Apparent Increase of Insulin Peak Area in HPLC Analysis of a Preparation Consisting of a Mixture of Insulin and Total Parenteral Nutrition

Etsuko ICHIKAWA,*[,a] Michio KIMURA,[a] Hiromi MORI,[a] Futoshi YAMAZAKI,[a] and Kazuyuki HIRANO[b]

[a] *Ogaki Municipal Hospital, Department of Pharmacy; 4–86 Minaminokawa-cho, Ogaki, Gifu 503–8502, Japan: and* [b] *Gifu Pharmaceutical University, Laboratory of Pharmaceutics; 5–6–1 Mitaborahigashi, Gifu, Gifu 502–8585, Japan.*
Received January 5, 2006; accepted April 13, 2006

The peak area of insulin in a mixture of K.C.L.® injection and hyperalimentation fluid was found to increase in a time dependent manner up to 24 h in measurement by a high-performance liquid chromatograph. The increase of peak area corresponding to the insulin was detected at wavelengths of both 210 and 280 nm. This increase was only observed in the presence of the sugars, tryptophan, riboflavin, and insulin, and ascorbate was shown to counteract the increase. These results suggest the possibility that insulin forms a mixture caused by the oxidation reaction in a hyperalimentation fluid.

Key words insulin; tryptophan; riboflavin; total parenteral nutrition; HPLC

Although insulin is well known to be adsorbed to infusion bags, infusion lines and filters,[1,2] studies on the stability of insulin in a HPN (Home Parenteral Nutrition) mixture, a hospital-prescribed hyperalimentation fluid, are rare. Since the mixture is prepared by adding insulin to a TPN (Total Parenteral Nutrition) fluid and can be stocked for up to one week, the quality control of insulin in the mixture must be examined. We previously reported the stability of insulin in a TPN fluid by high performance liquid chromatograph (HPLC) measurement.[3,4] During the course of the experiment, the peak area of insulin in the HPLC chromatogram was found to increase more than the amount theoretically calculated by measuring insulin in a hospital-prescribed HPN mixture composed of a K.C.L.® injection and a hyperalimentation fluid. In this report, we describe that the increase is based on the formation of the mixture consisting of insulin, tryptophan, and riboflavin in the presence of sugars.

Experimental

Medicinal Materials Insulin (Humulin® R, U-40) was purchased from Eli Lilly Japan K.K. and 10% glucose solution and Aminotripa® were obtained from Otsuka Pharmaceutical Co., Ltd. Moripron® F, and Hicaliq® No. 2 were purchased from Ajinomoto Pharma Co., Ltd., and Terumo Corporation, respectively. TPN multivitamin Sohvita® and vitamin C "Fuso" were purchased from Fuso Pharmaceutical Industries, Ltd. Flavin adenine dinucleotide (FAD) Flavitan® was obtained from Toa Eiyo Ltd. K.C.L.® injection and riboflavin sodium phosphate were purchased from Maruishi Pharmaceutical Co., Ltd.

The K.C.L.® injection (20 ml) contained 3 g of potassium chloride and 6 mg of riboflavin sodium phosphate as coloring agent.

Sohvita® (Table 1) consisted of No. 1, No. 2, and No. 3 components, and is usually used for patients when mixed with hyperalimentation formula.

Chemical and Reagent The 19 amino acids and 5 carbohydrates were special-grade reagents manufactured by Wako Pure Chemical Industries, Ltd.

Acetonitrile was of HPLC grade and perchloric acid was of analytical grade; both were from Wako Pure Chemical Industries, Ltd.

Special Preparation The hospital-prescribed TPN preparation (S60)[5] consisted of 400 ml of 50% glucose solution, 200 ml of AMIPAREN®, 170 ml of Veen®-F, 17 ml of K.C.L.® injection, 45 ml of 10%NaCl, and 128 ml of distilled water. Each solution was mixed in HICALIQ® IVH Bag (TERUMO® Corporation) using a TERUMO SYRINGE® (TERUMO® K.K.) and injector needle (NIPRO® Corporation). The entire preparation procedure for S60 was carried out in a clean room.

Apparatus The peak area of insulin was determined by a HPLC system equipped with LC-10A (Shimadzu) high-performance liquid chromatogram, Shim-pack column CLC-ODS (M) (4.6×150 mm) (Shimadzu) packed with spherical, porous, high purity silica particles, and a UV detector, SPD-M10A (Shimadzu). The UV wavelength detecting insulin was set at 210 and 280 nm.

Chromatographic Conditions The mobile phase was prepared with 1.4% perchloric acid–acetonitrile (70 : 30). The flow rate and the column temperature were set at 1.0 ml/min and 40 °C, respectively.

Data Processing Insulin concentration ratios were calculated from a calibration curve generated using standard insulin solution in accordance with the following formula: relative peak area of insulin=(apparent peak area at 0.01 U/ml of insulin)/(theoretical peak area at 0.01 U/ml of insulin) ratios were subsequently compared. The mean of 3 samples was calculated.

Results

We first determined which components in the mixture of insulin, K.C.L.® injection, and a TPN fluid participated in the increase of the relative peak area corresponding to insulin by HPLC analysis, since the relative peak area was observed to increase in our previous studies.[3,4] This increase was only found to occur in the mixture of insulin, K.C.L.® injection, glucose solution, and amino acid solution. Therefore, we investigated whether riboflavin sodium phosphate participated in the increase of the peak area or not, because K.C.L.® in-

Table 1. Composition of Sohvita®

No. 1 (powder)	No. 2 (solution)	No. 3 (solution)
Thiamin hydrochloride: 5 mg Riboflavin sodium phosphate: 5 mg Pyridoxine hydrochloride: 3 mg Cyanocobalamin: 0.03 mg Nicotinamide: 20 mg Folic acid: 1 mg Biotin: 0.2 mg	Ascorbic acid: 100 mg/2 ml Panthenol: 12 mg/2 ml	Retinol palmitate: 2500 I.U./2 ml Cholecalciferol: 200 I.U./2 ml Tocopherol acetate: 15 mg/2 ml Menatetrenone: 2 mg/2 ml

* To whom correspondence should be addressed. e-mail: f-oneriv@ogaki-tv.ne.jp
© 2006 Pharmaceutical Society of Japan

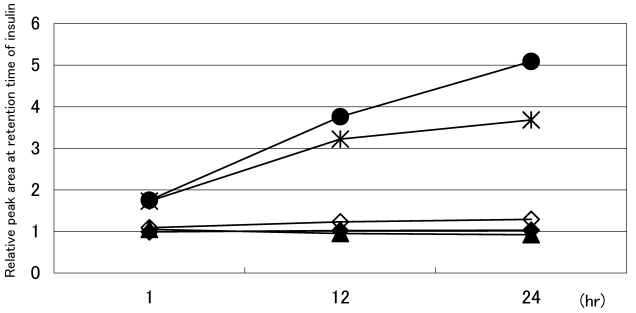

Fig. 1. Effect of Riboflavin Sodium Phosphate on the Peak Area at Retention Time of Insulin

Distilled water (◆) and 2 M KCL solution (▲) were prepared. Riboflavin sodium phosphate (5 mg/20 ml; ●) was dissolved in 2 M KCL solution. Flavin adenine dinucleotide (FAD) (5 mg/20 ml; ◇) was dissolved in 2 M KCL solution. Sohvita® No. 1 (✳) was dissolved in distilled water at 5 mg/20 ml as riboflavin sodium phosphate. One milliliter of the above solution was mixed with 50 ml of 10% glucose solution and 15 ml of Moripron® F. Insulin solution (0.01 U/ml) was dissolved in the mixture solution, respectively. Each insulin solution was stored in a glass test tube at room temperature under fluorescent light at time intervals of 1, 12 and 24 h after mixing. A 100 μl sample was injected to a column and measured by HPLC.

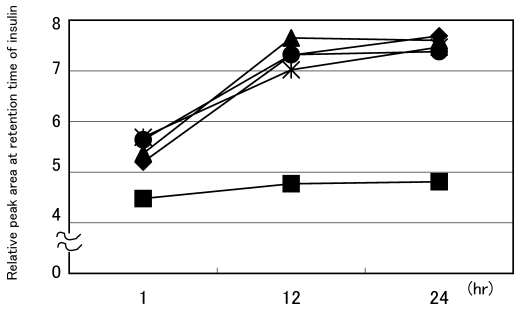

Fig. 2. Effect of Carbohydrates on the Peak Area at Retention Time of Insulin

Glucose (◆), maltose (▲), xylitol (✳), sorbitol (●), and fructose (■) were used as carbohydrates. Fifty milliliters of 10% carbohydrate solution was mixed with 15 ml of tryptophan solution (25 mg/15 ml) and 1 ml of riboflavin sodium phosphate solution (0.5 mg/ml). Insulin solution (0.01 U/ml) was prepared with the above mixed solutions, respectively. The experimental procedure are described in Fig. 1.

jection was colored with riboflavin sodium phosphate. The increase of the peak area was confirmed by the addition of K.C.L.® injection and Sohvita® No. 1 solution containing riboflavin sodium phosphate, but was not found by the addition of distilled water, KCl solution, and KCl solution containing FAD to the mixture of 10% glucose and Moripron® F (Fig. 1).

The effects of sugars, sugar alcohols, and amino acids were evaluated on the increase in the relative peak area corresponding to insulin. As shown in Fig. 2, the relative peak area increased in the presence of glucose, maltose, xylitol, and sorbitol, but not in the presence of fructose. Tryptophan was only shown to cause an increase of the relative peak area among amino acids containing a common infusion (Table 2).

In our previous study, we investigated the necessity of 3 components (glucose, riboflavin, tryptophan) on the increase in the relative peak area corresponding to insulin. We found that all 3 components were necessary to this phenomenon. In other words, in the absence of any one of the 4 components, there was no increase of the relative peak area corresponding to insulin. Therefore, the mixture of these components was analyzed using HPLC with detection wavelengths at 210 and 280 nm in order to obtain a clue to why the relative peak area increased when these components were mixed. As shown in

Table 2. Change of Relative Peak Area at Retention Time of Insulin Corresponding to Variety of Amino Acid

Amino acid	Relative peak area at retention time of insulin		
	1 h	12 h	24 h
L-Tryptophan	5.89	10.42	13.88
L-Proline	0.90	0.85	1.02
L-Phenylalanine	0.85	0.98	1.18
L-Tyrosine	1.06	1.01	1.02
L-Isoleucine	1.05	0.86	1.07
L-Alanine	0.96	0.85	1.04
L-Glutamic acid	0.95	0.77	0.88
L-Arginine	1.18	1.07	1.08
L-Methionine	1.09	1.06	1.08
L-Aspartic acid	1.08	1.05	1.05
L-Leucine	0.95	0.82	0.89
L-Lysine acetate	0.80	0.94	1.08
L-Serine	0.88	0.86	1.11
L-Cystine	0.90	1.04	1.15
L-Valine	0.99	0.83	1.14
L-Threonine	0.94	1.02	0.98
L-Cysteine	1.29	1.19	1.21
L-Histidine	0.95	1.00	0.87
L-Aminoacetate	1.04	0.89	0.61

The listed 19 reagents of constituent amino acid infusion were used as amino acid. Fifteen milliliters of each amino acid solution (25 mg/15 ml) was mixed with 50 ml of 10% glucose solution and 1 ml of riboflavin sodium phosphate solution (0.5 mg/ml), respectively. Insulin solution (0.01 U/ml) was prepared with the above mixed solutions, respectively. The experimental procedures are described in Fig. 1.

Fig. 3, the area increased at absorption wavelengths of not only 210 but also 280 nm in the presence of tryptophan. Increase of the area was found to be negated by the addition of ascorbic acid (Fig. 4). A typical TPN fluid used clinically commonly contains multi vitamins, for example, being prepared with complete sets of Sohvita® No. 1, 2, and 3. Sohvita® No. 1 contains riboflavin sodium phosphate and Sohvita® No. 2 contains ascorbic acid. Since the vitamin B_2 contained in these two formations caused the increase in the relative peak area, however, FAD did not show this phenomenon in spite of the vitamin B_2 group. On the other hand, complete Sohvita® sets did not show the increase in this area despite their content of vitamin B_2. Suppression of the increase in relative peak area was also observed with the TPN fluid containing ascorbic acid (complete Sohvita® sets) (Fig. 5).

Discussion

The peak area of insulin in a HPLC analysis of a HPN preparation prescribed in our hospital was found to increase and glucose, tryptophan, riboflavin, and insulin were identified as the sources of this increase. Chu *et al.*[6] reported that strongly-fluorescent tryptophan spontaneously attached to the A-chain of insulin. It is well known that riboflavin sodium phosphate produces a lumichrome in a neutral and acidic solution under light irradiation to yield fluorescence,[7] and a tryptophan–riboflavin complex is formed by oxidation reactions under light irradiation in an acidic alcohol solution.[8—12] These reports suggest the possibility that glucose, tryptophan, riboflavin, and insulin in the HPN preparation form a mixture which may contribute to the increase of the insulin peak area. As shown in Fig. 3, the peak area of insulin increase at 280 nm is similar to that at 210 nm in the mixture of the four above ingredients; this increase can be suggested to be based on the formation of the mixture, be-

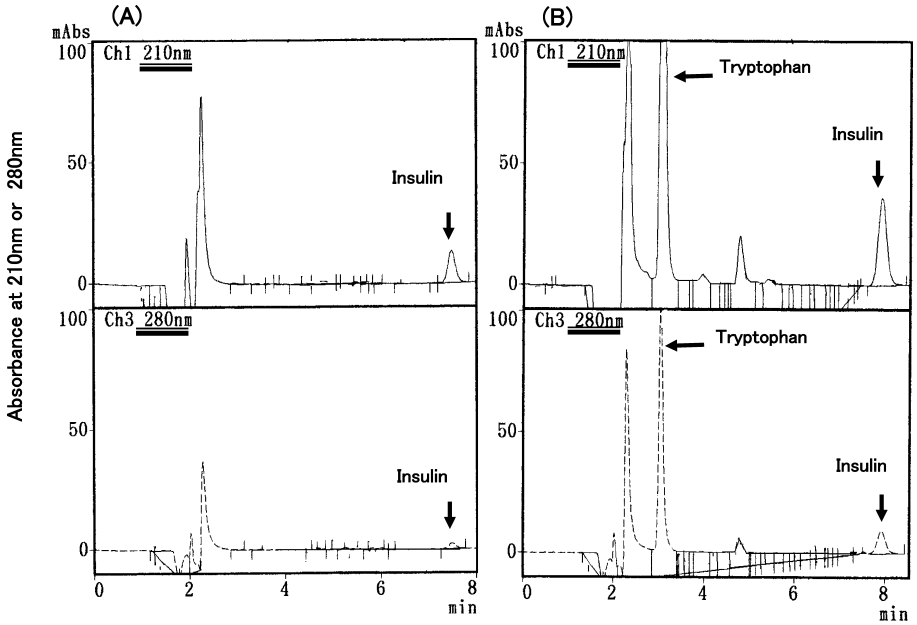

Fig. 3. Change of Absorbance at Retention Time Corresponding to Insulin

Insulin (0.01 U/ml) was dissolved in 10% glucose solution (A) or the mixture solution [tryptophan (25 mg/15 ml) : riboflavin sodium phosphate (0.5 mg/ml) : 10% glucose solution = 15 : 1 : 50] (B).

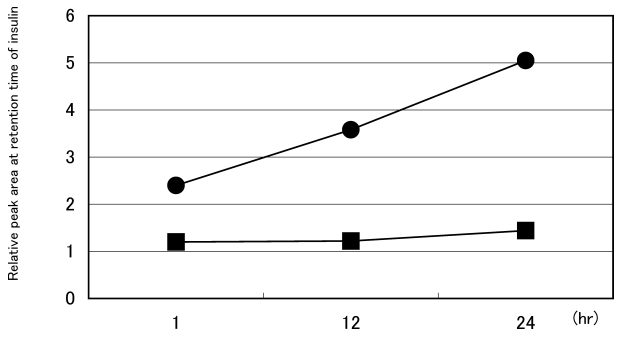

Fig. 4. Change of Relative Peak Area at Retention Time of Insulin Corresponding to Ascorbic Acid

One milliliter of riboflavin sodium phosphate (0.5 mg/ml) was mixed with 50 ml of 10% glucose solution and 15 ml of Moripron® F (●). Ten milligrams of ascorbic acid was mixed with the above solution (■). Insulin (0.01 U/ml) was dissolved in the mixed solution, respectively. The experimental procedures are described in Fig. 1.

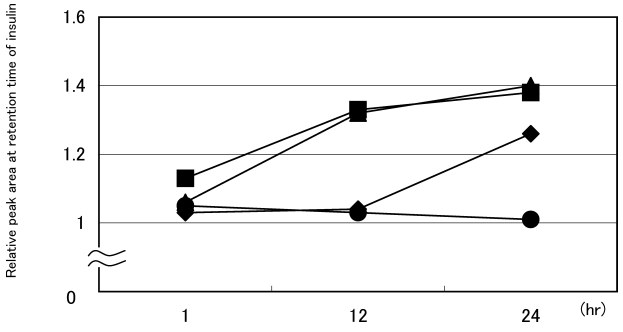

Fig. 5. Change of Relative Peak Area at Retention Time of Insulin Corresponding to Variety of Vitamin B_2 Formation

A complete set of Sohvita® (1 set/10 ml; ◆), riboflavin sodium phosphate (5 mg/10 ml; ▲), flavin adenine dinucleotide (FAD) (5 mg/10 ml; ●), and Sohvita® No. 1 (1 V/10 ml; ■) were used as vitamin B_2 containing formulation. Ten milliliters of each of these formulations was mixed with 700 ml of Hicalic® No. 2 and 200 ml of Moripron® F. Insulin solution (0.01 U/ml) was prepared with the above mixed solutions, respectively. The experimental procedures are described in Fig. 1.

cause native insulin does not contain a tryptophan residue and the absorbance of insulin at 280 nm is quite low. This possibility is further confirmed by our results that ascorbic acid, a reducing reagent, inhibits formation of the mixture by the oxidation reaction.

Since a TPN preparation clinically used is generally mixed with a multivitamin formulation containing ascorbic acid and is unlikely to mix merely riboflavin sodium phosphate with a TPN fluid, the formation of a tryptophan–riboflavin mixture rarely occurs in the TPN preparation within a short period after mixing. However, a K.C.L.® injection is commonly used to correct a serum potassium concentration and is frequently mixed with a TNP fluid. Therefore, it is necessary for pharmacists to pay attention to the formation of the insulin–tryptophan–riboflavin mixture if an infusion containing sugars, tryptophan, and insulin is prepared with a K.C.L.® injection. Pharmacists should also take account of the increase of the insulin peak area if they measure the insulin level in the TPN preparation by HPLC. Although several researchers have studied the hypoglycemic effect and other bioactivities of the tryptophan–insulin complex,[8,13,14] those of the insulin–tryptophan–riboflavin mixture have not yet been reported. It will be very interesting to determine what bioactivities the insulin–tryptophan–riboflavin mixture exhibits. Confirmation of the bioactivities of this mixture is our next goal.

References

1) Hirsh J. I., Fratkin M. J., Wood J. H., Thomas R. B., *Am. J. Hosp. Pharm.*, **34**, 583—588 (1977).
2) Whalen F. J., Lecain W. K., Latiolais C. J., *Am. J. Hosp. Pharm.*, **36**, 330—337 (1979).
3) Kimura M., Ichikawa E., Hiromi M., Yamazaki F., *Iryouyakugaku*, **28**, 108—115 (2002).
4) Kimura M., Ichikawa E., Ishii N., Yamazaki F., *Iryouyakugaku*, **29**, 230—236 (2003).
5) Ichikawa E., Yamazaki F., *Geka to Taisha, Eiyo*, **35**, 41—52 (2001).
6) Chu Y.-C., Burke G. T., Ross J. B., Katsoyannis P. G., *J. Protein Chem.*, **12**, 499—505 (1993).

7) Imahara K., "Basic Biochemical," Hirokawa, Tokyo, 1980, pp. 152—153.
8) Mitsuda H., Tsuge H., Kawai F., Tanaka K., *J. Vitaminol.*, **16**, 215—218 (1970).
9) Mitsuda H., Tsuge H., Kawai F., *J. Vitaminol.*, **16**, 219—224 (1970).
10) Salim-Hanna M., Edwars A. M., Silva E., *Int. J. Vitam. Nutr. Res.*, **57**, 155—159 (1987).
11) Silva E., Salim-Hanna M., Edwards A. M., Becker M. I., De Ioannes A. E., *Adv. Exp. Med. Biol.*, **289**, 33—48 (1991).
12) Tapia G., Silva E., *Radiat. Environ. Biophys.*, **20**, 131—138 (1991).
13) Geiger R., Geisen K., Summ H. D., *Hoppe. Seylers. Z. Physiol. Chem.*, **363**, 1231—1239 (1982).
14) Wang Z. Z., *Sci. Sin. Sin., Ser. B, Chem. Biol. Agric. Med. Earth Sci.*, **28**, 264—272 (1985).