

[Chem. Pharm. Bull.]
33(9)4037-4040(1985)

Heterogeneity of Human Epidermal Growth Factor/Urogastrone from Human Urine

SATOSHI NISHIMURO,* TOYOHICO NISHIMURA, NAOMI UCHIDA,
HIROYUKI SHIRONO and HAJIME HIRATANI

Laboratories, Japan Chemical Research Co., Ltd., 7-1-4,
Sakaguchi-dori, Chuo-ku, Kobe 651, Japan

(Received December 10, 1984)

Ten forms of human epidermal growth factor/urogastrone (hEGF/URO) were purified from human urine by using reverse-phase high performance liquid chromatography (RP-HPLC) following kaolin adsorption, gel filtration and ion-exchange chromatography. All of these forms competed with ^{125}I -mouse epidermal growth factor (mEGF) for binding to EGF receptors, cross-reacted with anti-hEGF and had the apparent molecular weight of 6000 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. However, the molecular heterogeneity of hEGF/URO was revealed by electrophoretic and RP-HPLC analysis.

Keywords—epidermal growth factor; urogastrone; heterogeneity; RP-HPLC; urine

Human epidermal growth factor/urogastrone (hEGF/URO) was first isolated from human urine as a mouse EGF-like substance, hEGF, by Cohen and Carpenter¹⁾ and as a gastric inhibitor, URO, by Gregory and Willshire.²⁾ Although a slight difference appeared to exist in amino acid composition between hEGF and URO,³⁾ they have been considered to be identical. Recently, the molecular heterogeneity of mouse epidermal growth factor (mEGF)

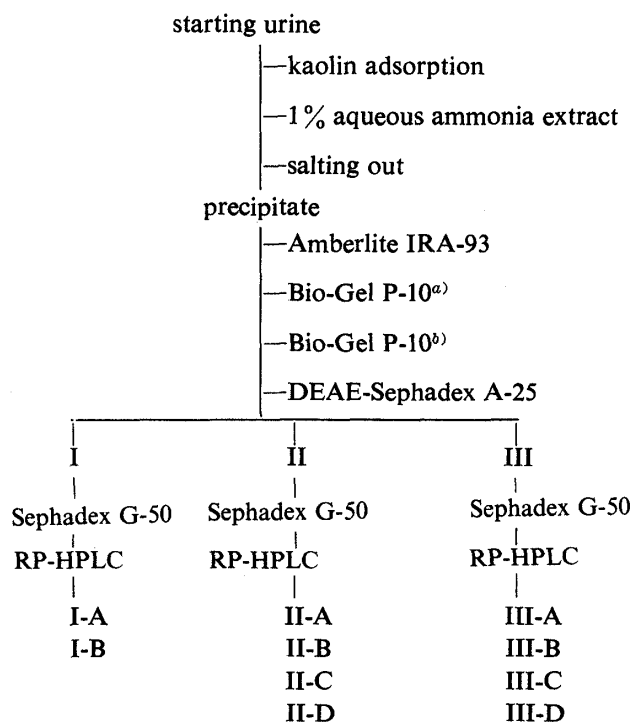


Chart 1. Purification Procedure for hEGF/URO

a) Bio-Gel P-10 gel filtration was carried out in 4% HCl saline.

b) Bio-Gel P-10 gel filtration was carried out in 0.05 M ammonium acetate.

TABLE I. Characterization of the Ten Sub-species of hEGF/URO

Sub-species	RP-HPLC (min) ^{a)}	Size-exclusion HPLC (min) ^{a)}	pI	Disc PAGE Rm ^{b)}	Cross-reactivity ^{c)} with anti-hEGF
I-A	23.5	42.5	4.6	0.48	+ ^{d)}
I-B	24.9	43.4	4.6	0.48	+
II-A	23.5	43.4	4.5	0.68	+
II-B	24.9	43.4	4.5	0.68	+
II-C	27.3	45.3	4.5	0.68	+
II-D	28.7	45.3	4.5	0.68	+
III-A	23.9	43.4	4.4	0.71	+
III-B	25.3	43.4	4.4	0.71	+
III-C	27.6	45.3	4.4	0.71	+
III-D	29.2	45.3	4.4	0.71	+
mEGF	26.8	52.5	4.6	0.35	- ^{e)}

a) Retention time. b) Relative mobility with respect to bromophenol blue. c) Determined by using double immunodiffusion (antigen; 1 µg/10 µl/well, anti-serum; 10 µl/well). d) Positive. e) Negative.

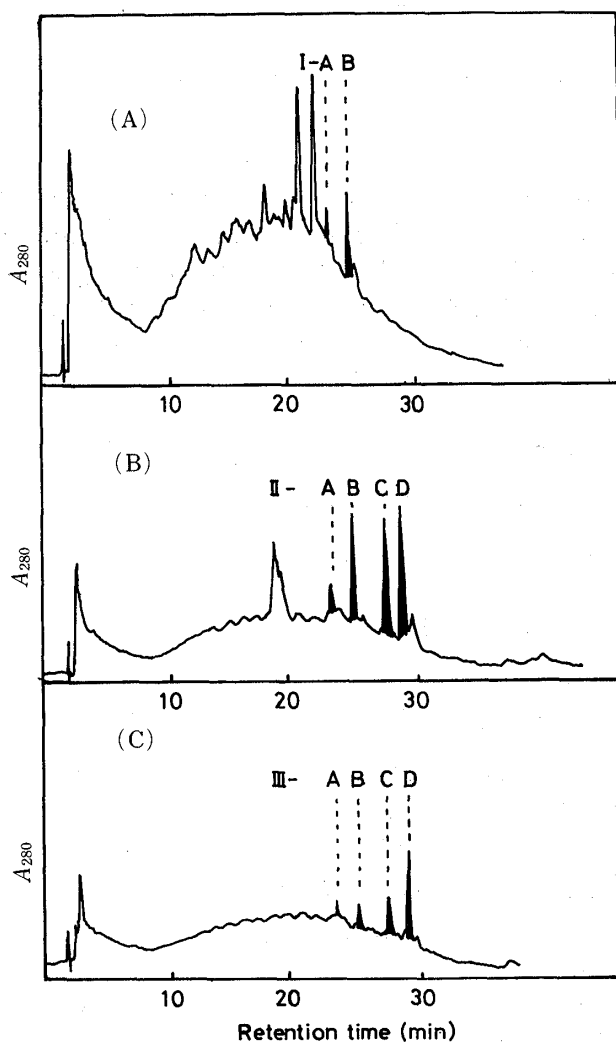


Fig. 1. RP-HPLC of Sephadex G-50 Fractions

Fractions I, II and III after Sephadex G-50 gel filtration were subjected to RP-HPLC monitored with UV detector at 280 nm and by RRA. EGF activity was found in the black peaks.

(A) fraction I, (B) fraction II, (C) fraction III.

was elucidated by means of reverse-phase high performance liquid chromatography (RP-HPLC).⁴⁾ However, the heterogeneity of hEGF/URO remains unclear. Thus, after several treatments of human urine as described in previous reports,^{1,2,5)} we carried out RP-HPLC for further purification of hEGF/URO. The purification revealed the presence of ten sub-species

of hEGF/URO in human urine, designated as I-A, I-B, II-A, II-B, II-C, II-D, III-A, III-B, III-C and III-D (Chart 1).

Each of the sub-species was confirmed to be hEGF/URO by demonstrating its abilities to compete with ^{125}I -mEGF in radioreceptor assay⁶⁾ and to form a single precipitin line with anti-EGF in double immuno-diffusion. The analytical profiles of the ten sub-species are shown in Table I.

The ten sub-species showed the same molecular weight as mEGF on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, but were eluted at somewhat earlier retention times on size-exclusion HPLC than mEGF and appeared individually at distinct positions. There seemed to be some interaction between EGF and the TSK gel matrix as described previously,⁷⁾ which changed the apparent molecular weight on the TSK column. By electrophoretic analysis, hEGF/URO was resolved into three groups (I-A, B, II-A, B, C, D and III-A, B, C, D) with pI's of 4.6, 4.5 and 4.4 and relative electrophoretic mobilities at pH 8.3 of 0.48, 0.68 and 0.71, respectively. Although each group appeared to be homogenous in charge and size, each was further resolved into several components on RP-HPLC (retention times of 23.5 and 24.9 min for I-A, B, 23.9, 25.3, 27.3 and 27.8 min for II-A, B, C, D and 23.9, 25.3, 27.6 and 29.2 min for III-A, B, C, D) as also found in the purification by using RP-HPLC (see Fig. 1).

The charge heterogeneity on electrophoretic analysis may be due to lack of COOH-terminal basic amino acid residues as suggested by Gregory^{2,3)} and Savage and Harper,⁵⁾ but this is not sufficient to explain the presence of ten receptor-active sub-species with a molecular weight of 6000. When human urine was subjected to RP-HPLC, several EGF active peaks which coincided with the sub-species, were detected (data not shown). Although our experiments demonstrated the previously undetected heterogeneity of hEGF/URO, it is not yet clear whether the ten sub-species all occur naturally in human urine. To determine this, it will be essential to analyze the amino acid sequences of the ten sub-species.

Experimental

RP-HPLC—The HPLC apparatus consisted of two Gilson model 302 pumps, a Gilson model 602 program controller, a Variator 311 UV detector set at 280 nm and a Shimadzu Chromatopac R-IB recorder. Samples were injected by means of a Rheodyne model 7125 injector into an M & S Pack C18 column (4 × 150 mm) equilibrated with 20% (v/v) acetonitrile in 0.05% (v/v) aqueous trifluoroacetic acid. The column was developed with a linear gradient of acetonitrile (0.7%/min) at a flow rate of 1.0 ml/min.

Size-Exclusion HPLC—The TSK3000SW column (0.75 × 60 cm, Toyo Soda) was equilibrated with 0.1 M phosphate buffer, pH 7.0, and eluted with the same buffer at a flow rate of 0.6 ml/min. Peptides were detected with a ultraviolet (UV) detector set at 280 nm.

Polyacrylamide Disc and SDS-Disc Gel Electrophoresis (Disc PAGE and SDS-PAGE)—Disc PAGE was performed on 15% (w/v) polyacrylamide gels, pH 8.3, by the method of Davis⁸⁾ with a minor modification. SDS-PAGE was carried out on 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS by the method of Weber and Osborn.⁹⁾

Isoelectric Focusing (IEF)—IEF was carried out on thin layer polyacrylamide gel (Ampholine[®] PAG PLATE range 4.0—6.5, LKB). pI was determined by using pI marker proteins (Pharmacia isoelectric focusing calibration kit).

Radioreceptor Assay (RRA)—RRA using HeLa cells was performed according to O'Keefe *et al.*¹⁰⁾ mEGF was purified by the method of Cohen and Taylor¹¹⁾ and radioiodinated with ^{125}I Na (Amersham) by the method of Hunter and Greenwood.¹²⁾ hEGF/URO was determined by using RRA as mEGF equivalent ($E_{50}^{\%} = 30.9$).

Double Immunodiffusion—Immunodiffusion in 1% agarose gel on the slide glass was carried out by the method of Ouchterlony¹³⁾ using anti-hEGF serum,¹⁴⁾ which was a kind gift from Dr. Hirata, Kobe University School of Medicine, Kobe, Japan.

Purification of hEGF/URO—Fractions in all purification steps were assayed by RRA. Fresh human male urine (1 t), collected under phenol, was adjusted to pH 3.0 and contacted with kaolin for 1 h. The mixture was allowed to stand, then the kaolin was collected by filtration and suspended in 1% aqueous ammonia. The supernatant was separated and, made up to 50% ammonium sulfate saturation, and finally the precipitate was collected by centrifugation. The precipitate was dissolved in water and subjected to Amberlite IRA-93 ion-exchange with 0.2 M

acetic acid and then to Bio-Gel P-10 gel filtration in 4% HCl saline. The eluate between 1.4—2.7 column volumes was concentrated and subjected to Bio-Gel P-10 gel filtration in 0.05 M ammonium acetate. The eluate between 0.6—0.8 column volume was subjected to DEAE-Sephadex A-25 ion-exchange chromatography with ammonium acetate gradient elution 0.05—1.0 M, pH 5.6). At this step, RRA activity was separated into three fractions, I, II and III, successively. After being passed through a column of Sephadex G-50 equilibrated with 1.0 M acetic acid, the fractions (I, II, III) were further separated by RP-HPLC to afford I-A, B (120, 160 μ g), II-A, B, C, D (90, 240, 320, 250 μ g) and III-A, B, C, D (80, 100, 150, 170 μ g).

Acknowledgement The authors are grateful to Dr. Hirata (Kobe University School of Medicine, Kobe, Japan) for providing anti-hEGF serum.

References

- 1) S. Cohen and G. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1317 (1975).
- 2) H. Gregory and R. Willshire, *Hoppe-Seyler's Z. Physiol. Chem.*, **356**, 1765 (1975).
- 3) H. Gregory, *Nature (London)*, **257**, 325 (1975).
- 4) A. W. Burgess, J. Knesel, L. G. Sparrow, N. A. Nicola and E. C. Nice, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 5753 (1982).
- 5) C. R. Savage, Jr. and R. Harper, *Anal. Biochem.*, **111**, 195 (1981).
- 6) M. D. Hollenberg and H. Gregory, *Life Sci.*, **20**, 267 (1976).
- 7) R. C. Sullivan, Y. W. Shing, P. A. D'Amore and M. Klagsbrun, *J. Chromatogr.*, **266**, 301 (1983).
- 8) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
- 9) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
- 10) E. O'Keefe, M. D. Hollenberg and P. Cuatrecasas, *Arch. Biochem. Biophys.*, **164**, 518 (1974).
- 11) S. Cohen and J. M. Taylor, *Recent Prog. Horm. Res.*, **30**, 533 (1974).
- 12) F. C. Greenwood and W. M. Hunter, *Biochem. J.*, **89**, 114 (1963).
- 13) O. Ouchterlony, *Prog. Allergy*, **6**, 30 (1962).
- 14) Y. Hirata and D. N. Orth, *J. Clin. Endocrinol. Metab.*, **48**, 667 (1979).