Stability of Insulin in Saline Containing Sodium Hydrogen Sulfite

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This study demonstrates that the stability of insulin is affected by the components in the preparation. The stability of human insulin was measured using high-performance liquid chromatography (HPLC) at various concentrations and temperatures (37.0°C, 30.0°C or 22.0°C) in the presence and absence of sodium hydrogen sulfite. Insulin in physiological saline at 37°C was stable for at least 24 hours. In the presence of sodium hydrogen sulfite, however, it was degraded, presumably due to cleavage of S-S bonds in the insulin molecules. Higher degradation rate constants were observed with increases in temperature. The degradation rate constant (k , observed value hr^{-1}) was 0.00619 at 22°C, 0.01649 at 30°C, and 0.03308 at 37°C. The activation energy of insulin (Arrhenius equation) calculated from these values was 20.3 kcal/mol. The daytime room temperature in hospitals is maintained at 22.0–27.0°C, and this study showed that, in this temperature range, the stability of insulin in physiological saline containing sodium hydrogen sulfite decreases. The residual percentage of insulin after 24 hours at 22.0°C and 25.0°C was 85.9% and 80.7%, respectively, based on the Arrhenius equation. These results indicate the importance of proper handling of insulin at medical facilities. They also show that appropriate temperature control is necessary for insulin dissolved in solutions containing sodium hydrogen sulfite.

Key words — insulin, sodium hydrogen sulfite, saline, stability, HPLC

Introduction

Insulin was one of the first bio-pharmaceutical products to be developed. A highly purified human insulin formulation was created by genetic recombination technology in the early 1980s, ensuring a stable supply of safe insulin formulations. The introduction of pen injectors made it possible to treat diabetes by insulin self-injection. Further breakthroughs in biotechnology have led to the clinical application of ultra-fast-acting and soluble long-acting human insulin analogues in diabetes treatment. It is anticipated that new insulin therapies tailored to a variety of lifestyles will be developed in the future.¹⁾

Current Japan Diabetes Society guidelines list insulin as one of the alternatives for early treat-

ment of type 2 diabetes. Because of its high efficacy, the biosimilar compound insulin glargine was approved for use in Japan in 2014. (Insulin Glargine BS Inj. [Lilly], Medical package insert, Mar 2015) New formulations are now on the market and have been listed in the Japanese Pharmacopoeia, 16th edition, Supplement II (JPXVI second supplement; The Ministry of Health, Labour and Welfare Ministerial Notification No. 47, 2014). However, for managing blood glucose levels in patients receiving intravenous (parenteral) nutrition, the adsorption of insulin administered by infusion can be problematic. Previous studies have reported differences in the amount of insulin delivered due to adsorption onto the surfaces of containers used for infusion or filters used in infusion lines, such that the residual ratio when the

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adsorbed amount reaches saturation becomes almost constant. The adsorption of insulin can be suppressed by the addition of vitamins to parenteral nutrition formulations containing a surfactant.²⁾ However, insulin is reportedly degraded by sodium hydrogen sulfite, which is often used to stabilize infusion components. This degradation is enhanced with increasing pH, but is suppressed by the addition of sugars.²⁾ In addition, other components (saccharides *et al*) in formulations have been shown to affect the stability of insulin.²⁻⁴⁾

In this study, high-performance liquid chromatography (HPLC) was used to evaluate the stability of human insulin at various temperatures and initial concentrations in the presence/absence of sodium hydrogen sulfite in order to better characterize the conditions affecting insulin stability. With regard to the stability of insulin preparations, there are descriptions of the storage temperature (2-8°C) in the package insert and the stress test at the time of application for manufacturing approval in the interview form. The temperature of hospital rooms is typically maintained at 22.0-27.0°C in the daytime.⁵⁾ There is already a report on the stability of insulin, but there are no reports on basic data. In this study, the stability of insulin at various room temperatures and time periods was predicted based on its activation energy.

Materials and Methods

1. Reagents

Insulin (Wako, insulin human, recombinant, for cell culture, minimum 27.5 units/mg) and sodium hydrogen sulfite (Wako, special grade) were used in the study. All other reagents used were commercially available (special grade).

Human insulin (Genetical Recombination): Contains not less than 27.5 Insulin Units per mg,

calculated on a dried basis. [The Japanese Pharmacopoeia, 17th edition (JPXVII; The Ministry of Health, Labour and Welfare Ministerial Notification No. 64, 2016)].

2. Preparation of test solutions

Insulin solution (A): insulin was dissolved in 0.01 mol/L hydrochloric acid to a concentration of 2 mg/mL (= 55 IU/mL). Diluted insulin solution (B): insulin solution (A) was diluted 10-fold in 0.01 mol/L hydrochloric acid (0.2 mg/mL = 5.5 IU/mL). Sodium hydrogen sulfite solution: sodium hydrogen sulfite was dissolved in distilled water (0.1 g / 10 mL). The actual concentration of sodium hydrogen sulfite in the reaction solution (25 mg / 100 mL) was the same as that in commercially available infusion formulations.

3. Calibration curves

Calibration curves were prepared based on the initial insulin concentration using the area value method, as follows:

- (1) Calibration curve (normal). Standard solution: insulin solution (A) (20 µL), saline (380 µL), and 0.02 mol/L HCl (400 µL), 800 µL total, prepared on ice. For HPLC, 50, 100 or 150 µL of the standard solution was injected (amount of insulin, 2.5, 5.0, or 7.5 µg, respectively).
- (2) Calibration curve (1/10 insulin initial concentration): Ten-fold diluted standard solution: diluted insulin solution (B) (20 µL), saline (380 µL), and 0.02 mol/L HCl (400 µL), 800 µL total, prepared on ice. For HPLC, 50, 100 or 150 µL of standard solution was injected (amount of insulin, 0.25, 0.5 or 0.75 µg, respectively).

4. Procedure for analysis of insulin degradation

Reaction solutions: sodium hydrogen sulfite was added (or not) to the insulin solution (initial amount of insulin, 5.0, 2.5, or 0.5 μg). At appropriate time intervals, the reaction mixture was sampled, and the amount of insulin remaining was determined by reversed-phase HPLC using a calibration curve prepared at the same time. Similar experiments were conducted using one-half or one-tenth the initial concentration of insulin at 37.0, 30.0, or 22.0°C.

5. Preparation of reaction solutions

Reaction solutions were prepared on ice as follows.

Reaction solution 1: saline (760 μL) and insulin solution (A) (40 μL), 800 μL total (insulin: 2.75 IU/mL).

Reaction solution 2: saline (720 μL), sodium hydrogen sulfate solution (40 μL) and insulin solution (A) (40 μL), 800 μL total.

Reaction solution 3: saline (740 μL), sodium hydrogen sulfate solution (40 μL) and insulin solution (A) (20 μL), 800 μL total.

Reaction solution 4: saline (720 μL), sodium hydrogen sulfate solution (40 μL) and insulin solution (B) (40 μL), 800 μL total.

6. Preparation of sample solutions for HPLC

Samples (60 μL) were taken from the reaction solutions at various time points (0, 2, 4, 6, 8, 10, 22.2 and 24 h, or 0, 1, 2, 3 and 4 h). Hydrochloric acid (0.02 mol/L; 60 μL) was added to the samples while on ice and mixed. A 100- μL aliquot of each sample was then injected for HPLC.

7. HPLC analysis

HPLC analysis was performed using a 515 HPLC pump (Waters, Milford, MA), equipped with a Rheodyne 7725i injector, Waters in-line degasser AF, 2487 dual-lambda absorbance detector (214 nm) (Waters), and Chromatocorder 21 (System Instruments Co, Ltd Tokyo, Japan). A reversed-phase column (TOSOH TSK-GEL ODS-80Ts; 4.6 mm ID $\times$ 150 mm) was used. The mobile phase was $\text{CH}_3\text{CN}/20 \text{ mM NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 7.0; 3/7), and the flow rate was 1.0 mL/min.

8. Other equipment and materials

Thermostatic baths: for 22.0°C, TB Braun Meisinger AG Thermomix_1441; W. Germany; for 30.0 or 37.0°C, TAITEC Dry Thermo Unit DTU-1C aluminum block heater. Reaction vessel: Chromacol Ltd. For HPLC/GC autosampler, Chromacol glass sample vials (screw top 1.1 mL) were used.

9. Method for calculating degradation rate constant

Activation energy was calculated from the Arrhenius equation using the degradation rate constant at three temperatures observed in this study.

Arrhenius equation: $k = A \exp(-E_a/RT)$, wherein A is the frequency factor, E_a is the activation energy; R is the gas constant, T is the absolute temperature, k is the kinetic rate constant obtained in experiments. Activation energy was calculated by an Arrhenius' plot of $\ln k$ versus $(1/T)$.

10. Significant differences

Significant differences were tested using Student's t -test.

Results and Discussion

A typical chromatogram for the reaction mixture is shown in **Fig 1**, indicating an elution time of 6.7 min for insulin. As shown in the chromatogram in **Fig 1** and in the Table, insulin with an initial amount of 5 μg was stable in the absence of sodium bisulfite after 24 hours at 37°C, but decreased to about 40% in the presence of sodium bisulfite. Good linearity was observed for calibration curves constructed using the area value and peak height methods for undiluted and 10-fold diluted insulin solutions, respectively. These results demonstrate that the numerical values in this study were appropriate.

No changes in the amount of insulin remaining in the solution without sodium hydrogen sulfite were observed after 24 hours at 37.0°C, indicating that insulin is stable under the conditions used in this experiment in the absence of sodium hydrogen sulfite.

The **Table** shows the amount of insulin remaining in solutions with/without sodium hydrogen sulfite at various time points, as determined by HPLC. As shown in **Fig 2** (0-24 hours), the amount of residual insulin declined significantly in the presence of sodium hydrogen sulfite. Significant differences between the presence and absence of sodium hydrogen sulfite with an initial amount of 5 μg of insulin after 24 hours at 37°C, as assessed

Fig 1 Typical chromatogram of insulin (initial amount 5 μg , t_R 6.7 min) in the presence (25 mg / 100 mL) / absence of NaHSO_3 (37.0°C, 24 h) and insulin solution calibration curve (a) peak area value method, b) peak height value method).

R^2 : coefficient of determination.

Table Average value (%) of remaining insulin calculated with the value at 0 h given as the initial value in solution in the presence (25 mg/100 mL)/absence of sodium bisulfite.

Time (h)	Average amount remaining (%) (n = 6)					
	37°C, 5 μg NaHSO_3 (-)	37°C, 5 μg NaHSO_3 (+)	37°C, 2.5 μg NaHSO_3 (+)	37°C, 0.5 μg NaHSO_3 (+)	30°C, 5 μg NaHSO_3 (+)	22°C, 5 μg NaHSO_3 (+)
0	100 $\pm$ 0.0	100 $\pm$ 0.0	100 $\pm$ 0.0	100 $\pm$ 0.0	100 $\pm$ 0.0	100 $\pm$ 0.0
2	103.6 $\pm$ 14.4	77.6 $\pm$ 16.1	111.1 $\pm$ 26.0	90.6 $\pm$ 15.0	102.9 $\pm$ 15.8	115.8 $\pm$ 26.8
4	104.2 $\pm$ 8.4	80.3 $\pm$ 6.1	100.6 $\pm$ 17.9	67.1 $\pm$ 7.7	91.7 $\pm$ 15.9	111.3 $\pm$ 21.4
6	101.1 $\pm$ 14.6	72.7 $\pm$ 6.1	93.3 $\pm$ 17.8	56.7 $\pm$ 7.6	89.8 $\pm$ 17.0	102.5 $\pm$ 13.7
8	97.3 $\pm$ 12.0	71.6 $\pm$ 6.3	87.3 $\pm$ 23.4	49.7 $\pm$ 4.6	92.2 $\pm$ 12.0	105.9 $\pm$ 24.9
10	100.3 $\pm$ 12.9	72.1 $\pm$ 7.8	83.0 $\pm$ 23.6	46.1 $\pm$ 6.5	87.0 $\pm$ 16.7	108.4 $\pm$ 22.6
22.2	94.2 $\pm$ 9.9	46.4 $\pm$ 8.9	48.1 $\pm$ 25.7	28.0 $\pm$ 7.1	69.8 $\pm$ 14.3	96.6 $\pm$ 19.0
24	99.7 $\pm$ 21.3	39.4 $\pm$ 8.2	49.8 $\pm$ 23.3	18.8 $\pm$ 4.0	70.5 $\pm$ 15.4	94.3 $\pm$ 25.2

mean $\pm$ SD.

Fig 2 Temporal variation in the amount of insulin when sodium hydrogen sulfite was added to the insulin solution (0-24 h) (average value, $n = 6$).

○ 37.0°C, 5 µg, ● 37.0°C, 5 µg (+), □ 37.0°C, 2.5 µg (+), △ 37.0°C, 0.5 µg (+), ◆ 30.0°C, 5 µg (+), ▲ 22.0°C, 5 µg (+). (+): sodium hydrogen sulfite added.

Fig 3 Effects of changing the initial insulin concentration on the amount of insulin remaining in the presence of sodium hydrogen sulfite (37.0°C) (24 h) (average value, $n = 6$).

● 37.0°C, 5 µg (+), □ 37.0°C, 2.5 µg (+), △ 37.0°C, 0.5 µg (+). (+): sodium hydrogen sulfite added.

by Student's t -test, were observed ($P < 0.01$). To confirm these results, changes in the amount of residual insulin were measured in samples to which sodium hydrogen sulfite was added. The results indicate that the addition of sodium hydrogen sulfite leads to the degradation of insulin.

The degradation of insulin in the presence of sodium hydrogen sulfite at 37.0°C progressed as the primary reaction (**Fig 3**). In addition, the degradation rate constant increased with decreasing initial insulin concentration.

Measurement of residual insulin was carried out

Fig 4 Relationship between reaction temperature and insulin stability in the presence of sodium hydrogen sulfite (average value, 0-24 h) ($n = 6$).

● 37.0°C, 5 µg (+), ◆ 30.0°C, 5 µg (+), ▲ 22.0°C, 5 µg (+). (+): sodium hydrogen sulfite added.

Fig 5 Arrhenius equation: $k = A\exp(-Ea/RT)$, wherein A is frequency factor, Ea is the activation energy; R is the gas constant, T is the absolute temperature, k is kinetic rate constant obtained in experiments. Activation energy was calculated by an Arrhenius' plot of $\ln k$ versus $(1/T)$.

R^2 : coefficient of determination.

at 37.0, 30.0 and 22.0°C in the presence of sodium hydrogen sulfite. The rate of insulin disappearance increased with reaction temperature (**Fig 4**).

In the presence of sodium hydrogen sulfite at 37.0°C, degradation of insulin was observed in the primary reaction. The degradation rate constant increased as the initial concentration of insulin decreased. The activation energy of insulin, as derived from an Arrhenius plot (Ea), was 85.1 kJ/mol (= 20.3 kcal/mol) (**Fig 5**). Reported activation energies for drug decomposition reactions are typically range from 14 to 23 kcal/mol.⁶⁾ The activation energy for insulin obtained in this study was therefore reasonable.

$$Ea = 10238 \times 8.3144621 = 85123.462 \text{ J/mol} =$$

$$85.1 \text{ kJ/mol}$$

$$\ln k = -10238 \times (1/T) + 29.641$$

(T : absolute temperature) (1)

The calculated value of k obtained from equation (1) and the observed value of k at each temperature were as follows (k : kinetic rate constant hr^{-1}):

22°C: 0.00632 (calculated value),

0.00619 (observed value)

25°C: 0.00896 (calculated value)

30°C: 0.01580 (calculated value),

0.01649 (observed value)

37°C: 0.03388 (calculated value),

0.03308 (observed value)

Disulfide bonds are known to be cleaved by reduction^{7, 8)} and sodium hydrogen sulfite is also used as a reducing agent. These facts suggest that disulfide bonds were cleaved by sodium hydrogen sulfite. The present results show that insulin is stable at 37.0°C in saline for up to 24 hours. However, the observed decrease in the amount of insulin remaining in solution in the presence of sodium hydrogen sulfite suggests that sodium hydrogen sulfite promotes the degradation of insulin via reduction of disulfide bonds in the insulin molecule. Examination of insulin stability at various temperatures (37.0, 30.0, and 22.0°C) showed that the rate of degradation increases with increasing temperature. The calculated activation energy (E_a) of insulin obtained from this experiment was 20.3 kcal/mol. The calculated percentage of insulin remaining after 24 hours at 22.0 and 25.0°C (representing typical hospital room temperatures) was 85.9 and 80.7%, respectively. The results of this study underscore the importance of ensuring proper use of insulin in medical facilities. The insulin dissolved in sodium bisulfite containing solution had a residual ratio of 80.7% after 24 hours at 25°C, suggesting that the effects of insulin are slightly attenuated by long-term storage under

the same conditions. Because of differences in the temperature settings of medical institutions, proper management strategies are necessary. In addition, insulin dissolved in sodium hydrogen sulfite-containing solutions must be maintained at an appropriate temperature after preparation.

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Competing Interests

The authors have no competing interests to declare.

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