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The Design of Analytical Methods for Use in Topical Epidermal Growth Factor Product Development

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Abstract—Data from a reverse phase gradient elution HPLC assay for human epidermal growth factor (EGF) was compared and correlated with data obtained from a competitive heterogeneous radioimmunoassay (RIA). The RIA was established to measure very low concentrations of EGF in formulation drug release and compatibility studies. The HPLC assay, capable of resolving parent and possible modified or fragmented forms of EGF isolated from human urine, was studied as a potential development tool for stability and final product evaluation. As independent analytical methods, the HPLC and the RIA procedures produced correlated results when quantifying freshly prepared and certain degraded samples of EGF. The capacity of the HPLC method to serve as a stability indicating assay was examined. Degradation of EGF was induced by storage in 0·05 M phosphate buffer pH 7·4 at 25, 37 or 50°C. The same three degradation products were detected at each temperature by the HPLC method, one of which was identified as L-isoaspartyl EGF.

Epidermal growth factor (EGF) is a 53-amino acid, mitogenic polypeptide (Fig. 1) present in many mammalian species (Cohen 1962; Starkey et al 1975; Yip et al 1986), in a variety of tissues and body fluids (Hirata & Orth 1979a, b). It is one of a number of growth factors being 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). Consequently, studies are in progress to develop pharmaceutical formulations that allow administration of EGF in a biologically active form to the wound. In order to develop these products successfully, information on the physicochemical and biological status of EGF in the formulation must be obtained and changes in that status that occur over a period of time must be assessed. There are a number of specific analytical methods published in the literature that are available for quantitative analysis of EGF. These assays include an in-vivo pig assay (Chvapil et al 1988), cell culture thymidine uptake (Yip et al 1986), radioreceptor (Hirata & Orth 1979a; Yip et al 1986), radioimmuno (RIA) (Hirata & Orth 1979a), and immunofluorimetric (Personen et al 1986) assays, ELISA (Abe et al 1987) and reverse phase HPLC (Smith & O'Hare 1984; Mount et al 1985).

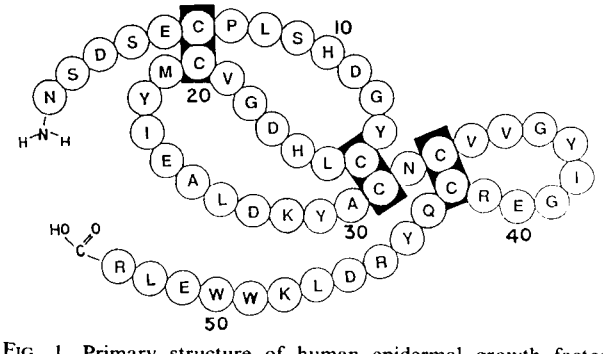

FIG. 1. Primary structure of human epidermal growth factor (Nascimento et al 1988). The single methionine residue can be found at position 21.

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During development of a topical product, appropriate analytical methods are needed to establish such product parameters as potency and content uniformity, drug release rate and product stability. HPLC has the advantages of speed, precision and accuracy and, unlike bioanalytical procedures, can differentiate between parent and biologically active degradation products. However, physicochemical methods such as HPLC do not necessarily measure biologically functional material. Small modifications in structure or conformation, undetectable by the HPLC method, may totally change the biological activity of the molecule. Unfortunately, wound healing assays designed to determine biological activity of EGF give subjective results of low precision and are difficult to quantify. Induced mitosis in cell culture is perhaps a more appropriate assay but is often time consuming and is too sensitive to formulation excipients for routine use in drug product development.

Thus, multiple analytical methods need to be used in the development of biotechnology derived pharmaceuticals. Assays based on different molecular characteristics or functions of the drug molecule can produce widely different quantitative results (Araki et al 1989); therefore results must be correlated and if possible, compared with a reliable indicator of biological activity. Such a comparison has been performed for insulin (Kroeff & Chance 1982), where an HPLC method was correlated with a rabbit hypoglycaemia bioassay. An appropriate assay for use on a formulation to measure direct biological function has yet to be developed for EGF and it is likely, as with many biotechnology derived products, that evidence of efficacy will not be available until the product enters the clinic. Until this time it is prudent to coordinate analytical development, alongside product development, to provide as much information as possible about the molecule under evaluation.

The objective of this study was to develop an HPLC assay for use as a development tool in final product evaluation and

stability analysis, and to correlate the assay with a competitive heterogeneous RIA. The RIA was established to determine very low concentrations of EGF in formulation drug release and compatibility studies and was not affected by any formulation excipients. In the reported study, analytical development has been performed using EGF isolated from human urine (Hirata & Orth 1979b). With recombinant EGF available, it is highly unlikely that a product containing an isolated protein would ever become marketed. However, yeast recombinant EGF has been reported to contain low concentrations of the same EGF fragments and oxidized forms that are found in the isolated preparation (Nascimento et al 1988). In recombinant production from yeast, the EGF fragments are produced by enzymes present in the fermentation media. For developmental studies the heterogeneous mixture of parent and related EGF molecules from urine has also provided us with a wider scope for investigating potential degradation products, and a basis for comparison of the recombinant molecules with the naturally occurring factor. Freshly voided urine has previously been subjected to reverse phase HPLC (Mount et al 1985) to establish that the different forms of EGF detected are not artifacts of the purification scheme.

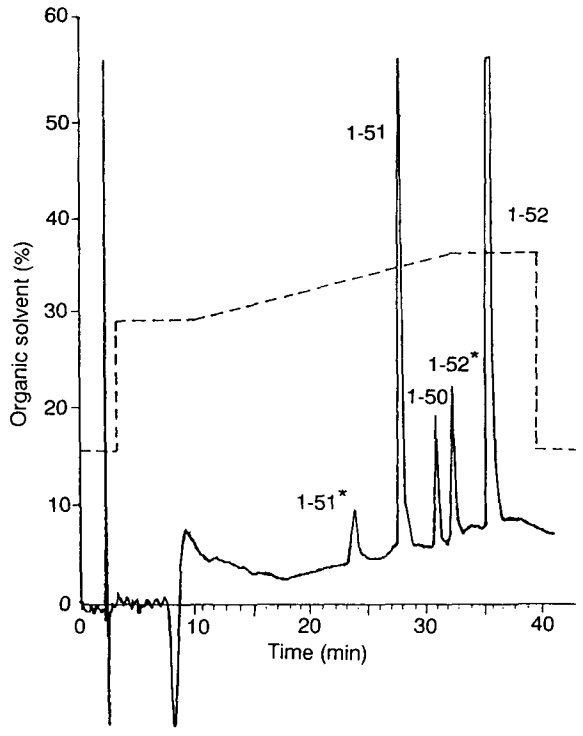

FIG. 2. HPLC of human isolated EGF showing gradient profile. 1-51* and 1-52* EGF have methionine sulphoxide at position 21. Individual peaks were identified by coinjection of standards, amino acid analysis (Table 2) and oxidation of 1-52 EGF and 1-51 EGF peak material with a freshly prepared 1 in 300 dilution of hydrogen peroxide USP.

Materials and Methods

Materials

EGF isolated from human urine was supplied by Serono Laboratories, Norwell, MA, and consisted of 1-52 EGF, 1-51 EGF, 1-50 EGF; and 1-52 EGF and 1-51 EGF with oxidized methionine at position 21. Purity of the supplied material was stated as >98% biologically active EGF. Lyophilized recombinant EGF, used as standard, was purchased from Boehringer Mannheim Biochemicals, Indianopolis, IN (*E. coli* EGF) and Upstate Biotechnology Inc., Lake Placid, NY (*Saccharomyces cerevisiae* EGF). Antibodies and sera required for RIA were supplied by Vanderbilt Medical Center, Nashville, TN. Iodinated EGF was processed by Vanderbilt Medical Center. Acetonitrile (HPLC grade) and 1-propranol were purchased from Fisher Scientific and trifluoracetic acid was purchased from Sigma Chemical Company.

High-performance liquid chromatography (HPLC)

The Waters (Milford, MA) HPLC system used consisted of: a model 680 automated gradient controller, a model 510 pump and a model 6000A solvent delivery system. Samples were injected using a model 710B Waters Sample Processor and detected with a model 480 LC spectrophotometer. Peak integration was performed on a model 730 data module. Samples were applied to a Vydac Separations Group (Hesperia, CA) 218TP54 C-18 300 Å column equilibrated in 16% solvent A and 84% solvent B, where solvent A was 0·1% trifluoroacetic acid and 1% 1-propanol in 80% acetonitrile, 20% water and solvent B was 0·1% trifluoroacetic acid and 1% 1-propanol in water. Samples were eluted by successive 3 min 16% isocratic, 9 min 28% isocratic and 20 min 28–36% linear gradient steps with a flow rate of 1·5 mL min^{-1}, the percentage referring to solvent A. Effluent was monitored at 276 nm. Injection volume was 100 μL and all injections were performed in 0·05 M phosphate buffer pH 7·4. All EGF standard concentrations were determined using UV absorption at 276 nm with an experimentally determined extinction coefficient of 27·4. Standard curves were produced using totalled areas of all five HPLC peaks (Fig. 2). Assay sensitivity was 2 μg and interassay variation showed a relative standard deviation (r.s.d.) of 4%.

RIA

Phosphate buffer (0·063 M pH 7·4) containing 0·02% sodium azide and 0·4737% EDTA (Fisher Scientific) was used as diluent throughout the experiments. All assay components were added in buffer diluent containing 0·1% Triton X-100 (Fisher Biotech) and 24 trypsin inhibitor units aprotinin (Sigma) per 100 mL, in 100 μL vol. Samples for analysis were prepared in 0·05 M phosphate buffer pH 7·4. Analyte or standard was equilibrated at 4°C with anti-EGF antibody for one day and a further two days following addition of ^{125}I-labelled antigen. Precipitation of the antigen-antibody complex was achieved by addition of a second antibody, incubation for 3 h, addition of 1·6 mL bovine serum albumin (Sigma, 20 mg mL^{-1} in buffer diluent), centrifugation and aspiration. Radioactivity of the resulting pellet was determined in a gamma counter. Analysis of results was effected using logit transformations of standard curve data. At intervals throughout the study, results were procured to ensure parallelism between sample curves and competition curves generated by dilutions of each sample. Assay sensitivity was 5 pg/100 μL. Interassay variation showed an r.s.d. of 13% and intra-assay variation a mean r.s.d. of 7%.

Comparison of RIA and HPLC analyses

Direct comparison. Samples were analysed by HPLC and appropriately diluted to a concentration range for detection by the RIA. Dilutions were performed six independent times to obtain estimates of dilution error.

Indirect comparison. Indirect comparison was achieved by RIA analysis of the isolated EGF peaks separated by HPLC (Fig. 2). Known concentration samples were assayed by HPLC; 0·75 mL fractions of effluent were collected from the column, appropriately diluted and analysed by RIA. RIA chromatograms were constructed for comparison with HPLC data.

Induction of EGF degradation by thermal stress

Samples of human isolated EGF, 12 μg/100 μL in 0·05 M phosphate buffer pH 7·4, ionic strength = 0·15, were placed on stability at −80, 4, 25, 37 and 50°C. Samples at each temperature were evaluated by HPLC at regular intervals over 50 days (−80°C samples were evaluated over longer periods). When sample HPLC chromatograms showed significant changes, individual peaks were collected, diluted and analysed by RIA.

Amino acid analysis

HPLC fractions were individually collected from the column and dried under nitrogen. Repeated injections, sample collections and drying steps were necessary to collect sufficient sample for analysis. Analysis was performed using a Beckman model 6000 ion exchange amino acid analyser. Samples were hydrolysed in 6 M HCl with 0·2% phenol at 110°C for 24 h.

Amino acid sequencing

Sequencing of five amino terminal amino acids was performed on certain degradation product peaks collected as for amino acid analysis. Automated Edman degradations were performed on an Applied Biosystems model 470 protein sequencer.

Results

Comparison of HPLC and RIA

Table 1 shows results of the comparative HPLC and RIA evaluation of two samples of human isolated EGF. Less

Table 1. Direct comparison of HPLC and RIA analysis of samples of human isolated EGF.

Sample 1		Sample 2	
HPLC (μg)	RIA (μg)	HPLC (μg)	RIA (μg)
6·24 ± 0·34	5·91 ± 0·72	12·84 ± 0·17	11·28 ± 0·35
	5·58 ± 0·46		13·19 ± 1·08
	7·05 ± 0·24		10·79 ± 0·44
	4·82 ± 0·35		11·57 ± 0·97
	5·34 ± 0·48		11·01 ± 0·34
	5·85 ± 0·55		12·84 ± 0·80
	5·76 ± 0·75*		11·78 ± 1·00*

Samples analysed by HPLC were appropriately diluted to be within range for detection by RIA. Six independent dilutions were performed to obtain estimates of dilution error. Results show mean ± s.d. HPLC mean concentration and overall RIA mean concentration are equivalent by two-sided *t*-tests n = 6 for each analysis. * shows s.d. due to dilution.

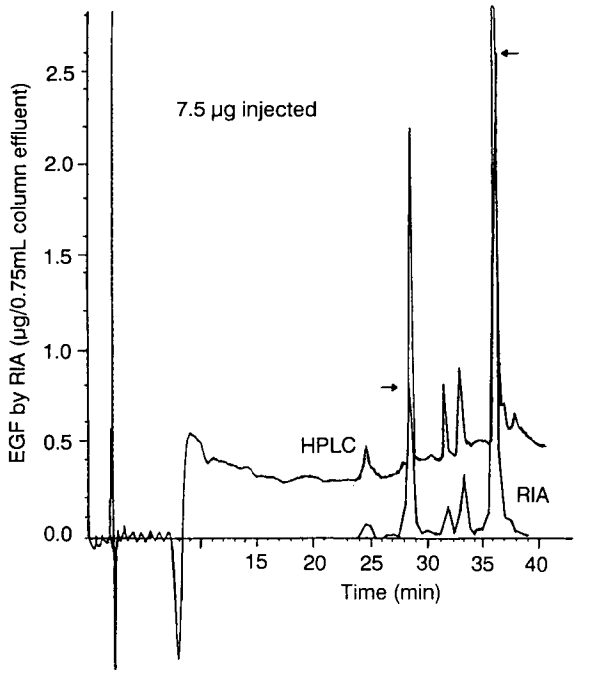

FIG. 3. HPLC chromatogram compared with an RIA profile created by analysis of column effluent. Horizontal arrows show locations of the RIA peak maxima.

error than expected was encounted in RIA values due to the significant dilutions required to assay μg samples by RIA. Good linear correlation can be seen between the HPLC analysis and the results obtained by RIA. An overall equation for the regression analysis of RIA results (x) against corresponding HPLC results (y) for twelve independent samples diluted once for RIA analysis was: $y = 1{\cdot}1464 + 0{\cdot}9800x$ ($r = 0{\cdot}9690$). Further evidence of correlation is shown by the results of indirect HPLC and RIA comparison in Fig. 3. RIA analysis of eluted HPLC peaks showed comparative EGF concentration results and indicated that all EGF components of the human isolated material show comparable binding activity. Total recovery of EGF as measured by RIA was 75·6% of injected material.

Induction of degradation by thermal stress

The reverse phase HPLC method was able to detect degradation of EGF over the period studied. Significant degradation was seen only with samples stored at 50°C (Fig. 4). Degradation peaks were detected after six days at 50°C as shoulders on peaks 2 (EGF 1-51) and 5 (EGF 1-52). Samples stored 15 days at 50°C also showed three further small degradation peaks, two eluting slightly later than the shoulder peaks of EGF 1-51 and EGF 1-52, the third slightly later than EGF 1-50. Although samples stored at 37°C did not undergo extensive degradation, shoulder peaks on EGF 1-51 and EGF 1-52 were apparent after 15 days and three further degradation peaks, having the same retention time as those seen at 50°C, were seen after 36 days. Samples stored at −80, 4 and 25°C showed no signs of degradation after 50 days, although 25°C samples evaluated after 133 days showed evidence of shoulder peaks on EGF 1-51 and EGF 1-52. Samples stored at −80°C for six months or more show a

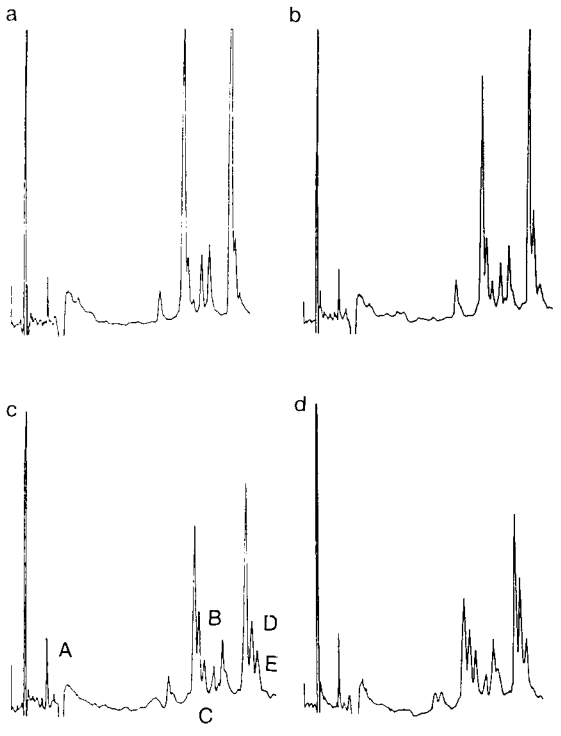

FIG. 4. HPLC showing degradation of human isolated EGF at 50°C after storage for (a) 6, (b) 15, (c) 25 and (d) 36 days. Upper case notation shown on (c) identifies degradation peaks isolated and collected for amino acid analysis and sequencing.

new, early peak. A similar peak is seen in samples stored at 50°C for short periods (Fig. 4). Fig. 5 shows the degradation data collected from the 50°C study and defines a first order degradation rate constant k of 0·024 days^{-1}.

The amino acid analyses of degradation peaks from the sample stored at 50°C for 36 days are shown in Table 2 (A, B, C, D, E) with the corresponding analysis of EGF peaks present in the sample before degradation (1, 2, 3, 4, 5). The degradation peaks analysed are identified in Fig. 4c. High values for serine and glycine residues are the result of

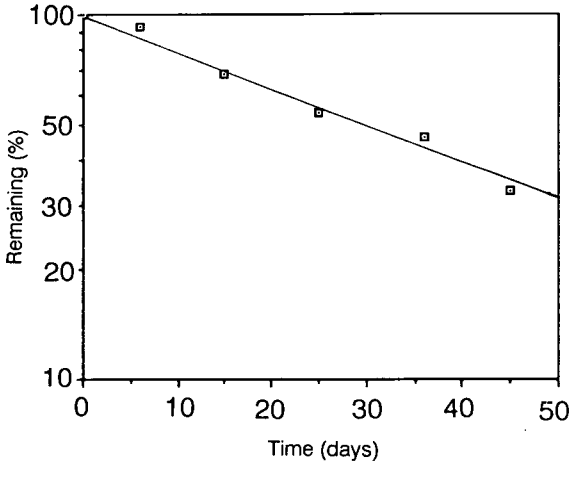

FIG. 5. Kinetic analysis of data collected for degradation of human isolated EGF at 50°C. Percent remaining data was calculated using total area of the five peaks present in the original sample. Degradation rate constant k = 0·024 days^{-1} (r^2 = 0·9722).

elevated background levels due to small sample size. The low recovery of valine residues is due to only 50% hydrolysis of the Val–Val bond between positions 34 and 35. The analyses confirm the absence of parent EGF 1-53 and show that the degradation taking place does not involve any breakage of peptide bonds. Peaks B and C appear to be degradation products of EGF 1-51 and peaks D and E degradation products of EGF 1-52. The low levels of tyrosine recorded have not been explained.

Amino terminal sequencing performed on degradation products B and D produced less than 0·5% theoretical yield. Hydrolysis of the sequencer filter showed more than sufficient material for analysis suggesting that the EGF amino terminal was blocked and resistant to sequencing. These results indicate deamidation of the amino terminal asparagine, forming L-isoaspartyl EGF as documented by DiAugustine et al (1987) with a variant of mouse EGF and by Araki et al (1989) with human recombinant EGF.

In an attempt to further identify degradation peaks, denaturation of non-degraded samples was attempted in sodium phosphate pH 7 with two volumes of 0·4 M Tris buffer pH 8·5, containing 6 M guanidine hydrochloride, 0·1% EDTA and 15 mM dithiothreitol. Analysis of treated samples by HPLC revealed minimal peaks of EGF 1-51 and EGF 1-52 but no other peaks.

All degradation peaks detected showed immunoreactivity equivalent to that of non-degraded peaks when assayed by RIA, except for the early eluting peak A which may have been assayed at a concentration below RIA sensitivity.

Discussion

Although the HPLC and RIA procedures measure different properties of the EGF molecule it appears that, as independent analytical methods, they can produce equivalent results when quantifying freshly prepared samples of EGF. This equivalency applies whether the EGF sample is homogeneous or the heterogeneous mixture of recognized fragments and oxidized forms. The same EGF fragments and oxidized forms, including parent EGF 1-53, have previously shown identical placental membrane receptor binding activity (Mount et al 1985) and, when found in yeast culture medium during recombinant production, have shown the same specific activity in stimulating mitotic activity of human foreskin fibroblasts (Nascimento et al 1988). Ideally, the HPLC and RIA need to be further correlated with an independent measure of biological activity to provide clinically meaningful information. However, the use of RIA to detect low concentrations in EGF formulation release and compatibility studies, and the use of HPLC in content uniformity, drug recovery and stability studies will provide invaluable correlated data during initial product development.

As a macromolecule, EGF can undergo both chemical and physical degradation. Such types of degradation have been documented (Giddins et al 1987; Manning et al 1989), but the number of different reactions that can occur, and the dependence of degradation mechanism on environment, make identification of both degradation pathways and products extremely difficult. Chemical polypeptide degradation is dependent on temperature, pH and ionic strength

Table 2. Amino acid analysis of the components of human isolated EGF and their degradation products.

	Human isolated EGF peaks					Thermal degradation peaks					
	1	2	3	4	5	A	B	C	D	E	[c]
Asx	6·3	7·2	7·0	6·7	7·4	4·3	5·7	6·1	7·1	4·5	7
Thr	1·1	0·0	0·5	0·7	0·0	0·5	0·7	0·8	0·4	0·7	0
Ser	6·2	3·0	5·2	4·8	3·1	8·4	5·1	5·5	3·2	4·7	3
Glx	6·4	4·9	4·2[a]	5·8	5·0	6·6	5·7	5·9	5·3	4·8	5
Pro	1·3	1·3	1·2	1·0	0·9	1·0	1·2	1·3	1·0	1·1	1
Gly	5·2	4·1	6·0	6·9	4·2	7·1	6·8	7·5	5·3	6·4	4
Ala	2·6	2·0	2·3	2·2	2·1	2·4	2·2	2·4	2·0	2·0	2
Cys	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	6
Val	1·7	2·4	1·9	2·5	2·2	1·7	2·1	2·3	2·9	1·8	3
Met	0·4	0·8	0·4	0·5	0·7	0·3	0·5	0·5	0·5	0·4	1
Ile	1·6	1·9	1·7	1·7	2·0	1·4	1·7	1·7	2·0	1·5	2
Leu	3·2[a]	3·6[a]	3·4[a]	4·2	4·9	2·6[a]	3·5[a]	3·6[a]	4·7	4·4	5
Tyr	2·2	4·6	3·2	2·8	4·5	1·3	2·8	2·5	4·2	2·2	5
Phe	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0
Lys	2·0	1·9	1·9	2·1	1·9	1·8	2·0	1·9	1·8	1·6	2
His	1·4	1·8	1·7	1·5	1·9	0·8	1·3	1·4	1·8	1·0	2
Trp	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2
Arg	2·0[a]	2·1[a]	2·0[a]	2·2[a]	2·2[a]	1·9[a]	2·3[a]	2·2[a]	2·2[a]	2·0[a]	3

[a] There is at least one amino acid residue less than in the intact molecule. [b] Not determined. [c] Integer values based on the proposed sequence of human EGF (Nascimento et al 1988).

(Manning et al 1989); indeed, our stability studies with EGF at pH 2 showed significantly different degradation as detected by this HPLC method. Thermal stress was chosen to induce degradation for this study because physiological constraints would restrict the use of varying pH in a wound treatment preparation and degradation products produced by pH changes would not be indicative of potential product degradation. It appears from the data collected that the mechanism of degradation is the same for each fragment of EGF, and for all samples stored at 25, 37 and 50 °C. Accelerated studies may therefore give useful information on long term stability at lower temperatures during EGF product development.

The resistance of samples B and D to Edman degradation provides strong evidence of a deamidated terminal asparagine. Deamidation of asparagine to form the free carboxylic acid has been documented as a degradation mechanism for a number of proteins and model peptides (Manning et al 1989; Bhatt et al 1990; Patel & Borchardt 1990). Deamidation at neutral pH occurs through a cyclic intermediate and results in the formation of L-aspartyl and L-isoaspartyl isomers. The L-isoaspartyl isomer is resistant to Edman degradation and due to the extremely low sequencing yield in this case, probably constitutes the major part of the EGF degraded sample. Serine or glycine residues adjacent to the asparagine residue, as found in human EGF, are reported to aid in the formation of the cyclic imide intermediate and subsequent deamidation (Manning et al 1989). Degradation samples A, C and E have not been identified. Among possible explanations that do not involve cleavage of a peptide bond are deamidation of a glutamine or the second asparagine residue, oxidation, and changes in one or more disulphide bonds.

The clinical impact of chemical degradation of protein pharmaceuticals can only be evaluated once the biological activity of the degradation products, compared with the parent molecule, is known. All peaks detected after thermal stressing of samples, with the possible exception of peak A, demonstrated immunoreactivity. Additionally, previous reports have indicated that deamidation of the amino terminal asparagine does not have a deleterious effect on the receptor binding or the mitogenic activity of the molecule (DiAugustine et al 1987; Araki et al 1989).

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References

Abe, Y., Sagawa, T., Sakai, K., Kimura, S. (1987) Enzyme-linked immunosorbent assay (ELISA) for human epidermal growth factor (hEGF). Clin. Chim. Acta 168: 87–95

Araki, F., Nakamura, H., Nojima, N., Tsukomo, K., Sakamoto, S. (1989) Stability of recombinant human epidermal growth factor in various solutions. Chem. Pharm. Bull. 37: 404–406

Bhatt, N. P., Patel, K., Borchardt, R. T. (1990) Chemical pathways of peptide degradation. I. Deamidation of adrenocorticotropic hormone. Pharm. Res. 7: 593–599

Brown, G. L., Nanney, L. B., Griffen, J., Cramer, A. B., Yancey, J. M., Curtsinger, L. J., Holtzin, L., Schultz, G. S., Jurkiewicz, M. J., Lynch, J. B. (1989) Enhancement of wound healing by topical treatment with epidermal growth factor. New Engl. J. Med. 321: 76–79

Chvapil, M., Gaines, J. A., Gilman, T. (1988) Lanolin and epidermal growth factor in healing of partial thickness pig wounds. J. Burn Care Rehab. 9: 279–285

Cohen, S. (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem. 237: 1555–1562

DiAugustine, R. P., Gibson, R. W., Aberth, W., Kelly, M., Ferrua, C. M., Tomooka, Y., Brown, C. F., Walker, M. (1987) Evidence for isoaspartyl (deamidated) forms of mouse epidermal growth factor. Analyt. Biochem. 165: 420–429

Giddins, M. D., Dabbah, R., Grady, T., Rhodes, C. T. (1987) Scientific and regulatory aspects of macromolecular drugs and devices. Drug Devel. Ind. Pharm. 13: 873–968

Gregory, T. J. F. H., Morris, W. P. (1986) Acceleration of wound

healing by recombinant human urogastrone (epidermal growth factor). J. Lab. Clin. Med. 108: 103–107

Hirata, Y., Orth, D. N. (1979a) Epidermal growth factor (urogastrone) in human tissues. J. Clin. Endocrinol. Metab. 48: 667–672

Hirata, Y., Orth, D. N. (1979b) Epidermal growth factor (urogastrone) in human fluids: size heterogeneity. Ibid. 48: 673–679

Kroeff, E. P., Chance, R. E. (1982) Applications of high-performance liquid chromatography for analysis of insulins. In: The United States Pharmacopoeial Convention Inc., Hormone Drugs: Proceedings of the FDA-USP Workshop on Drug and Reference Standards for Insulins, Somatotropins and Thyroid-axis Hormones. Mack Printing Co., Easton Pennsylvania, pp 148–162

Manning, M. C., Patel, K., Borchardt, R. (1989) Stability of protein pharmaceuticals. Pharm. Res. 6: 903–918

Mount, C. D., Lucas, T. J., Orth, D. N. (1985) Purification and characterization of epidermal growth factor (beta-urogastrone) and epidermal growth factor fragments from large volumes of human urine. Arch. Biochem. Biophys. 240: 33–42

Nascimento, C.-G., Gynes, A., Mitcyhell Hallorn, S., Merryweather, J., Valenzuela, P., Steimer, K. S., Masiarz, F. R., Randolph, A. (1988) Characterization of recombinant human epidermal growth factor produced in yeast. Biochemistry 27: 797–802

Patel, K., Borchardt, R. T. (1990) Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. Pharm. Res. 7: 703–711

Personen, K., Alfthan, H., Stenman, U.-H., Viinikka, L., Perheentupa, J. (1986) An ultrasensitive time-resolved immunofluorometric assay of human epidermal growth factor. Anal. Biochem. 157: 208–211

Schultz, G. S., White, M., Mitchell, R., Brown, G., Lynch, J., Twardzik, D. R., Todaro, G. J. (1987) Epithelial wound healing enhanced by transforming growth factor-alpha and vaccinia growth factor. Science 235: 350–352

Smith, J. A., O'Hare, M. J. (1984) Reversed phase high performance liquid chromatography of mouse epidermal growth factor and its congeners: mobile phase optimization with ion-pairing additives. J. Chromat. 299: 13–28

Starkey, R., Cohen, S., Orth, D. N. (1975) Epidermal growth factor: identification of a new hormone in human urine. Science 189: 800–802

Yip, T. T., Tsao, S. W., Ko, K. M., Xin, J.-X., Kong, Y. C. (1986) Shrew submaxillary gland epidermal growth factor: homologous radioreceptor assay and mitogenic activity. Mol. Cell. Endocrinol. 47: 131–135