# Identification of Insulin Variants in Patients with Hyperinsulinemia by Reversed-Phase, High-Performance Liquid Chromatography

SUSUMU SEINO, AKIHIRO FUNAKOSHI, ZU ZHI FU, AND AARON VINIK

**SUMMARY**

**We have characterized the molecular forms of circulating insulins in patients with hyperinsulinemia of diverse etiology. We have also compared the efficacy of various chromatographic conditions using reversed-phase (RP) HPLC. Using 0.2% trifluoroacetic acid (TFA) and triethylamine (TEA) with acetonitrile as the organic modifier, at an elution rate of 0.17%/min, porcine, bovine, and human insulins could be easily separated as well as abnormal insulins in the plasma of a patient (J.R.) with hyperinsulinemia of unknown etiology. When the reversed-phase C18 column was changed and a gradient of 0.33%/min was used, the abnormal insulin in patient J.R. could not be separated. By changing the solvent system to acetonitrile and isopropanol (vol:vol, 3:1) containing 0.1% TFA, omitting the TEA, and using a gentle gradient of 0.1%/min, various semisynthetic analogues of human insulin could be easily separated and the abnormal insulin could be identified in the plasma of the patient J.R. Abnormal insulin was also found in a patient with MEN-I, but in contrast, the insulins in eight patients with benign sporadic insulinomas appeared to be normal.**

**These results suggest that certain hyperinsulinemic states may be associated with an abnormal insulin and that RP-HPLC is useful for identification of insulin variants in the circulation. However, the conditions of RP-HPLC may be critical if the abnormalities of the insulin are subtle. DIABETES 1985; 34:1–7.**

Since the discovery that insulin was synthesized via a large single-chain precursor, preproinsulin, and its cleavage product, proinsulin, in the B-cells of the pancreas,[1-3] the possibilities of mutational change in insulin, as in all proteins, have become apparent.[4] Familial hyperproinsulinemia constitutes failure of cleavage of either the A-chain–C-peptide linkage site[5] or of the B-chain–C-peptide linkage site,[6] but neither present with diabetes. Tager et al.[7] have reported a patient with a structurally abnormal insulin that contained a leucine substituted for phenylalanine in position 24 or 25 of the insulin B-chain in a diabetic patient with hyperinsulinemia, suggesting that the abnormality of the insulin molecule per se contributed to the development of diabetes. The introduction of reversed-phase, high-performance liquid chromatography (RP-HPLC) as an analytic tool has made it possible to identify minor changes in the structure of various peptides in the circulation,[8-12] and Shoelson et al.[13] identified abnormal insulins in the serum of three diabetic patients with hyperinsulinemia.

In patients with insulin-secreting tumors, the proportion of proinsulin usually exceeds 22% of the total immunoreactive insulin.[14-16] Reports on the abnormal processing of hormones in hormone-producing tumors have accumulated in recent years.[17-18] Weintraub et al.[19] reported that there is a difference in the amino acid composition between tumor HCG and standard urinary HCG, suggesting that a structurally abnormal form of hormone may be produced in endocrine tumors. There are no reports of abnormal insulins in patients with insulin-secreting tumors. We have used RP-HPLC to characterize the circulating forms of insulin in patients with hyperinsulinemia: benign sporadic insulinoma, multiple endocrine neoplasia type I (MEN-I), and familial hyperinsulinemia with insulin resistance of unknown etiology.

## MATERIALS AND METHODS

Peripheral blood samples were obtained from normal, healthy subjects and patients with hyperinsulinemia of diverse etiology after an overnight fast and 30–180 min after ingestion of either 100 g of glucose or a meal. The characteristics of these patients are summarized in Table 1. Patient J.R. (familial hyperinsulinemia) presented with reactive hypoglycemia. Plasma counterregulatory hormones were normal and antibodies to insulin and insulin receptor were absent. Insulin binding to the patient's cellular receptors was

From the Department of Internal Medicine, Division of Endocrinology and Metabolism, The University of Michigan, Ann Arbor, Michigan.
Address reprint requests to A. I. Vinik, M.D., University Hospitals, Box 046 MPB, Room 2226, Ann Arbor, Michigan 48109.
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TABLE 1
Characteristics of patients studied

| Patient | Fasting IRI | Peak IRI | G/IRI Ratio | %PLC | Diagnosis |
|---|---|---|---|---|---|
| J.R. | 14 (98) | 643 (232)† | 7 | 2 | Familial hyperinsulinemia of unknown etiology |
| P.N. | 36 (131) | 384 (199)* | 3.6 | 25 | MEN-I |
| D.S. | 46 (66) | 104 (91)† | 1.4 | 22 | Benign sporadic insulinoma |
| A.S. | 16 (94) | 278 (101)† | 5.9 | 31 | Benign sporadic insulinoma |
| D.T. | 45 (44) | — | 1.0 | 22 | Benign sporadic insulinoma |
| S.P. | 23 (73) | 136 (125)† | 3.2 | 66 | Benign sporadic insulinoma |
| A.S. | 20 (57) | 145 (134)† | 2.0 | 67 | Benign sporadic insulinoma |
| B.N. | 244 (100) | | 0.41 | 66 | Benign sporadic insulinoma |
| M.S. | 141 (52) | — | 0.37 | 21 | Benign sporadic insulinoma |
| B.T. | 14 (117) | 158 (234)† | 8.4 | 41 | Benign sporadic insulinoma |

Abbreviations: IRI, immunoreactive insulin (μU/ml); G, blood glucose (mg/dl); PLC, proinsulin-like component in plasma (% of total IRI in fasting plasma); and MEN-I, multiple endocrine neoplasia type I. Samples obtained after meal* or glucose ingestion†. Concomitant blood glucose values are shown in parentheses. G/IRI ratio was calculated from the values in the fasting state.

also normal. The fasting insulins and insulin responses to glucose were 14–46 μU/ml and 475–1393 μU/ml, respectively. He became diabetic over a period of 3 yr. Three offspring of the patient had marked hyperinsulinemia without hypoglycemia or diabetes. Patient P.N. (MEN-I) presented with hypercalcemia with elevated parathyroid hormone and was proven to have tumors of the parathyroid and pituitary gland with markedly elevated serum gastrin levels indicative of gastrinoma. The patient underwent subtotal ($2\frac{1}{2}$ glands) parathyroidectomy, vagotomy, and pyloroplasty. The fasting insulin levels ranged from 25 to 45 μU/ml without accompanying hypoglycemia. Plasma counterregulatory hormones were all within the normal range. Glucose tolerance gradually became diabetic over a period of 4 yr, but the patient remained sensitive to exogenous insulin.

All the patients with benign sporadic insulinomas had symptoms of organic hyperinsulinism and hypoglycemia. The sample containing the highest concentration of insulin obtained during percutaneous, transhepatic, portal venous sampling[20] for tumor localization was chosen for the characterization of insulin, except in patient J.R. in whom peripheral venous samples were used. Blood was withdrawn into heparinized syringes and immediately centrifuged and the plasma was stored at −20°C until analysis.

The series of standard insulin, proinsulin, and C-peptide preparations used in this study were kindly donated by Dr. R. Chance of Eli Lilly and Company, Indianapolis, Indiana: biosynthetic human insulin, lot 615-2H2-86-1 and lot 615-2H2-270-3; crystalline porcine insulin, lot 615-07J-256; crystalline bovine insulin, lot 615-70N-80; biosynthetic human proinsulin, lots 759-0B6-201 and -205; porcine proinsulin, lot 759-70N-279-1; bovine proinsulin, lot 615-2H2-62; and human C-peptide, lot 759-0B6-200. Semisynthetic analogues of insulin were kindly provided by Dr. H. Tager, University of Chicago and Dr. K. Inouye, Shionogi Research Laboratories, Osaka, Japan: insulin with serine substituted for phenylalanine in B24 and B25 positions, and insulin with leucine substituted for phenylalanine in the B24 and B25 positions, respectively.

### REVERSED-PHASE, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

The apparatus used for HPLC (Model 332, Beckman Instruments, Inc., Berkeley, California) consisted of two pumps (A and B, model 110A), a programmer (model 420), and an injector (model 210) equipped with a model 160 absorbance detector with a Zn lamp and 214-nm filter connected to a BD 40 recorder (Kipp and Zonen, Delft, Holland). Two kinds of reversed-phase columns were used: an Ultrasphere ODS (250 × 4.6 mm, 5-μm particle size, Beckman Instruments) and a Bondapak C18 (300 × 3.9 mm, 10-μm particle size, Waters Associates, Milford, Massachusetts). An Ultrasphere ODS precolumn was attached to the column. Organic solvents and water were all HPLC grade, purchased from J. T. Baker Chemical Co. (Phillipsburg, New Jersey). Trifluoroacetic acid (TFA) was purchased from Sigma Chemical Co. (St. Louis, Missouri) and triethylamine (TEA) from Pierce Chemical Co. (Rockford, Illinois). All chromatography was carried out at room temperature. The flow rate was kept constant at 1 ml/min throughout each experiment. The column eluates were collected every 30–60 s and were immediately lyophilized or dried under nitrogen gas. Two different experimental procedures, A and B, were used as described below.

#### PROCEDURE A

**Sample preparation by Sep-Pak extraction.** A Sep-Pak C18 cartridge (Waters Associates) was used for sample preparation. Each cartridge was prewashed with 5 ml of 80% acetonitrile in 0.1% TFA (vol/vol) and then washed with water. One milliliter of each plasma sample was diluted in water (vol:vol, 1:5) and then applied to the cartridge. Plasma was then passed through the Sep-Pak cartridge and retained insulin was eluted with 1 ml of 60% acetonitrile in 0.1% TFA. The recovery of insulin with the Sep-Pak method was 65–75%. The eluates containing insulin were lyophilized and 2–3 samples were combined and reconstituted in 0.1 M acetic acid before application to the HPLC. As a control, a biosynthetic insulin preparation with and without the process of Sep-Pak extraction was used. Further controls included insulin extracted by the Sep-Pak cartridge method from the plasma of normal subjects both with and without the addition of the synthetic human insulin standard.

**Conditions of RP-HPLC.** A gradient elution was established using two solvents. Solvent A consisted of 0.2% TFA and 0.2% TEA in water (vol/vol) and solvent B was acetonitrile as an organic modifier. After injection of a 1-ml sample

into the access port, the column (Ultrasphere ODS, 5-μm particle size) was eluted with an initial concentration of solvent B in A + B of 32% and a final concentration of 45% with either step-wise or linear gradients using various incremental rates, as indicated in the figures. Fractions were collected every 30–60 s and dried either by lyophilization or under nitrogen gas and stored at −20°C for determination of immunoreactive insulin (RIA) content.

PROCEDURE B

**Sample preparation by immunoabsorbent purification.** The immunoaffinity column for sample preparation was prepared as follows. An insulin antiserum raised in a guinea pig was treated with 33% $(NH_4)_2SO_4$ to precipitate the immunoglobulin fraction. The immunoglobulin fraction was immobilized on a cyanogen bromide-activated Sepharose 4B column, according to the recommendation of the manufacturers, and the conjugate was packed in a column with a bed volume of 2 ml and a binding capacity of 10 μg of insulin. Five to ten milliliters of each plasma sample was diluted 1:2 with 0.4 M, pH 7.4, phosphate-buffered saline (PBS), applied to the immunoaffinity column, and recycled for 2–3 h at 4°C. The column was extensively washed with PBS and subsequently washed with distilled water. Insulin bound to the affinity column was desorbed with 1 M acetic acid and then lyophilized. The recovery of insulin from the immunoaffinity column was 90–95%. The immunopurified insulin was reconstituted with 0.1 M acetic acid before application to the HPLC column. As controls, insulin in plasma of normal subjects, normal human plasma fortified with human insulin, and biosynthetic human insulin standard in buffer, with and without the process of immunoaffinity purification, were used.

**Conditions of RP-HPLC.** During the course of the experiments using procedure A, we found that eluates from the HPLC column were not completely dry after lyophilization or nitrogen gas evaporation because of the concentration of TEA employed. Although this did not interfere with RIA of insulin after reconstitution with insulin assay buffer, pH 8.6, 0.1 M barbital-saline containing 0.1% merthiolate, and 0.25% bovine serum albumin,[21] the presence of TEA did affect further characterization of the biologic activity of insulin in receptor binding and glucose transport in isolated fat cells. We therefore modified the solvent system by eliminating TEA, according to the method of Tarr and Crabb.[22] Solvent A consisted of 0.1% TFA in water (vol:vol) and solvent B consisted of a combination of acetonitrile and isopropanol (MeCN:2-PrOH, 3:1, vol:vol) containing 0.1% TFA. After injection of 1-ml sample, the column (10-μm particle size, Bondapak) was eluted with 30% of B for an initial 10 min and then eluted with a linear gradient to 40% of solvent B for 100 min. Fractions were collected at 1-min intervals and lyophilized for RIA of insulin.

**Determination of insulin, proinsulin, and C-peptide.** Insulin was determined by radioimmunoassay previously described by Hayashi et al.[21] The insulin antiserum has been shown to cross-react slightly (1.6% ) with C-peptide, but reacts with human proinsulin on a mole-for-mole basis with insulin. The insulin antiserum also reacts on a mole-for-mole basis with porcine insulin and bovine insulin. The C-peptide antiserum used does not cross-react with either insulin or proinsulin. Iodination of insulin and C-peptide were performed by a modification of the chloramine-T method of Hunter and Greenwood.[23] $^{125}$Iodine was purchased from New England Nuclear Pharmaceuticals (Boston, Massachusetts).

## RESULTS

**HPLC profile of standard insulins, proinsulins, and C-peptide of various species and various semisynthetic insulin analogues.** Bovine, human, and porcine insulins and proinsulins were easily separated using RP-HPLC and a step-wise gradient elution using the solvent system in procedure A (Figure 1A). Four different semisynthetic insulin analogues substituted with leucine or serine at either the 24 or 25 position in the B-chain could be clearly separated by RP-HPLC under the conditions and the solvent system in procedure B (Figure 1B). The retention time of human insulin

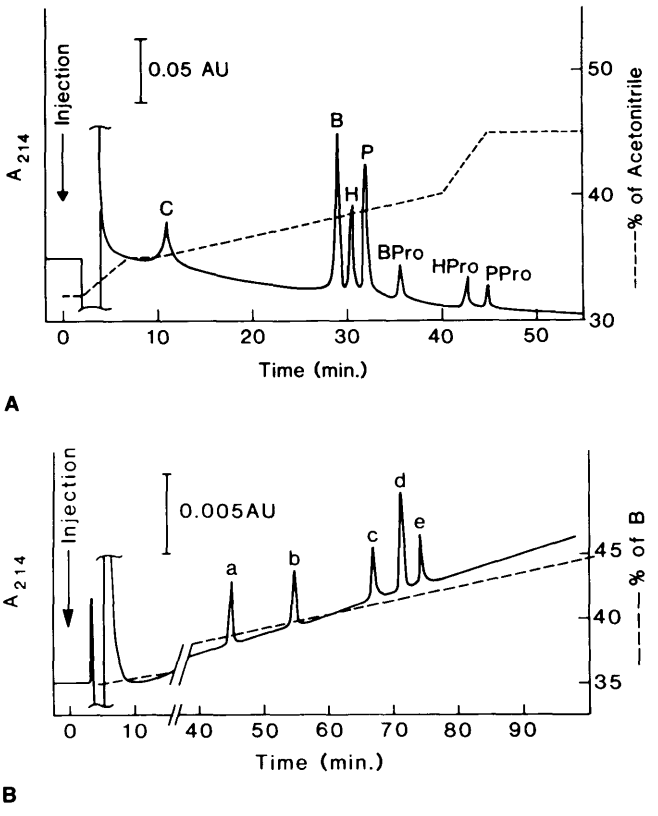


**FIGURE 1. (A) Separation of standard insulins, proinsulins, and C-peptide of various species by RP-HPLC. Abbreviations: C, human C-peptide; B, bovine insulin; H, human insulin; P, porcine insulin; BPro, bovine proinsulin; HPro, human proinsulin; and PPro, porcine proinsulin. A mixture of standard preparations containing 2–4 μg of each peptide was applied to the RP-HPLC. The column (Ultrasphere ODS, 5 μm) was eluted with 32% of solvent B in procedure A for an initial 2 min and then eluted with a linear gradient of 0.6%/min followed by isocratic elution for 3 min and then again eluted with a linear gradient of 0.17%/min to a concentration of 40% B, followed by a gradient of 1.0%/min to a final concentration of 45% of B. The continuous line represents the absorbance units detected at 214 nm. The interrupted line represents the gradient used. (B) Separation of semisynthetic analogues of human insulin by RP-HPLC using procedure B. Abbreviations: a, [Ser B$^{25}$]insulin; b, [Ser B$^{24}$]insulin; c, [Leu B$^{25}$]insulin; d, human insulin; and e, [Leu B$^{24}$]insulin. A mixture of standard preparations containing 1–2 μg of each peptide was applied to RP-HPLC. The column (Ultrasphere ODS, 5 μm) was eluted with 35% of solvent B in procedure B for an initial 5 min, and then eluted with a linear gradient of 0.1%/min to a final concentration of 45% of B. The continuous line represents the absorbance units. The interrupted line represents the gradient.**

was clearly distinguished from that of the insulin variants and porcine and bovine insulins, which differ structurally from human insulin by 1–3 amino acids, indicating that minor structural changes in the insulin molecule could be detected by RP-HPLC.

**HPLC profile of insulin in normal subjects and in patients with hyperinsulinemia.** As shown in Figure 2, insulin in plasma of normal subjects eluted with the same retention time as that of the human insulin standard detected by RIA. The elution volume of the insulin peak of a patient with a benign sporadic insulinoma (D.S.) corresponded to that of the human insulin standard and insulin in plasma of normal subjects that eluted in fraction 30. The insulin peak in other patients with benign sporadic insulinomas also corresponded to that in the controls (data not shown). In contrast, the insulin peaks in patient P.N. with multiple endocrine neoplasia type I and patient J.R. with familial hyperinsulinemia of unknown etiology eluted later, in fractions 40 and 36, respectively, suggesting that the insulins in both patients P.N. and J.R. are structurally abnormal and are more hydrophobic than normal human insulin.

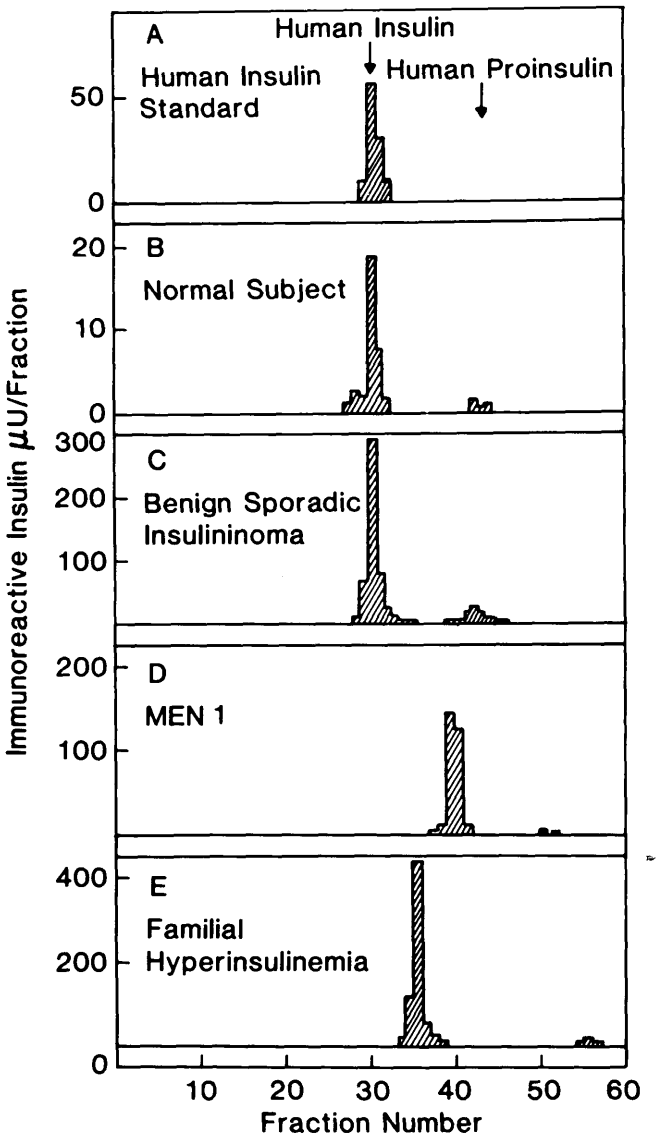


**FIGURE 2. Separation of insulins in a normal subject and in patients with various causes of hyperinsulinemia by RP-HPLC using procedure A. Panel A, standard human insulin; panel B, normal subject; panel C, one of eight patients with benign sporadic insulinomas; panel D, MEN-I; and panel E, familial hyperinsulinemia. The conditions of HPLC in these experiments were as described in Figure 1. All samples were prepared by Sep-Pak extraction of plasma.**

**Separation of normal and abnormal insulins by RP-HPLC using different linear gradients.** We examined the effect of changing the rate of gradient development on separation of mutant insulin from normal insulin. (Note: a new column was used for these experiments.) Using a linear increase in the gradient rate of 0.33%/min, the mutant insulin in patient J.R. eluted in the same fraction position as that of the human insulin standard and insulin extracted from plasma of normal subjects both with and without the addition of exogenous human insulin (Figure 3, left panel), indicating that the abnormal insulin could not be distinguished from normal human insulin under these conditions. However, with a reduction in the rate of increase in the linear gradient to 0.15%/min, the abnormal insulin in patient J.R. eluted in fraction 47, corresponding with a retention time of 23.5 min, which was later than that of the human insulin standard that eluted in fraction 45, corresponding to a retention time of 22–22.5 min (Figure 3, middle panel). Moreover, using a more gradual rate of increase of the gradient of 0.1%/min, the abnormal insulin peak in patient J.R. eluted in fraction 60 after 29.5–30 min, considerably later than that of the human insulin standard in fraction 55, after 27–27.5 min (Figure 3, right panel). On the other hand, insulin extracted from the plasma of normal subjects either with or without the addition of exogenous human insulin always eluted in the same position as the human insulin standard irrespective of the differences in gradients used. These results suggested that the use of the relatively slower gradient resulted in better separation of the abnormal insulin in patient J.R. and normal human insulin. Figure 3 also demonstrates that the Sep-Pak extraction method did not alter the retention time of insulin, indicating that the insulin molecule remained intact through this extraction procedure.

**HPLC profile of insulin in normal subjects and in patient J.R.** Using procedure B, the peak of the human insulin standard eluted with a retention time range of 72.5–75 min with day-to-day variation (73.6 ± 1.1 min, mean ± SD, N = 8, Figure 4). Furthermore, immunoabsorbent-purified insulin from plasma of normal subjects with or without the addition of exogenous human insulin standard eluted in a fraction that corresponded to that of the human insulin standard applied directly to the HPLC without prior extraction. However, using this procedure, insulin purified from the plasma of patient J.R. eluted in a peak at 85.5–86 min, suggesting that the insulin was structurally abnormal.

## DISCUSSION

In the present study, we have examined the circulating forms of insulin in patients with hyperinsulinemia of diverse etiology by a combination of reversed-phase, high-performance liquid chromatography (RP-HPLC) and radioimmunoassay (RIA). Using this method, we identified structurally abnormal insulins in a patient with multiple endocrine neoplasia type I (MEN-I) and in a patient with familial hyperinsulinemia of

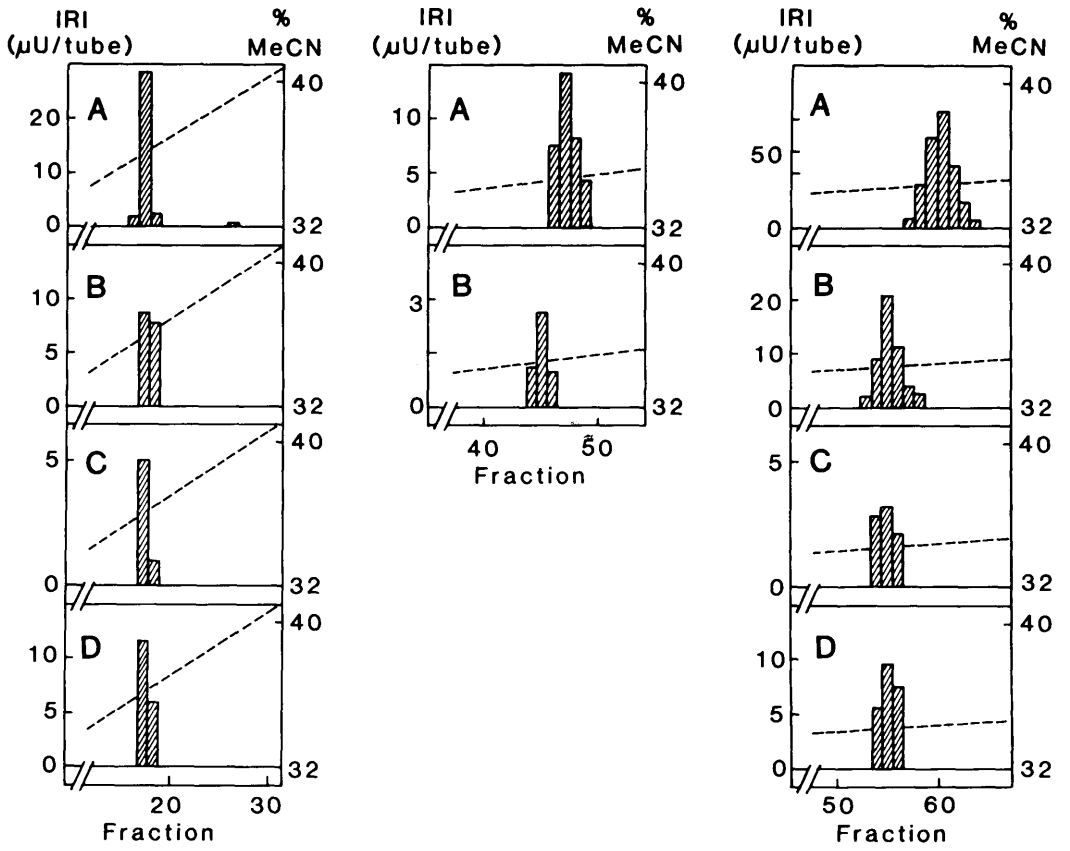


**FIGURE 3. Separation of normal insulin and abnormal insulin by RP-HPLC using different gradients in procedure A. For this experiment, after injection of the sample, the column (Ultrasphere ODS, 5 μm) was initially eluted with 32% of solvent B for 5 min and then eluted with a linear gradient of 0.33%/min (left panel), 0.15%/min (center panel), or 0.1%/min (right panel) to a final concentration of 45% of solvent B. Panel A, patient J.R.; panel B, human insulin standard; panel C, normal subject; and panel D, normal subject's plasma fortified with human insulin standard. Samples A, C, and D were prepared by Sep-Pak extraction. Interrupted lines represent the gradient of solvent B.**

unknown etiology, but not in 8 patients with benign insulinomas.

Since Berson and Yalow established the use of antibodies in the RIA for determination of the circulating insulin concentration,[24] this method has been extensively used and has been a crucial tool for the evaluation of quantitative aspects of B-cell function. On the other hand, detection of mutational change in insulin using RIA alone has not been successful. Apart from the limited material that can be obtained from blood, the conventional immunochemical methods have failed to identify abnormal insulins in the circulation, mainly because most of the polyclonal antibodies produced for RIA of insulin are incapable of distinguishing minor structural differences in the insulin molecule. Although RIA using monoclonal insulin antibodies in recently reported studies[25] may have high specificity for epitopes of the insulin molecule, the assays may be too insensitive to directly determine the presence of abnormal forms of insulin in plasma.

With the introduction of RP-HPLC as an analytic tool, it has become possible to isolate and purify large as well as small peptides,[8-11] based on the hydrophobic interaction between the hydrocarbonaceous column and the peptides to be separated.[26] Until recently, there have been some difficulties in separation of larger peptides and proteins by HPLC mainly because of insolubility, aggregation, and irreversible adsorption of peptides to the reversed-phase column.[27,28] In addition to the improvement of the columns, the application of the ion-pairing principle to RP-HPLC by several investigators has improved the resolution and the recovery of polypeptides and some proteins.[29-31] We initially established the RP-HPLC method for insulin separation by using a 5-μm particle size column and the addition of TEA to the mobile phase. In this system, the finding that the retention time of human insulin was clearly distinguished from that of porcine and bovine insulin, which differ structurally from human insulin by 1 and 3 amino acids, respectively, was striking and confirmed previous observations.[9,12,13,30,32,33] During the course of the experiments characterizing insulin in various patients with hyperinsulinemia, we found that the insulins in a patient with MEN-I (P.N.) and in a patient with hyperinsulinemia and insulin resistance of unknown etiology (J.R.) eluted considerably later than the human insulin standard and insulin in normal subjects on the RP-HPLC, suggesting that these insulins were structurally abnormal. When we attempted to examine the reproducibility of the elution profile of insulin in patient J.R. using a new column, the patient's insulin could not be distinguished from normal insulin when using a relatively steep gradient, whereas with the use of a relatively slower gradient it was possible to separate the patient's insulin from normal insulin, suggesting that the conditions of separation of abnormal insulin are delicate. To further confirm the structural abnormality of insulin in patient

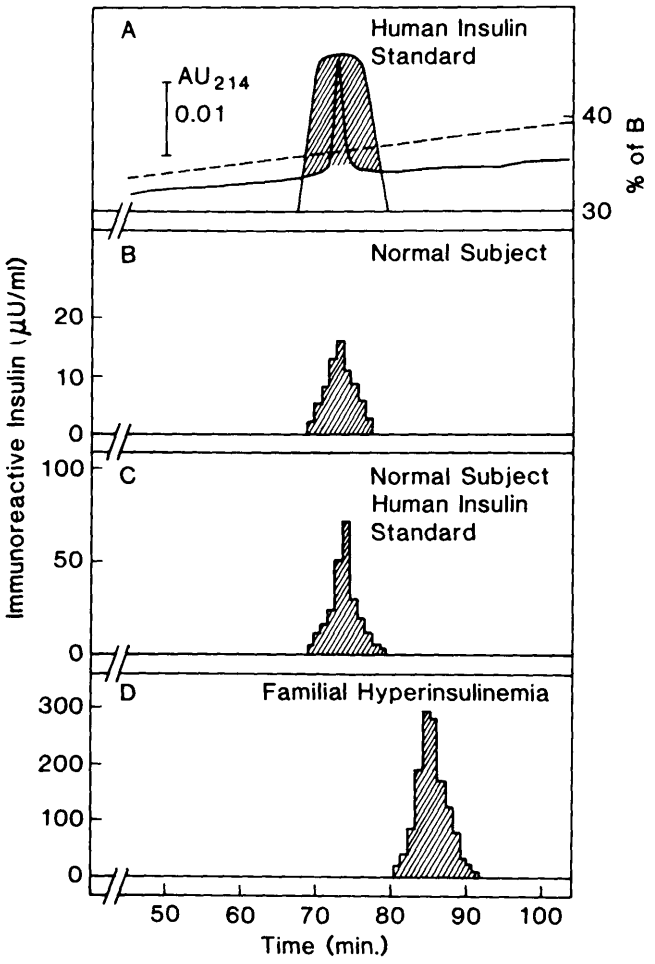


**FIGURE 4. Separation of normal and abnormal insulin by RP-HPLC in procedure B. Panel A represents the peak of standard human insulin with day-to-day variations (shaded area); panel B, normal subject; panel C, human insulin standard in normal subject's plasma; and panel D, familial hyperinsulinemia (J.R.). The detailed description of the method used for this experiment appears in the text.**

J.R., we attempted to purify sufficient insulin for biologic studies. Because of incomplete drying of eluates after lyophilization or evaporation by nitrogen gas, we modified the original solvent system. Omission of TEA caused broadening of the insulin peak and poor recovery. We, therefore, applied the method using a combination of acetonitrile and isopropanol reported by Tarr and Crabb[22] for membrane-associated proteins and large peptides to the separation of insulin. Using this system, various semisynthetic insulin analogues could be easily separated. Moreover, the eluates were found to be completely dried after lyophilization or drying with nitrogen gas, an advantage for further characterization of the biologic activity of insulin. Under these conditions, the abnormal insulin in patient J.R. could again be clearly distinguished from the human insulin standard and insulin purified from plasma of normal subjects. We also found that, under the HPLC conditions in procedure B, the separation of the patient's insulin from normal insulin was dependent on the initial concentration of the organic modifying solvent B as well as the rate of the increase of the gradient used (data not shown).

The abnormal insulin in patient J.R. with familial hyperinsulinemia was associated with initial reactive hypoglycemia culminating 4 yr later with diabetes and resistance to the action of exogenous insulin. The binding of insulin isolated from plasma to isolated rat fat cells was markedly reduced and its ability to stimulate glucose transport and oxidation was 10% of that of normal human insulin.[34] An abnormal insulin was also found in two of three sons (data not shown) without hypoglycemia or diabetes.

An abnormal form of insulin was also found in a patient with MEN-I. The biologic significance of this finding remains unclear. She had normal glucose tolerance initially and became diabetic after 4 yr of observation, but remained sensitive to exogenous insulin. We have not yet determined the biologic activity of the abnormal insulin in this patient. However, considering the possibility that the patient has an abnormal molecular form of gastrin,[35] there may be a common abnormality in the processing of several hormones at the transcriptional, translational, or posttranslational level in MEN-I. Whether or not the present finding is universal in MEN-I requires clarification. In contrast, in 8 patients with benign insulinomas, the circulating insulin was found to be normal.

Thus, the application of HPLC to the characterization of circulating insulin in hyperinsulinemic states has led to the discovery that patients with abnormal insulins may present in a variety of ways. These findings may have relevance to the understanding of the impact of mutations on the biologic activity of insulin.

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