Stability of Human Insulin in Solutions Containing Sodium Bisulfite¹⁾

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The stabilities of human insulin (HI) in aqueous solutions were investigated in the pH range of 4.0–7.0 in the presence ($1.0\text{--}3.0 \times 10^{-3}$ M) and absence of sodium bisulfite (SBS) both in the dark and under scattered light (1000 lux) using high performance liquid chromatography. Increasing concentrations of SBS tended to degrade HI. In the presence of SBS, with an increase in the pH value, the stability of HI decreased in the pH range of 4.0–7.0. There was a partial difference in the stability of HI in the presence of SBS in the dark and that under scattered light.

HI was stabilized by glucose in the presence of SBS in the dark, and the stability of HI was revealed to depend on the concentration of free SBS. The reason for this phenomenon was postulated to be the formation of bisulfite–glucose adduct.

Keywords sodium bisulfite; human insulin; degradation; stabilization; bisulfite–glucose adduct

Sodium bisulfite (SBS) used as a stabilizer in injectable preparations is known to degrade various drugs including thiamine,^{2,3)} fursultiamine,³⁾ gabexate mesilate,⁴⁾ nafamostat mesilate,⁵⁾ urokinase⁶⁾ and bovine insulin.^{7,8)} Considering the pharmaceutical stability of insulin injection in an intravenous admixture (mixed infusion) containing SBS, however, there are no reports on detailed kinetic studies for the degradation reaction on bovine and porcine insulin with SBS. These insulins and human insulin have two disulfide inter-chain bonds linking the A and B chains and an intra-chain bond linking residues 6 and 11 on the A chain.⁹⁾

Recently, a human insulin (HI) having fewer adverse reactions (allergy *etc.*) than other insulins was developed. HI is becoming a major insulin preparation, and is often added to various infusion solutions. In view of the compatibility of the mixed infusion, the stability of HI injection in aqueous solution containing SBS was investigated by high-performance liquid chromatography (HPLC) in the pH range of 4.0–7.0 in the presence and absence of SBS both in the dark and under scattered light (1000 lux).

In addition, many infusion solutions contain glucose, which has an aldehyde group and forms bisulfite (BS)–glucose adduct^{3,10)} in the presence of SBS. Therefore, the stabilization of HI in the presence of SBS by glucose was attempted. In order to clear the stabilization mechanism, the relationship between the stability of HI in the mixed solution of SBS and glucose and the free SBS concentration was studied. The free SBS concentrations were measured stoichiometrically by the complementary tristimulus colorimetry (CTS method)^{3,11–13)} using pyridoxal phosphate (PLP).

Experimental

Materials HI injection (Humalin R U-40[®], lot No. 3DF58B) was supplied by the Shionogi Co., Ltd. HI, SBS, glucose, PLP and the other agents were commercial products of special grade. Buffer solutions, *i.e.*, 0.05 M acetate buffer (pH 4.0 and 5.0), 0.05 M phosphate buffer (pH 6.0 and 7.0) were adjusted to an ionic strength (μ) 0.5 with sodium chloride.

Samples and Storage Conditions The stability of HI injection in aqueous solution (approximately 0.08 unit/ml, *ca.* 0.5 μ M) was studied in the pH range of 4.0–7.0 in the presence ($1.0\text{--}3.0 \times 10^{-3}$ M) and absence of SBS in the dark and under scattered light (indoor 1000 lux) at 25°C. Test solutions were stored in glass bottles of 500 ml in inner volume with each glass stopper.

To evaluate the influence of glucose on the stability of HI in the presence

of SBS, glucose was added to the solution of HI injection (approximately 0.08 unit/ml) buffered at pH 6.0 and 7.0 in the presence of SBS (2.0×10^{-3} M).

Determination of HI HI was measured by HPLC. Equipment: a Hitachi 655-11 high-performance liquid chromatograph, a Hitachi 655A UV detector, and a Shimadzu C-R3A chromatopack were used. Measurement conditions: column, Zorbax TMS (4.6 mm i.d. $\times$ 250 mm); mobile phase, 0.1 M ammonium phosphate buffer (pH 2.0)–acetonitrile (74 : 26); flow rate, 1.0 ml/min; column temperature, 40°C; and detection wavelength, 210 nm. As an internal standard, ethyl *p*-hydroxybenzoate was employed, and a 10 μ l aliquot was injected into the HPLC.

Measurement of the Equilibrium Constant for the BS–Glucose Adduct

1) Measurement of Absorption Spectra: PLP and glucose have an aldehyde group, respectively, so form each 1 : 1 adduct of BS^{3,10,11)} in the presence of SBS. The BS addition reaction with PLP and glucose is a competition reaction. PLP and BS–PLP adduct have an absorption maximum at 386 and 328 nm, respectively. Glucose, however, has only slight absorption in this ultraviolet (UV) region.

To evaluate the equilibrium constants of the BS–PLP adduct, various amounts of SBS ($0\text{--}3.6 \times 10^{-3}$ M) were added to the PLP solutions (9.0×10^{-4} M) buffered at pH 6.0 and 7.0 at 25°C in 0.1 M phosphate buffer ($\mu = 0.5$). The UV spectra of these solutions were measured using a Hitachi model 200-20 spectrophotometer with an isothermal cell holder (25°C) in an air-conditioned room at $25 \pm 1^\circ\text{C}$. Subsequently, various amounts of glucose (5–20%) were added to the mixed solutions of PLP (9.0×10^{-4} M) and SBS (1.0 or 2.0×10^{-4} M) buffered at pH 6.0 and 7.0. These UV spectra were measured similarly to calculate the equilibrium constants of the BS–glucose adduct based on the same constants of the BS–PLP adduct.

2) CTS Method: The three ranges for the CTS method were selected (*u* range 323–332 nm, *v* range 345–354 nm, *w* range 381–390 nm) from the obtained UV spectra. From the sum of 10 absorbance readings in each range, the color point and molar fraction were calculated by the CTS method^{12,13)} based on previous papers.^{3,11)} The main complementary tristimulus parameters and the equilibrium constants obtained in the present study were listed in Table I.

Results and Discussion

Determination of HI by HPLC Method for the determination of HI by HPLC have been reported by Tokunaga *et al.*¹⁴⁾ This method was not suitable for the

TABLE I. The Equilibrium Constants of the BS–PLP and BS–Glucose Adduct at pH 6.0 and 7.0^{a)}

pH	$E_{\text{PLP}}^b)$	$E_{\text{BS-PLP}}^b)$	$K_{\text{BS-PLP}}^c)$	$K_{\text{BS-glucose}}^d)$
6.0	1.035×10^5	1.105×10^5	25310 ± 288	$2.01 \pm 0.367^e)$
7.0	9.910×10^4	1.006×10^5	7579 ± 113	$1.17 \pm 0.193^f)$

a) At 25°C; 9.0×10^{-4} M PLP in 0.1 M phosphate buffer ($\mu = 0.5$). b) *E* represents each overall absorbivity. c) $0\text{--}3.6 \times 10^{-3}$ M SBS was used. d) 5–20% glucose was used. e) 1.0×10^{-4} M SBS was used. f) 2.0×10^{-4} M SBS was used.

Fig. 1. HPLC Chromatograms of HI

(A) HI injection. (B) HI (approximately 0.08 unit/ml) in the presence of SBS (2×10^{-3} M) in 0.05 M phosphate buffer (pH 6.0) in the dark at 25 °C and $\mu=0.5$ (6 h incubation). Peak 1, *m*-cresol; peak 2, internal standard (ethyl *p*-hydroxybenzoate); peak 3, HI.

Fig. 2. Semilogarithmic Plots for the Degradation of HI in the Absence of SBS in the pH Range of 4.0–7.0 in the Dark at 25 °C and $\mu=0.5$

Initial concentration of HI, approximately 0.08 unit/ml; ●, in 0.05 M acetate buffer (pH 4.0); ○, in 0.05 M acetate buffer (pH 5.0); □, in 0.05 M phosphate buffer (pH 6.0); △, in 0.05 M phosphate buffer (pH 7.0).

separation of HI regarding the HI solution containing SBS. The reason for this seemed to be attributed to the fact that the inter-chain disulfide bonds of insulin are cleaved by SBS.^{7,8,15} Therefore, new conditions for the determination of HI were studied. The conditions described in Experimental allowed determination of HI in the presence of SBS (Fig. 1). Peak 1 corresponded to *m*-cresol which was contained in HI injection as a preservative. The degradation products of HI were eluted jointly in the first peak. A calibration curve was produced using 0.04–0.16 unit/ml solutions of HI based on the peak area obtained by the HPLC method. The calibration curve showed a linear relationship in the concentration range ($y = 583x - 0.9118$) $r = 0.9957$.

Stability of HI in the Presence and Absence of SBS in the Dark or under Scattered Light No significant degradation was observed in the time courses of the residual % of HI in the absence of SBS in the pH range of 4.0–7.0 and in the presence of SBS (2.0×10^{-3} M) at pH 4.0. An adsorption of insulin on the surface of the container was well known.^{16–18} The residual ratios of HI in the pH range

Fig. 3. Semilogarithmic Plots for the Degradation of HI in the Presence of SBS in Phosphate Buffer (pH 7.0) in the Dark at 25 °C and $\mu=0.5$

Initial concentration of HI, approximately 0.08 unit/ml; ●, [SBS] = 0 M; ■, [SBS]_{total} = 1×10^{-3} M; ▲, [SBS]_{total} = 2×10^{-3} M; ▼, [SBS]_{total} = 3×10^{-3} M.

of 4.0–7.0 after 0 h storage were $87.6 \pm 1.94\%$ in this experiment, and this extent was considered to be rather small.¹⁸ Based on these results, HI concentration after 0 h storage was regarded as 100% residual in view of the kinetic analysis for the degradation of HI by SBS. No significant degradation was observed in the time courses of the residual % of HI in the absence of SBS in the pH range of 4.0–7.0 in the dark (Fig. 2). Therefore, the adsorption of HI was considered to be momentarily equilibrated.¹⁸

In contrast, significant degradation of HI was observed in the presence of SBS in the pH range of 5.0–7.0. The semilogarithmic plots for the time course of the degradation of HI in the presence of SBS (0 – 3.0×10^{-3} M) at pH 7.0 in the dark are shown in Fig. 3. The semilogarithmic plots for the degradation of HI in the presence of SBS (0 – 2.0×10^{-3} M) gave straight lines in the course of 6 h (Fig. 3). Nevertheless, the same plots for the degradation of HI in the coexistence of 3.0×10^{-3} M SBS did not give any straight lines in the course of 6 h (Fig. 3).

Sulfitolysis *i.e.* the scission of disulfides by BS or sulfite has frequently been used to cleave disulfide bonds in proteins. Referring to the scission of disulfide bond in a number of proteins by sulfite alone, the inter-chain disulfide bond generally reacts with SBS more easily than with the intra-chain bond.⁸ It was reported that the two inter-chain bonds of insulin react with sulfite causing the insulin to split into its component A and B chains, and the 6–11 intra-chain bond on the A chain does not react with sulfite alone.^{7,8}

It is well known that the scission of disulfides by sulfitolysis forms *S*-sulfonated derivative ($R-S-S-R + SO_3^{2-} \rightleftharpoons R-S^- + R-S \cdot SO_3^-$).^{19,20} The reaction is reversible and will proceed under conditions where the concentration of $R-S^-$ is small.⁸ Therefore, the degradation of disulfides by SBS was shown to obey a reversible biomolecular reaction.²¹ However, it was also reported that the initial reaction of some disulfides by SBS below pH 9 was apparently a first-order reaction.²¹ From these considerations, the initial degradation of HI by SBS (0 – 2.0×10^{-3} M) was practically regarded as an apparent first-order reaction.

On the other hand, the time courses of the residual % of HI in the presence of SBS under scattered light (Fig. 4)

Fig. 4. Semilogarithmic Plots for the Degradation of HI in the Presence of SBS in 0.05 M Phosphate Buffer (pH 7.0) under Scattered Light at 25 °C and $\mu=0.5$

Initial concentration of HI, approximately 0.08 unit/ml; $\circ$, [SBS]=0 M; $\square$, [SBS]_{total} = 1×10^{-3} M; $\triangle$, [SBS]_{total} = 2×10^{-3} M; ∇ , [SBS]_{total} = 3×10^{-3} M.

Fig. 5. Relationship between the Total SBS Concentration and Apparent First-Order Degradation Rate Constant for the Degradation of HI in the Presence of SBS in the Dark in 0.05 M Phosphate Buffer (pH 7.0, $\mu=0.5$) at 25 °C

Initial concentration; HI, approximately 0.08 unit/ml.

were complicated, especially at 3.0×10^{-3} M of SBS concentration under the influence of light. The existence of a free radical chain mechanism for the aerobic oxidation of BS to sulfate had been well documented.²²⁾ It has also been reported that the oxidative radicals formed during autooxidation of BS were regulated by light.^{6,23,24)} For these reasons, the difference in the stabilities of HI in the presence of SBS under scattered light and in the dark seemed attributable to the light-induced chain oxidation of BS.

Effects of SBS Concentrations The apparent first-order degradation rate constants (k_{obs}) of HI at an initial concentration of approximately 0.08 unit/ml at various SBS concentrations was evaluated at 25 °C in the pH range of 5.0–7.0 in the dark. Typical plots for SBS concentration vs. apparent first-order degradation rate constants of HI at pH 7.0 yielded a straight line as shown in Fig. 5. The second-order rate constant in the degradation of HI by SBS (k_{SBS}) obtained from the slope of the line was $79.0 \text{ M}^{-1} \text{ h}^{-1}$. The intercept of this line on the ordinate, which corresponded to the rate constant of HI in the absence of SBS (k_0) was regarded as 0, therefore the rate constant of HI in the presence of SBS (k_{obs}) can be represented as in Eq. 1 in this kinetic study.

$$k_{\text{obs}} = k_{\text{SBS}} \cdot [\text{SBS}]_{\text{total}} \quad (1)$$

Fig. 6. Second-Order Rate Constant for the Degradation of HI by SBS in the Dark at 25 °C and $\mu=0.5$ as a Function of pH

Initial concentration of HI, 0.08 unit/ml.

pH-Profiles At various pHs, the degradation of HI at approximately 0.08 unit/ml in $0\text{--}2.0 \times 10^{-3}$ M SBS concentration was studied at 25 °C in the dark. The second-order rate constants in the degradation of HI by SBS (k_{SBS}) at various pHs obtained from the slope of the lines by the same treatment as for pH 7.0 (Fig. 5). The pH-profiles of the second-order rate constants in the degradation of HI by SBS (k_{SBS}) are shown in Fig. 6. Figure 6 shows that the lower the pH value was, the lower the rate constants of HI degradation by SBS became in the pH range of 5.0–7.0 in the dark.

The dissociation constants of SBS were reported as $K_1 = 1.72 \times 10^{-2}$ (ca. $\text{p}K_1 = 1.8$) and $K_2 = 6.24 \times 10^{-8}$ (ca. $\text{p}K_2 = 7.2$),²⁵⁾ therefore the equilibrium between BS and sulfite was considered to be formed in the pH range of 5.0–7.0.

Though the three dimensional structure of HI and the charge distribution in the vicinity of the disulfide bonds of HI were considered to be changed with the change of pH, it was assumed for practical kinetic analysis that these factors change only slightly in a relatively small pH range of 5.0–7.0.

From these considerations, the stability of HI in the presence of SBS was regarded to depend on the equilibrium between BS (HSO_3^-) and sulfite (SO_3^{2-}). Therefore k_{SBS} must be divided to $k_{\text{HSO}_3^-}$ and $k_{\text{SO}_3^{2-}}$, and Eq. 2 can be obtained from the kinetic equation and the dissociation equilibrium SBS.

$$k_{\text{SBS}} = \frac{k_{\text{HSO}_3^-} \cdot K_1 \cdot [\text{H}^+] + k_{\text{SO}_3^{2-}} \cdot K_1 \cdot K_2}{[\text{H}^+]^2 + K_1 \cdot [\text{H}^+] + K_1 \cdot K_2} \quad (2)$$

where $k_{\text{HSO}_3^-}$ is the apparent second-order rate constant for the degradation of HI by BS, $k_{\text{SO}_3^{2-}}$ is the apparent second-order rate constant for the degradation of HI by sulfite. These rate constants were estimated by the least-squares method (Simplex method²⁶⁾), and $k_{\text{HSO}_3^-} = 9.50 \text{ M}^{-1} \text{ h}^{-1}$ and $k_{\text{SO}_3^{2-}} = 196.6 \text{ M}^{-1} \text{ h}^{-1}$ were obtained. These results show that the effect of sulfite on the degradation of HI was 20 times greater than that of BS. The degradation of HI in the presence of SBS in the pH range of 5.0–7.0 can be represented as in Eq. 3.

$$k_{\text{obs}} = [\text{SBS}] \cdot \frac{k_{\text{HSO}_3^-} \cdot K_1 \cdot [\text{H}^+] + k_{\text{SO}_3^{2-}} \cdot K_1 \cdot K_2}{[\text{H}^+]^2 + K_1 \cdot [\text{H}^+] + K_1 \cdot K_2} \quad (3)$$

Employing Eq. 3, the residual % of HI stored for a

constant time (t) at a condition was estimated by Eq. 4. Stability estimation is useful for evaluating the compatibility of an intravenous admixture (mixed infusion) of HI containing SBS as an additive.

$$\text{residual \%} = 10^{-(k_{\text{obs}}/2.303) \times 100} \quad (4)$$

Effects of Glucose on the Degradation of HI We studied the stabilization effects of glucose on HI in the presence of SBS in the pH range of 6.0–7.0, in which HI was relatively unstable. The semilogarithmic plots for the degradation of HI (initial concentration approximately 0.08 unit/ml) in the coexistence of glucose (0–20%) and SBS (2.0×10^{-3} M) at pH 7.0 in the dark gave straight lines (Fig. 7). The residual of HI after storage for 2 h in the coexistence of SBS (2.0×10^{-3} M) and glucose (20% (w/v), *ca.* 1.1 M) at pH 7.0 and 6.0 were 90.9 and 98.2%, respectively. From these findings, the mixed infusion of HI containing SBS with glucose (about 20%) was considered to be rather stable even in the pH range 6.0–7.0.

We subsequently studied the stabilization mechanism of glucose for HI in the presence of SBS in the pH range of 6.0–7.0. The degradation rate constants for HI incubated at pH 7.0 and 25°C in the coexistence of SBS (2.0×10^{-3} M) and glucose (0–20%) in the dark were plotted against the concentration of glucose (Fig. 8). As shown in Fig. 8, HI

Fig. 7. Semilogarithmic Plots for the Degradation of HI in the Coexistence of Glucose and SBS in Phosphate Buffer (pH 7.0) in the Dark at 25°C and $\mu=0.5$

Initial concentration of HI, approximately 0.08 unit/ml; $[\text{SBS}]_{\text{total}} = 2 \times 10^{-3}$ M; ●, glucose = 0%; ■, glucose = 5%; ▲, glucose = 10%; ▼, glucose = 20%.

Fig. 8. Relationship between the Glucose Concentration and the Apparent First-Order Degradation Rate Constant of HI in the Presence of SBS in the Dark in 0.05 M Phosphate Buffer of pH 7.0 with $\mu=0.5$ at 25°C

Initial concentration; HI, approximately 0.08 unit/ml; SBS, 2×10^{-3} M.

was stabilized by glucose concentration dependently. However, a linearity was not observed in these plots.

We previously revealed that stability for thiamine, fursultiamine³⁾ and urokinase⁶⁾ in the coexistence of SBS and glucose depended on the concentration of free SBS. The degradation rates of HI were assumed to depend mainly on the concentrations of free SBS.

The equilibrium of the BS-glucose addition reaction is represented as Eq. 5, and the equilibrium constant of the BS-glucose adduct ($K_{\text{BS-glucose}}$) is represented as Eq. 6. The equilibrium of the chemical species are represented by Eqs. 7 and 8. The concentration of the BS-glucose adduct was much smaller than the concentration of free glucose in this experimental condition, therefore the total glucose concentration can be represented approximately as in Eq. 8'. From Eqs. 6–8', the concentration of free SBS can be expressed as Eq. 9.

$$K_{\text{BS-glucose}} = \frac{[\text{BS-glucose}]}{[\text{SBS}]_{\text{free}} \cdot [\text{glucose}]_{\text{free}}} \quad (6)$$

$$[\text{SBS}]_{\text{total}} = [\text{SBS}]_{\text{free}} + [\text{BS-glucose}] \quad (7)$$

$$[\text{glucose}]_{\text{total}} = [\text{glucose}]_{\text{free}} + [\text{BS-glucose}] \quad (8)$$

$$[\text{glucose}]_{\text{total}} \div [\text{glucose}]_{\text{free}} \quad (8')$$

$$[\text{SBS}]_{\text{free}} = \frac{[\text{SBS}]_{\text{total}}}{1 + K_{\text{BS-glucose}} \cdot [\text{glucose}]_{\text{total}}} \quad (9)$$

Fig. 9. Relationship between the Free SBS Concentration and Apparent First-Order Degradation Rate Constant of HI in the Dark in the Coexistence of Glucose and SBS in 0.05 M Phosphate Buffer (pH 7.0 and 6.0, $\mu=0.5$) at 25°C

(A) pH 7.0, (B) pH 6.0. Initial concentration; HI, approximately 0.08 unit/ml; $[\text{SBS}]_{\text{total}} = 2 \times 10^{-3}$ M; ●, glucose = 0%; ■, glucose = 5%; ▲, glucose = 10%; ▼, glucose = 20%.

where $K_{\text{BS-glucose}}$ means the equilibrium constant of the BS-glucose adduct (BS-glucose), and $[\]$ expresses the molar concentration of each chemical species. The measurement of the equilibrium constants for the BS-glucose adduct at pH 6.0 and 7.0 was described in Experimental.

The relationship between the degradation rate constants of HI at pH 7.0 in the coexistence of SBS ($2.0 \times 10^{-3} \text{ M}$) and glucose (0–20%) in the dark and the concentrations of free SBS is shown in Fig. 9A, and the plots gave a straight line. Similarly, the plots between the degradation rate constants of HI at pH 6.0 and the concentrations of free SBS gave a straight line (Fig. 9B). From these results, the stability of HI in the solution containing SBS and glucose was inferred to depend largely on the concentration of free SBS.

Another stabilization mechanism for HI was also suggested from the fact that each regression line in Fig. 9 did not pass through each origin. Brange *et al.* reported that the physical stability of insulin was slightly increased by the addition of glucose.²⁷⁾ Nevertheless, this stabilization mechanism was not discussed in the report. A study by Loughheed *et al.* showed that the alcohols such as glycerol (30–50%) and isopropanol (10–50%) are moderately effective stabilizers for insulin.²⁸⁾ The mechanism for this phenomenon had been postulated to be the reduction in the polarity of the environment of the insulin molecule.²⁸⁾ Moreover, glucose also slightly reduces the polarity of the aqueous solution.²⁹⁾ For these reasons, it was suggested that the reduction of the polarity by the addition of glucose also contributed to the stabilization of HI in the presence of SBS.

Considering the practical stability estimation, the concentration of free SBS in the coexistence of glucose could be estimated by Eq. 9 as previously described. The degradation rate constant of HI could be estimated practically from the concentration of free SBS and regression lines at pH 7.0 and pH 6.0 shown in Fig. 9A and 9B, respectively. Therefore, the residual % of HI in a mixed infusion stored for a chosen constant time (t) at a constant glucose concentration in the coexistence of SBS (about $2.0 \times 10^{-3} \text{ M}$) at pH 6.0 or 7.0 can be estimated by Eq. 4.

From these kinetic studies, it may be possible to evaluate the compatibility of a mixed infusion of HI containing SBS in the absence or presence of glucose, and to find the optimal conditions.

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References and Notes

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