Degradation of Synthetic Salmon Calcitonin in Aqueous Solution

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INTRODUCTION

Calcitonin (CT) is a polypeptide comprised of 32 amino acid residues arranged in a single chain. There is a disulfide bridge between cysteine residue 1 and cysteine residue 7 that forms a ring at the N terminus of the molecule and a proline amide moiety at the C terminus. Among the CT available for clinical use (e.g., human, salmon, porcine, chicken, and eel CT, etc.), salmon CT (sCT) is one of the most potent forms and is used primarily for the treatment of postmenopausal osteoporosis and Paget's disease and in the management of hypercalcemia (1). sCT has a short biological half-life, approximately 14 min (2). Preparations in liquid and freeze-dried forms are currently available, and recently prolonged controlled-release dosage forms have been considered as a novel sCT delivery system (3,4). Although, there have been some investigations on the stability of sCT in rat plasma and tissue homogenates (5–7), there is little fundamental information on the chemical stability of sCT. This note describes the kinetics of degradation of sCT as a function of pH and temperature by reversed-phase gradient high-performance liquid chromatography.

MATERIALS AND METHODS

Materials

Synthetic salmon calcitonin (cyclic) was obtained from BACHEM Inc. (Torrence, CA). Reduced sCT was prepared by treating sCT with 2-mercaptoethanol (Sigma) and purified using Sep-Pak C_{18} cartridges (Waters) (8). HPLC-grade trifluoroacetic acid (TFA) and acetonitrile were obtained from Pierce and Fisher, respectively. All solutions and buffers were prepared with distilled deionized water that was filtered with a 0.22-μm membrane (Milli-Q system, Millipore) prior to use. All other materials were of reagent grade.

HPLC Method

The HPLC system consisted of two pumps (Model 501, Waters), a gradient controller (Model 680, Waters), an injector (U6K, Waters), an ODS column (10 μm, 4.6 × 25 cm, Bio-Rad), a turnable UV detector (Model 484, Waters), and a computing integrator (C-R6A, Shimadzu).

sCT and its degradation products were eluted from the column and detected at 220 nm. A linear gradient was employed: 30% A:70% B to 55% A:45% B over 25 min. Mobile phase A was a 0.1% TFA/acetonitrile solution and mobile phase B was 0.1% TFA/distilled water. The injection volume was 10 μl and the flow rate was 1.5 ml/min (4).

A standard curve was constructed for each series of determinations over a range of 1 to 15 μg of sCT. The initial concentration of each sCT solution was designated 100%; all subsequent concentrations are expressed as a percentage of the initial concentration.

Kinetic Studies

Stock sCT solutions were 5 mg/ml. Test solutions were prepared by adding 0.1 ml of stock sCT solution to amber vials containing 0.4 ml of buffer solutions.

A citrate–phosphate buffer system containing 0.01 *M* citric acid and 0.02 *M* dibasic sodium phosphate (pH 2.2 to 6.0) was used and these buffer solutions were in the range of 0.010–0.016 *M* with respect to the mixture of citrate and phosphate. A borate–hydrochloric acid buffer system containing 0.01 *M* HCl and 0.02 *M* sodium borate (pH 9.0) was also used.

The reaction vial was then placed into a constant-temperature water bath (±0.1°C, Gallenkamp). Samples were periodically removed from the vials with a syringe (Hamilton) and injected into the HPLC directly.

RESULTS AND DISCUSSION

Figure 1 illustrates the HPLC chromatograms of sCT kept in different pH solutions at 70°C for a specific time. All of the degradation compounds detected in this HPLC system eluted before sCT. Four main degradation products were detected, as shown in Fig. 1 (peaks 2–5). Similar chromatograms of sCT and reduced sCT (8) and degradation products of sCT (9) have been observed previously by reversed-phase HPLC.

With the exception of the reduced sCT assigned as peak 4, the degradation products have not been identified in this study. The degradation products might be a form of sCT deamidated at the asparagine residues or the glutamine residue (10). Although sCT can undergo degradation via a variety of chemical reactions including beta-elimination, disulfide exchange, racemization, and oxidation, which are specific to certain amino acid residues, deamidation of asparagine and glutamine residues is the most common chemical pathway of peptide degradation over the pH range studied and probably occurs by different degradation mechanism (11–15). The sCT degradation pattern showed a marked dependence on pH and temperature as is observed with general deamidation reaction (16). Figure 1 also suggests that sCT may degrade by several specific pathways. Degradation products 2, 3, and 5 were found at pH 3.7 and became more apparent as the pH was increased to more alkaline values. Product 4 (assigned as the reduced sCT) was detected only at pH 2.2. The major degradation products

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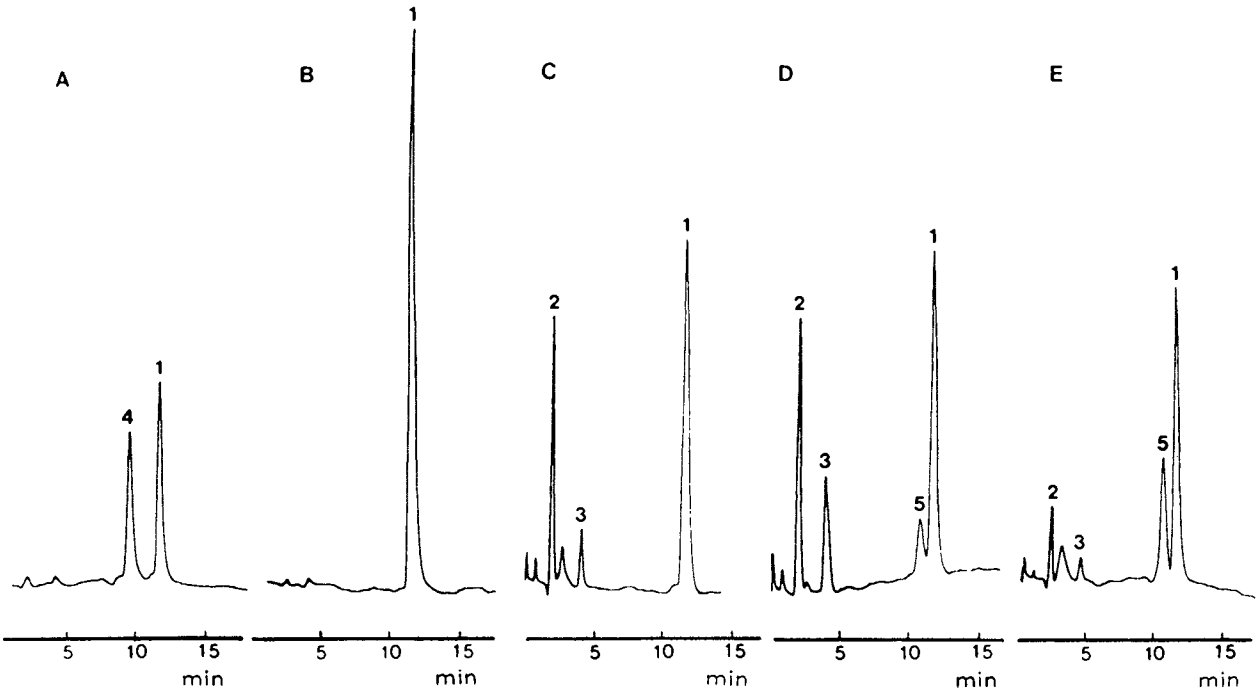

Fig. 1. HPLC chromatograms of salmon calcitonin solution (10 μg sCT) prepared in 0.01 *M* citric acid/0.02 *M* phosphate (pH 2.2–6.0) and 0.01 *M* HCl/0.02 *M* borate (pH 9.0) buffers at 70°C. (A) pH 2.2, 3h; (B) pH 3.3, 10h; (C) pH 3.7, 5h; (D) pH 6.0, 10h; (E) pH 9.0, 1h. 1, sCT; 2, unknown; 3, unknown; 4, reduced sCT; 5, unknown.

(peaks 2, 3, and 5) appear to be relatively stable since their concentration increases as the degradation of sCT progresses. No sCT degradation products were detected at pH 3.3.

The degradation kinetics of sCT was also studied. Figure 2 shows a semilogarithmic plot of the residual percentage amounts of sCT vs time in various pH solutions at 70°C. It was found that the pH affected the degradation rate of sCT and that the observed degradation reaction rates approximately followed first-order kinetics.

The observed reaction rate constants (k) were obtained from the slopes of the semilog plots of concentration vs time

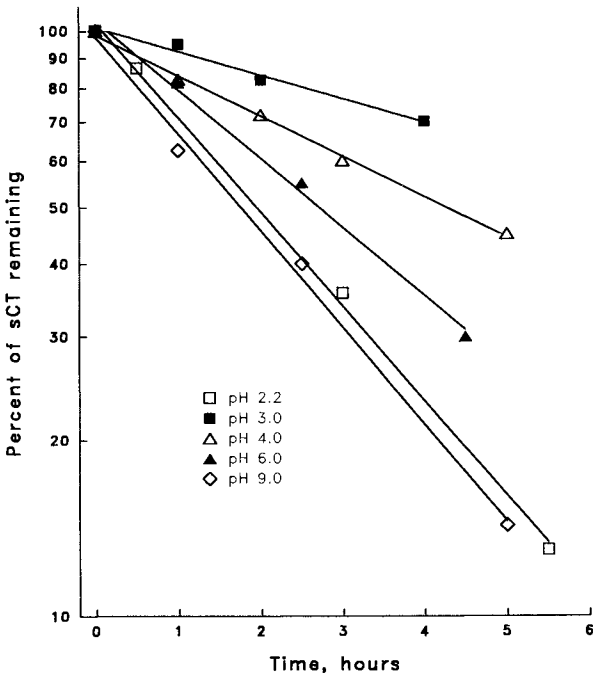

Fig. 2. First-order plot for the degradation of salmon calcitonin in 0.01 *M* citric acid/0.02 *M* phosphate (pH 2.2–6.0) and 0.01 *M* HCl/0.02 *M* borate (pH 9.0) buffer solutions at 70°C.

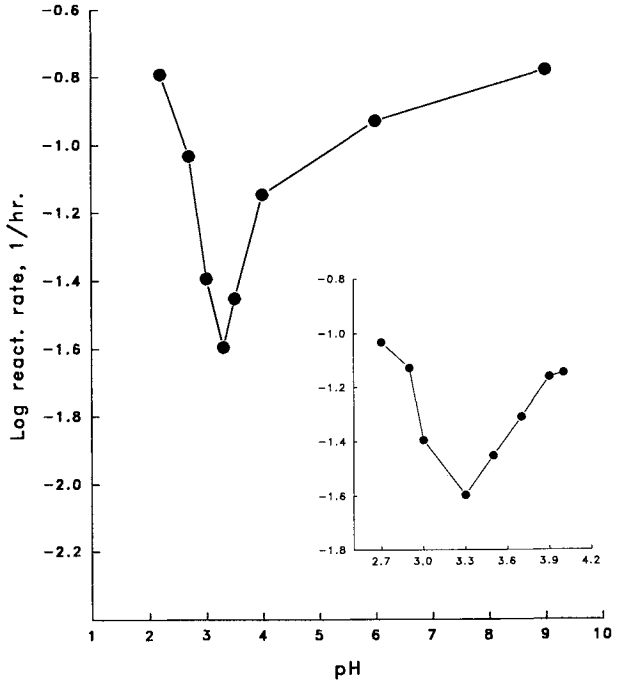

Fig. 3. pH–rate profile of the degradation of salmon calcitonin in 0.01 *M* citric acid/0.02 *M* phosphate (pH 2.2–6.0) and 0.01 *M* HCl/0.02 *M* borate (pH 9.0) buffer solutions at 70°C.

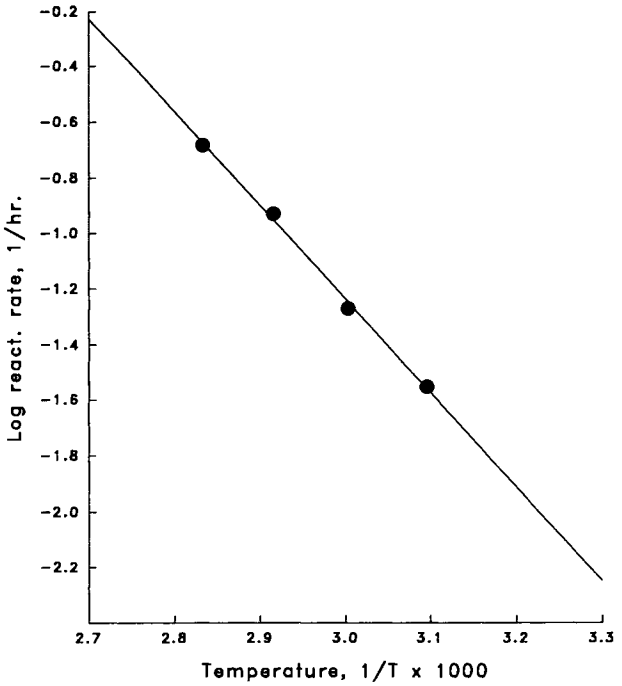

Fig. 4. Arrhenius plot of log rate constant vs $1/T$ for the degradation of salmon calcitonin in pH 6.0 buffer.

by statistical regression analysis. The pH–rate profiles for the degradation of sCT were obtained by plotting the value of log$k$ against pH (Fig. 3). sCT was most stable at pH 3.3. The result is more acidic than those of previous studies, which recommend a slightly acid pH (pH 4.5–6.0) for minimal decomposition of peptides in solution (17–20).

Arrhenius plots of the data observed in kinetic studies at different temperatures ranging from 50 to 80°C and at a constant pH of 6.0 were reasonably linear, with a correlation coefficient greater than 0.99 (Fig. 4). The slope gives an activation energy for the overall reaction of 15.42 kcal/mol at pH 6.0.

In conclusion, we observed the kinetics of degradation of sCT as a function of pH and temperature by reversed-phase gradient high-performance liquid chromatography and detected four main degradation products of sCT. The degradation reaction of sCT followed first-order kinetics and maximum stability was achieved by adjusting the pH to 3.3.

REFERENCES

1. M. Azria. *The Calcitonins. Physiology and Pharmacology,* Karger, Basel, 1989.
2. G. Segre and P. Dal Pra. In A. Pecile (eds.), *Calcitonin,* Excerpta Medica, Amsterdam, 1985, pp. 99–107.
3. M. Hanson, G. Gazdick, J. Cahill, and M. Augustine. In S. S. Davis, L. Illum, and E. Tomlinson (eds.), *Delivery Systems for Peptide Drugs,* Plenum, New York, 1986, pp. 233–242.
4. K. C. Lee, E. E. Soltis, P. S. Newman, K. W. Burton, R. C. Mehta, and P. P. DeLuca. In vivo assessment of salmon calcitonin sustained release from biodegradable microspheres. *J. Control. Release* 17:199–206 (1991).
5. M. DeLuise, T. J. Martin, and R. A. Melick. Inactivation and degradation of porcine calcitonin by rat liver, and relative stability of salmon calcitonin. *J. Endocrinol.* 48:181–188 (1970).
6. F. E. Newsome, R. K. O'Dor, C. O. Parkes, and D. H. Copp. A study of the stability of calcitonin biological activity. *Endocrinology* 92:1102–1106 (1973).
7. S. B. Baylin, A. L. Bailey, T.-H. Hsu, and C. V. Foster. Degradation of human calcitonin in human plasma. *Metabolism* 26:1345–1354 (1977).
8. M. L. Heinitz, E. Flanigan, R. C. Orlowski, and F. E. Regnier. Correlation of calcitonin structure with chromatographic retention in high-performance liquid chromatography. *J. Chromatogr.* 443:229–245 (1988).
9. I. H. Lee, S. Pollack, S. H. Hsu, and J. R. Miksic. Influence of the mobile phase on salmon calcitonin analysis by reversed-phase liquid chromatography. *J. Chromatogr. Sci.* 29:136–140 (1991).
10. M. C. Manning, K. Patel, and R. T. Borchardt. Stability of protein pharmaceuticals. *Pharm. Res.* 6:903–918 (1989).
11. V. B. Riniker, R. Neher, R. Maier, F. W. Kahnt, P. G. H. Byfield, T. V. Gudmundsson, L. Galante, and I. MacIntyre. Human calcitonin. I. Isolation and characterization. *Helv. Chim. Acta.* 51:1738–1742 (1968).
12. R. P. Diaugustine, B. W. Gibson, W. Aberth, M. Kelly, C. M. Ferrua, Y. Tomooka, C. F. Brown, and M. Walker. Evidence for isoaspartyl (deamidated) forms of mouse epidermal growth factor. *Anal. Biochem.* 165:420–429 (1987).
13. T. Geiger and S. Clarke. Deamidation, isomerization and racemization at asparaginyl and aspartyl residues in peptides. *J. Biol. Chem.* 262:785–794 (1987).
14. N. P. Bhatt, K. Patel, and R. T. Borchardt. Chemical pathways of peptide degradation. I. Deamidation of adrenocorticotropic hormone. *Pharm. Res.* 7:593–599 (1990).
15. K. Patel and R. T. Borchardt. Chemical pathways of peptide degradation. III. Effect of primary sequence on the pathways of deamidation of asparaginyl residues in hexapeptides. *Pharm. Res.* 7:787–793 (1990).
16. X. H. Zhou and A. Li Wan Po. Peptide and protein drugs. I. Therapeutic applications, absorption and parenteral administration. *Int. J. Pharm.* 75:97–115 (1991).
17. D. M. Johnson, R. A. Pritchard, W. F. Taylor, D. Conley, G. Zuniga, and K. G. McGreevy. Degradation of the LH-RH analog nafarelin acetate in aqueous solution. *Int. J. Pharm.* 31:125–129 (1986).
18. J. W. VanNispen. In D. D. Breimer and P. Speiser (eds.), *Topics in Pharmaceutical Sciences 1987,* Elsevier, Amsterdam, 1987, pp. 293–307.
19. V. J. Helm and B. W. Müller. Stability of gonadorelin and triptorelin in aqueous solution. *Pharm. Res.* 7:1253–1255 (1990).
20. V. J. Helm and B. W. Müller. Stability of the synthetic pentapeptide thymopentin in aqueous solution: Effect of pH and buffer on degradation. *Int. J. Pharm.* 70:29–34 (1991).