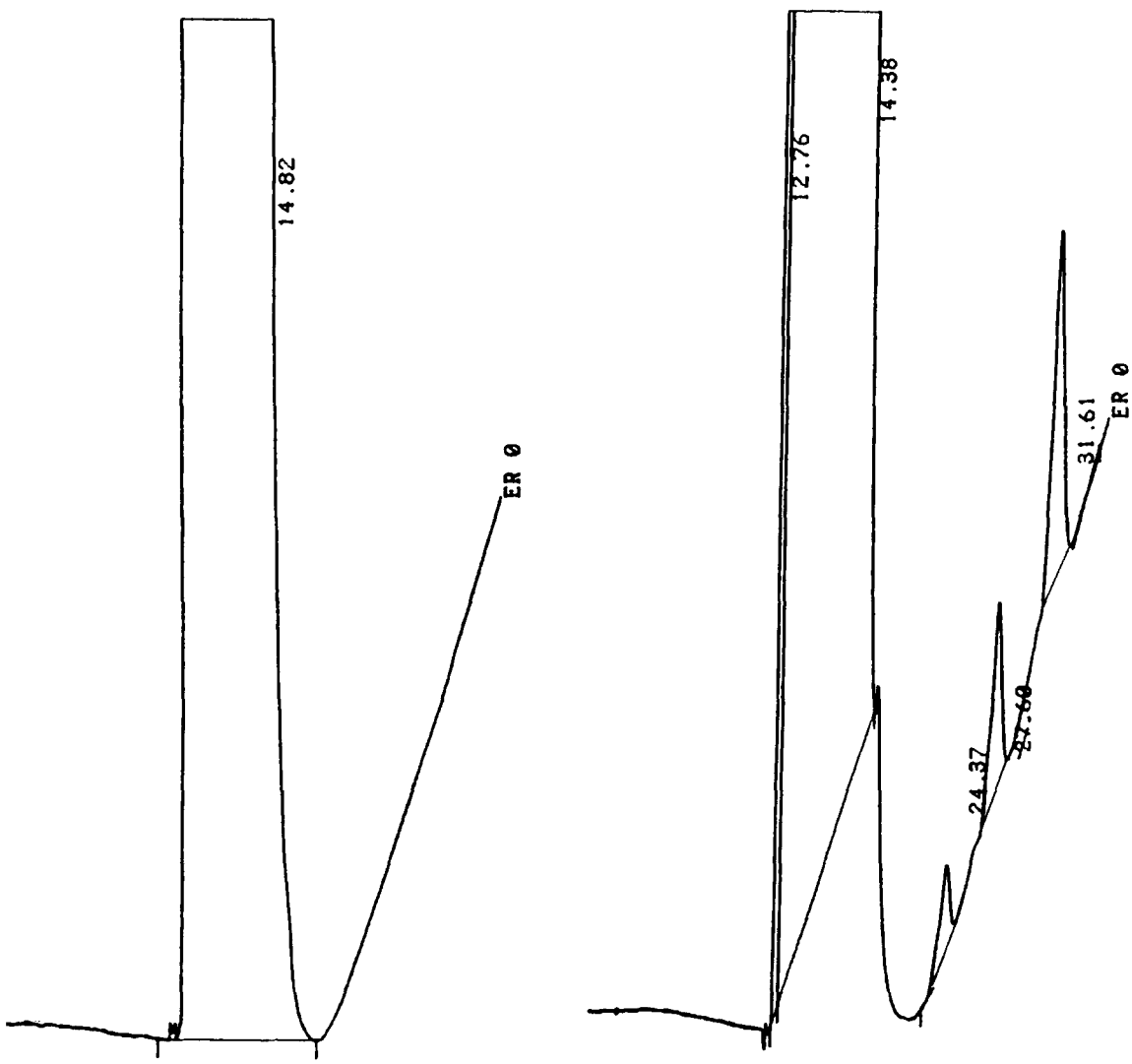

Fig. 3. Chromatograms of 200 μl injection into the tandem IAC-SCX system: (a) blank human plasma; (b) human plasma spiked with hEGFs 1-47 (0.5 μg ml^{-1}), 1-48 and 1-53 (1 μg ml^{-1}) in order of elution. See Table 1 for chromatographic conditions.

tion with a much lower back-pressure (maximum approx. 1200).

Using the tandem method developed, no UV active interferences were observed for plasma samples spiked with hEGFs 1-53, 1-48, and 1-47 (Fig. 3). Insufficient supplies of hEGF 1-46 precluded its inclusion in these studies. However, based on its resolution from hEGF 1-48 ($R = 4.7$) in buffer using only SCX chromatography, it is feasible that it also could be resolved from hEGFs 1-48 and 1-53 in plasma samples in the tandem system.

A feature evident in chromatograms using UV absorbance detection (i.e. Fig. 3) is the prominent rise in the base-line occurring with increasing KCl concentrations during the KCl gradient. Because this rise is caused by KCl absorbance at 220 nm, it was not anticipated as a problem using either fluorescence, of an immunoassay, for detection.

The tandem IAC-SCX method appeared to be suitable for fraction collection. The sample run time was 42 min, injection-to-injection, with greater than base-line resolution of ana-

lyte peaks. Peak widths were less than 1.5 min, and analyte within-day retention times varies by no more than 18 s; frequent minor modifications of the method precluded accurate determination of day-to-day variability. Additionally, recovery studies on a limited number of samples were encouraging, as plasma spiked with 1 μg ml^{-1} of hEGF 1-48 and 1-53 produced recoveries greater than 90%.

The tandem IA-SCX system also appeared suitable for immunoassay detection. hEGF 1-48 concentrations were indistinguishable (±10%) in ELISA for equivalent samples (1 μg ml^{-1} in PBS) either injected into the tandem system and fraction collected, or diluted to the volume of the fractions collected. This indicated that significant analyte immunoreactivity was not altered irreversibly by tandem IAC-SCX chromatography.

Finally, our preliminary study suggests that tandem IAC-SCX may be reliable and efficient. No change in capacity was apparent after injections of 18 plasma samples and 63 buffer samples.

3.4. Attempted analyte detection via derivatization/fluorescence

Initially, the strategy planned to detect trace levels of hEGF and fragements involved post-column derivatization of analyte primary amino groups with naphthalene-2,3-dicarboxyaldehyde (NDA)/sodium cyanide, to produce highly fluorescent 1-cyanobenz[*f*]isoindole (CBI) derivatives [9]. The fluorescent properties of CBI products make them amenable to helium–cadmium laser-induced fluorescence detection.

In a model study for the derivatization of hEGF lysine side chains, Nα-Boc-Lys-OMe treatment with NDA–sodium cyanide produced an intense fluorescence (ex. 433 nm, em. 490 nm) in cuvettes. However, the analogous reaction of hEGF 1-48 appeared more complex than anticipated initially. Therefore, an alternative detection method was evaluated for trace quantification.

3.5. Analyte detection via fraction collection/ELISA

Analytes were detected by ELISA of collected fractions. The ELISA limits of quantitation for solution standards of hEGFs 1-48 or 1-53 were 8 or 125 pg ml^{-1}, respectively. However, preliminary studies of hEGF 1-48 spiked into the SCX mobile phase (Table 1) achieved a detection limit of only approximately 1 μg ml^{-1}. The 100 000 fold loss in sensitivity was attributed to analyte adherence to the polypropylene fraction collection tubes. The addition of 0.2 ml PBS–0.1% porcine gelatin to the tube decreased the detection limit to approximately 500 pg ml^{-1}, as the gelatin afforded effective competition for the protein binding sites on the tube, without interfering with the ELISA.

Later studies revealed that analyte concentrations of less than 500 pg ml^{-1} oculd be detected, but required additional sample dilution to minimize perturbations caused by the SCX mobile phase. Samples containing greater than 500 pg ml^{-1} had to be diluted with an appropriate volume of PBS–0.1% porcine gelatin to bring analyte concentrations within the log-linear portion of the standard curve. Less concentrated samples were initially assayed directly in the SCX mobile phase (i.e. pH 3, 300 mM KCl, 25% acetonitrile). Subsequent studies demonstrated that all three conditions (low pH, high salt and organic concentrations) were detrimental to the ELISA. A two-fold dilution with PBS (pH 7.4)–gelatin was sufficient to increase the pH, and dilute the salt and organic concentrations, so that detection limits of 75 pg ml^{-1} in plasma appear feasible.

The imprecision (RSD) associated with replicate (n = 3) determinations of human plasma sample, spiked with 1 μg ml^{-1} of hEGF 1-48, was 10%, suggesting that fraction collection/ELISA detection affords suitable reproducibility. Further optimization of ELISA detection limits should allow a recovery study of trace levels of hEGF. These studies are in progress.

4. Conclusions

IAC in tandem with SCX afforded greater than base-line resolution of hEGFs 1-53, 1-48, and 1-47 in samples from direct injection of human plasma. No interferences were observed. The advantage of the specificity of the IA column to recognize only HEGF 1-48 and related compounds (i.e. potential metabolites) was demonstrated (Fig. 3) by the absence of any contaminating peaks (detection at 220 nm). The potential for the durability of the system was noted, as no apparent loss in capacity occurred after injections of 18 plasma samples and 63 buffer samples. Preliminary recovery studies demonstrated greater than 90% recovery from plasma of analytes at approximately

1 μg ml^{-1}. More extensive studies are in progress.

An important issue in tandem IAC-HPLC method development is finding conditions that are both strong enough for analyte elution from the IA column, yet mild enough to prevent distortion of the second chromatographic technique [10]. One approach reported for tandem systems using IAC-RP HPLC is to introduce an additional pre-column after the IA column, to concentrate the sample prior to RP HPLC [11]. For these hEGF analytes, SCX chromatography not only produced better resolution than RP HPLC, but was also more compatible with IAC elution conditions, as no pre-column was required.

Finally, interfacing the SCX with ELISA posed some problems, but comparable problems (i.e. pH, salt) might be anticipated had derivatization been used for detection.

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