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Degradation kinetics of L-glutamine in aqueous solution

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Abstract

The degradation kinetics of L-glutamine (Gln) in aqueous solution was studied as a function of buffer concentration, pH and temperature. Stability tests were performed using a stability-indicating high-performance liquid chromatographic assay. The degradation product of Gln was 5-pyrrolidone-2-carboxylic acid. The reaction order for Gln in aqueous solution followed pseudo-first-order kinetics under all experimental conditions. The maximum stability of Gln was observed in the pH range from 5.0 to 7.5. The pH–rate profile described by specific acid–base catalysis and hydrolysis by water molecules agreed with the experimental results. Arrhenius plots showed the temperature dependence of Gln degradation, and the apparent activation energy at pH 6.41 was determined to be 9.87×10^4 J mol^{-1}.

Keywords: L-Glutamine; Degradation; 5-Pyrrolidone-2-carboxylic acid

1. Introduction

L-Glutamine (Gln) is an important substrate for renal ammonia production (Welbourne, 1987), hepatic gluconeogenesis (Kimura et al., 1988), modulation of muscle protein turnover (Wu and Thompson, 1990), and is a metabolic fuel for enterocytes (Horvath et al., 1996; Wilmore and Shabert, 1998). During total parenteral nutrition (TPN), changes in the morphology and function of the small intestine occur (Li et al., 1994), and the risk of bacteremia and endotoxemia increase (Frankel et al., 1993; Bai et al., 1996). The addition of Gln to TPN solution maintains the integrity of the gut mucosa in postoperative patients (Hulst et al., 1993; Morlion et al., 1998). Formerly, Gln was thought to be 'non-essential' and was not included in TPN solution because of its relative instability in solution. Several studies under simulated usage patterns have shown that Gln is stable if the correct pharmaceutical techniques are followed. At a pH just below neutrality, the concentration of Gln in TPN solution decreased by 5% per day at 37°C (Souba et al., 1985). At room temperature, Gln degradation was 0.7–0.9% per day in different parenteral solutions (Khan et al., 1991), at ≈4°C, it was minimal (Wilmore, 1994). However, there has been no report of any precise kinetic study concerning the degradation of Gln in aqueous solution. The purpose of this study was to investigate the effects of pH and temperature on the stability of Gln using a stability-indicating high-performance liquid chromatographic (HPLC) assay method.

2. Experimental procedures

2.1. Materials

Gln (>99.0%, reagent grade) was purchased from Takara Kohsan (Tokyo, Japan). All other chemicals used in this experiment were of analytical or HPLC grade. The water was double-distilled.

2.2. Stability studies of Gln

In the stability studies, several different pH values of citrate buffer (pH 1.21–6.41) and borate buffer (pH 7.39–11.01) were used. The pH values were measured at the experimental temperature using a pH meter equipped with a combination electrode. The concentration of the total citrate buffer was 13.8–110 mM and that of total borate buffer was 25–140 mM. The ionic strength was adjusted to 0.5 by using potassium chloride. Weighed amounts of Gln were dissolved in the buffer solutions preheated to a

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desired temperature to produce a final Gln concentration of 0.2 mg ml^{-1}. Aliquots (200 ml) of the solutions were poured into 200-ml glass vials and sealed. The sealed solutions were stored in a constant temperature water bath at 70°C. At pH 6.41, the samples were studied at four different temperatures (40, 50, 60 and 70°C). Aliquots of the solution were withdrawn at appropriate time intervals and assayed immediately.

2.3. Analytical procedures

The HPLC system consists of a solvent delivery pump (model 655-15, Hitachi, Tokyo, Japan), a high-pressure sampling valve (model 655, Hitachi), and an integrator (model 655-71B, Hitachi). Column effluents were monitored with a fluorimeter (model RF-530, Shimazu, Kyoto, Japan) and the excitation and emission wavelength were set to 330 and 470 nm, respectively. The separation was carried out using a C_{18} column (200×4 mm I.D., Nucleosil $5C_{18}$, Macherey-Nagel, Duren, Germany). The mobile phase was a 65:35 mixture (v/v) of 12.5 mM di-sodium hydrogenphosphate and methanol. The mobile phase was delivered at a constant rate of 1.0 ml min^{-1}. The remaining Gln was determined by a pre-column derivatization with *o*-phthalaldehyde (OPA) (Lindroth and Mopper, 1979). The concentration of Gln was determined by a method of peak area ratio compared to the peak area ratio of samples of standard solutions from the calibration curve (internal standard: L-asparagine).

2.4. Degradation product of Gln

The degradation product of Gln was isolated by the HPLC system (solvent delivery pump: model 655-15, sampling valve: model 655, integrator: model 655-71B, Hitachi). The mobile phase (0.1% perfluorobutyric acid solution) was delivered at a constant rate of 0.7 ml min^{-1} and the column eluent was monitored at 210 nm (UV-detector: model 655, Hitachi). The mass spectra of the degradation product was recorded by a Frit FAB-MS spectrometry system (model MS-LX2000, JEOL, Tokyo, Japan) via a direct inlet. The FAB-MS matrix was methanol containing 3% of glycerol and the flow rate was 0.2 ml min^{-1}. The FAB-MS measurement was done at a xenon acceleration voltage of 3 kV and an ion acceleration voltage of 2 kV. The degradation product of Gln was identified as 5-pyrrolidone-2-carboxylic acid (pGlu) (MH^+ ion at m/z 130) by the authentic compound.

The concentration of pGlu was determined by a pre-column derivatization with 4-nitrophenacyl bromide. The method used was established in earlier research (Bousquet et al., 1983).

2.5. Measurement of dissociation constants of Gln

The apparent dissociation constants of Gln were determined potentiometrically by measuring the pH solutions prepared by mixing Gln solution with 0.1 mol l^{-1} hydrochloric acid or 0.1 mol l^{-1} sodium hydroxide solution at 70°C. The initial concentration of Gln was 5.0×10^{-4} mol per 50 ml. The same method has been used in previous research (Takeuchi et al., 1995). The dissociation constants of Gln, pK_1 and pK_2, were 2.21 and 9.01, respectively.

3. Results and discussion

3.1. Degradation product

A typical chromatogram of the reaction mixture in 27.5 mM citrate buffer of pH 6.41 is shown in Fig. 1. The peak designated as **2** is the parent compound Gln, and that designated as **1** is the internal standard L-asparagine. There have been several reports on the degradation products of Gln. During storage, Gln hydrolyzed to L-glutamic acid (Glu) and ammonia (Heller et al., 1967), while at higher temperature, the amino-group of Gln was labile and yielding pGlu (Stehle et al., 1984). In this study, the OPA-reactive substance such as Glu (retention time=3.2 min) was not observed. This finding indicated that hydrolytic deamination of Gln to Glu was not the cause of Gln loss. In the analysis of reaction mixtures pH 1.93, 6.41 and 11.01, the decrease of Gln was 6.32×10^{-4} M (70°C, 0.6 h), 4.78×10^{-4} M (70°C, 10 h) and 5.71×10^{-4} M (70°C, 3 h), respectively, and the formation of pGlu was 6.18×10^{-4}, 4.72×10^{-4}, and 5.60×10^{-4} M, respectively. There was close agreement between the loss of Gln and the

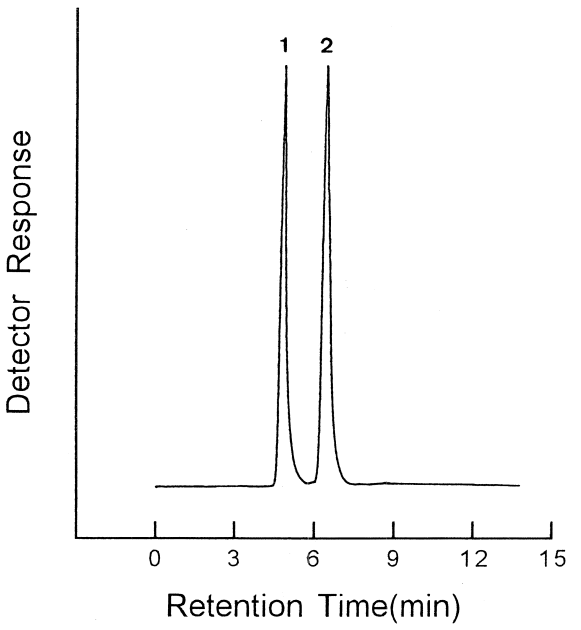

Fig. 1. A typical chromatogram of a partially degraded sample of Gln at 70°C in buffer solution. (1) internal standard, (2) Gln.

appearance of pGlu. Extensive loss of Gln was the result of the intramolecular cyclization to pGlu.

3.2. Order of reaction

The time-course of the concentration of Gln at various pH, at constant temperature (70°C) and constant ionic strength (0.5) is shown in Fig. 2. In all determinations, there was a linear relationship between storage time and logarithmic remaining percent of Gln, and the degradation of Gln followed pseudo-first-order reaction kinetics in aqueous solution. The observed first-order rate constant, K_{obs}, was determined from the slope of the graph by the method of least squares. The first-order plots under all experimental conditions yielded coefficients of over $r=-0.99$.

3.3. pH–rate profile of Gln

Fig. 3 shows the effects of citrate and borate on the degradation of Gln at various pH. K_{obs} increased linearly with the increase of buffer concentrations; thus, K, the apparent rate constant which depends purely on pH, is obtained by the extrapolation to zero buffer concentration. Fig. 4 shows the pH–rate profile constructed from the buffer-free first-order rate constants and the pH values. The observed rate in this pH–rate profile was a summation of a series of rates of catalytic reactions by the hydrogen and hydroxyl ions and water molecules. In the isoelectric regions, Gln is more stable and the degradation rate constant approaches a plateau. Therefore, the degradation of neutral species of Gln is due mainly to hydrolysis by water molecules (Fig. 4a). The degradation of Gln is rapid in acidic regions, and the rate decreases with an increase in pH, which indicates specific hydrogen-ion catalysis (Fig. 4d) and water hydrolysis (Fig. 4b) of the cationic species of Gln. In the alkaline regions, the rate indicates specific hydroxide-ion catalysis (Fig. 4e) and water hydrolysis (Fig. 4c) of the anionic species of Gln. Similar degradation

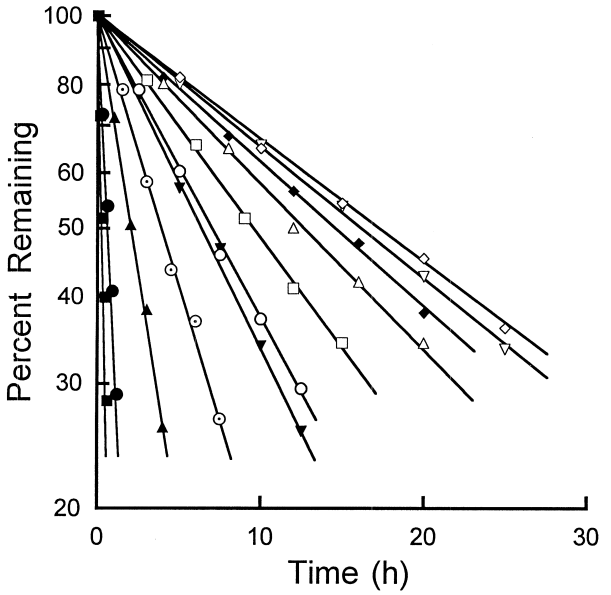

Fig. 2. Apparent first-order plots for the degradation of Gln at 70°C in buffer solution. pH values: (■) 1.21, (●) 1.93, (▲) 2.87, (▼) 3.93, (♦) 4.80, (▽) 5.61, (◇) 6.41, (△) 7.39, (□) 8.31, (○) 9.31, (○ dot) 11.01.

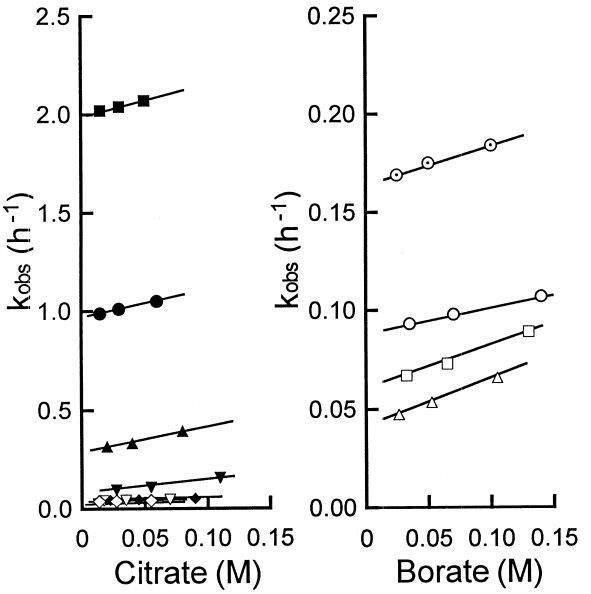

Fig. 3. Plots of the K_{obs} versus the total buffer concentration at 70°C and $\mu=0.5$ for the degradation of Gln. pH values: (■) 1.21, (●) 1.93, (▲) 2.87, (▼) 3.93, (♦) 4.80, (▽) 5.61, (◇) 6.41, (△) 7.39, (□) 8.31, (○) 9.31, (○ dot) 11.01.

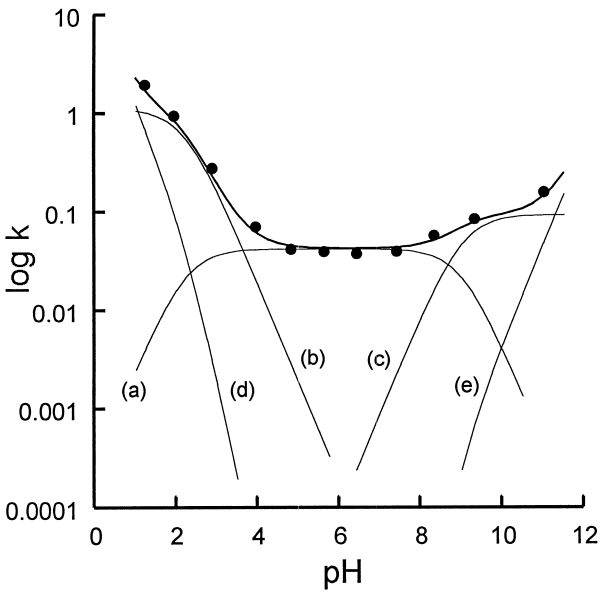

Fig. 4. pH–rate profile for the degradation of Gln in aqueous solution at 70°C. The points are the experimental values, and the lines are the theoretical curves. (a) Water-catalyzed degradation of neutral species, (b) water-catalyzed degradation of cationic species, (c) water-catalyzed degradation of anionic species, (d) hydrogen-ion-catalyzed degradation of cationic species, and (e) hydroxide-ion-catalyzed degradation of anionic species.

profiles were obtained for L-alanyl-L-glutamine (Arii et al., 1998) and orbifloxacin (Morimura et al., 1995). Thus, the overall velocity is equal to the sum of the rates of these reactions, as shown in the following equation

$$K = K'_{H} \cdot [a_{H}] \cdot f_1 + K'_{H_2O} \cdot f_1 + K_{H_2O} \cdot f_2 + K''_{H_2O} \cdot f_3 + K''_{OH} \cdot [a_{OH}] \cdot f_3 \quad (1)$$

where K'_{H} is the second-order rate constant for the hydrogen-ion-catalyzed degradation of the cationic species; K''_{OH} is the second-order rate constant for hydroxide-ion-catalyzed degradation of the anionic species; K'_{H_2O}, K_{H_2O} and K''_{H_2O} are the first-order rate constants for the water-catalyzed degradation of cationic, neutral and anionic species, respectively; and a_{H} is the hydrogen-ion activity and a_{OH} is the hydroxyl-ion activity. By introducing the fractions of Gln in the cationic (f_1), neutral (f_2) and anionic (f_3) forms, the dissociation constants of Gln, K_1 and K_2, and the dissociation constant of water, $K_W = 12.80$ (Harned and Hamer, 1933), the following overall rate expression, Eq. (2), was obtained:

$$K = K'_{H} \cdot [a_{H}]^3 + K'_{H_2O} \cdot [a_{H}]^2 + K_{H_2O} \cdot [a_{H}] \cdot K_1 + K''_{H_2O} \cdot K_1 \cdot K_2 + K''_{OH} \cdot K_1 \cdot K_2 \cdot K_W \cdot [a_{H}]/([a_{H}]^2 + K_1 \cdot [a_{H}] + K_1 \cdot K_2) \quad (2)$$

The rate constants were estimated by the least-squares method by fitting Eq. (2) to the experimental rate constants at various pH: $K'_{H} = 1.45 \times 10\ M^{-1}\ h^{-1}$, $K''_{OH} = 3.52\ M^{-1}\ h^{-1}$, $K'_{H_2O} = 1.29\ h^{-1}$, $K''_{H_2O} = 1.09 \times 10^{-1}\ h^{-1}$, $K_{H_2O} = 4.87 \times 10^{-2}\ h^{-1}$. The calculated values are in good fit with the experimental data ($r = 0.99$) and this equation adequately describes the degradation kinetics in the pH ranges studied.

3.4. Dependence of degradation rate on temperature

The temperature dependence of Gln degradation in buffer solution at pH 6.41 (27.5 mM citrate) was studied in the temperature range of 40–70°C. Arrhenius plots of log rate versus the reciprocal of the absolute temperature were constructed to describe the results of this investigation. Linearity ($r = 0.99$) of the regression line in this plot was observed in the temperature range of 40–70°C. The activation energy for degradation was determined to be 9.87×10^4 J mol^{-1} from the slope of this plot and the frequency factor (log A) was determined to be 31.5 h^{-1}.

References

Arii, K., Kai, T., Kokuba, Y., 1998. Degradation kinetics of L-alanyl-L-glutamine and its derivatives in aqueous solution. Eur. J. Pharm. Sci. 7, 107–112.

Bai, M.X., Jiang, Z.M., Liu, Y.W., Wang, W.T., Li, D.M., Wilmore, D.W., 1996. Effects of alanyl-glutamine on gut barrier function. Nutrition 12, 793–796.

Bousquet, E., Guarcello, V., Morale, M.C., Rizza, V., 1983. Analysis of 5-pyrrolidone-2-carboxylate ester by reverse phase high-performance liquid chromatography. Anal. Biochem. 131, 135–140.

Frankel, W.L., Zhang, W., Afonso, J., Klurfeld, D.M., Don, S.H., Laitin, E., Deaton, D., Furth, E.E., Pietra, G.G., Naji, A., Rombeau, J.L., 1993. Glutamine enhancement of structure and function in transplanted small intestine in the rat. J. Parenter. Enter. Nutr. 17, 47–55.

Harned, H.S., Hamer, W.J., 1933. The ionization constant of water and the dissociation of water in potassium chloride solutions from electromotive forces of cells without liquid junction. J. Am. Chem. Soc. 55, 2194–2206.

Heller, L., Becher, A., Beck, A., Müller, F., 1967. On the problem of utilization of infused amino acid solutions. Klin. Wochenschr. 45, 317–318.

Horvath, K., Jami, M., Hill, I.D., Papadimitriou, J.C., Magder, L.S., Chanasongcram, S., 1996. Isocaloric glutamine-free diet and the morphology and function of rat small intestine. J. Parenter. Enter. Nutr. 20, 128–134.

Hulst, R.R.W.J., Kreel, B.K., Meyenfeldt, M.F., Brummer, R.J.M., Arends, J.W., Deutz, N.E.P., Soeters, P.B., 1993. Glutamine and the preservation of gut integrity. Lancet 341, 1363–1365.

Khan, K., Hardy, G., McElroy, B., Elia, M., 1991. The stability of L-glutamine in total parenteral nutrition solutions. Clin. Nutr. 10, 193–198.

Kimura, R.E., LaPine, T.R., Johnston, J., Ilich, J.Z., 1988. The effect of fasting on rat portal venous and aortic blood glucose, lactate, alanine, and glutamine. Pediatr. Res. 23, 241–244.

Li, J., Langkamp-Henken, B., Suzuki, K., Stahlgren, L.H., 1994. Glutamine prevents parenteral nutrition-induced increases in intestinal permeability. J. Parenter. Enter. Nutr. 18, 303–307.

Lindroth, P., Mopper, K., 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. Anal. Chem. 51, 1667–1674.

Morimura, T., Ohno, T., Matsukura, H., Nobuhara, Y., 1995. Degradation kinetics of the new antibacterial fluoroquinolone derivative, orbifloxacin, in aqueous solution. Chem. Pharm. Bull. 43, 1052–1054.

Morlion, B.J., Stehle, P., Wachtler, P., Siedhoff, H.P., Koller, M., Konig, W., Fürst, P., Puchstein, C., 1998. Total parenteral nutrition with glutamine dipeptide after major abdominal surgery: a randomized, double-blind, controlled study. Ann. Surg. 227, 302–308.

Souba, W.W., Smith, R.J., Wilmore, D.W., 1985. Glutamine metabolism by the intestinal tract. J. Parenter. Enter. Nutr. 9, 608–617.

Stehle, P., Pfaender, P., Fürst, P., 1984. Isotachophoretic analysis of a synthetic dipeptide L-alanyl-L-glutamine. Evidence for stability during heat sterilization. J. Chromatogr. 294, 507–512.

Takeuchi, Y., Sunagawa, M., Isobe, Y., Hamazume, Y., Noguchi, T., 1995. Stability of a 1β-methylcarbapenem antibiotic, meropenem (SM-7338) in aqueous solution. Chem. Pharm. Bull. 43, 689–692.

Welbourne, T.C., 1987. Interorgan glutamine flow in metabolic acidosis. Am. J. Physiol. 253, F1069–F1076.

Wilmore, D.W., 1994. Glutamine and the gut. Gastroenterology 107, 1885–1886.

Wilmore, D.W., Shabert, J.K., 1998. Role of glutamine in immunologic responses. Nutrition 14, 618–626.

Wu, G., Thompson, J.R., 1990. The effect of glutamine on protein turnover in chick skeletal muscle in vitro. Biochem. J. 265, 593–598.