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Article

Determination of Insulin in Humans with Insulin-Dependent Diabetes Mellitus Patients by HPLC with Diode Array Detection

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A simple and reliable high-performance liquid chromatographic method with diode array detection has been developed and validated for the determination of insulin in human plasma. A good chromatographic separation was achieved on a C18 column with a mobile phase consisting of acetonitrile and 0.2M sodium sulfate (pH 2.4), 25:75 (*v*/*v*). Its flow rate was 1.2 mL/min. Calibration curve was linear within the concentration range of 0.15–25 μg/mL. Intra-day and inter-day relative standard deviations for insulin in human plasma were less than 6.3 and 8.5%, respectively. The limits of detection and quantification of insulin were 0.10 and 0.15 μg/mL, respectively. Also, this assay was applied to determine the pharmacokinetic parameters of insulin in eight insulin-dependent diabetes mellitus patients after subcutaneous injection of 25 IU of Actrapid HM.

Introduction

Insulin (Figure 1) is a protein hormone synthesized by pancreatic β-cells and used in controlling the blood-glucose level. Insulin monomer contains 51 amino acid residues in two chains (chain A with 21 residues and chain B with 30 residues) linked together by two disufide bridges. Since its discovery in 1921, insulin has remained a major clinic drug for the treatment of diabetes mellitus (1).

Several immune and non-immune methods have been reported for the determination of human insulin. Immune methods such as radioimmunoassay (2, 3), enzyme immunoassay (4) and luminescent immunoassay (5), and non-immune methods, especially high-performance liquid chromatography (HPLC) (6–14) and capillary electrophoresis (15–18) have been widely used for insulin determination.

The disadvantages of the enzymatic methods are high costs, short shelf life of kits and inability to distinguish between endogenous and exogenous insulin and levels of circulating pro-insulin and true levels of circulating insulin. All of these disadvantages make these methods ineffective for estimating endogenous β-cell insulin reserve (9). Non-immune methods for insulin detection have insufficient sensitivity compared with immune methods because of the interferences from the sample matrix. However, HPLC can be a rapid and accurate method for quantifying peptide and protein in solution form (11).

In addition, no method has been reported to date for determination of insulin by HPLC–diode array detection (DAD) in human plasma. It is known that HPLC–DAD is a highly effective screening method. The criterion for identification of the analyte is that the maximum absorption wavelength in the ultraviolet (UV) spectrum of the analyte should be the same as that of the standard material within ±2 nm. The use of photo-DAD also confers the advantage of identifying the analyte both by retention time and UV spectrum. Therefore, this report describes a simple and specific HPLC procedure with DAD detection for determining insulin in insulin-dependent diabetes mellitus patients. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines (19). Also, the advantages of the present method include simplicity and a single step extraction procedure using inexpensive chemicals.

Experimental

Chemicals and reagents

Recombinant human insulin was provided by Eli Lilly & Co. (Indianapolis, IN). Water was Milli-Q grade and all other chemicals and solvents used were of analytical grade. Actrapid HM (100 U/mL) was obtained from the Department of Endocrinology and Metabolism, Faculty of Medicine, Ataturk University. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany).

HPLC system and chromatographic conditions

Chromatographic analysis was carried out on an HPLC system equipped with a Thermoquest Spectra System P 1500 isocratic pump, Spectra System UV 6000 LP photodiode array detection, SCM 1000 vacuum membrane degasser and Chromquest software. Separation was achieved using an Ace C18 column (5 μm, 4.6 × 250 mm i.d.; Merck) at a flow rate of 1 mL/min with asetonitril–0.2M Na_2SO_4 buffer solution (adjusted to pH 2.4 with H_3PO_4), 25:75 (*v*/*v*) as mobile phase. The mobile phase was freshly prepared every day. The mobile phase was premixed, filtered through a 0.45-μm membrane filter to remove any particulate matter and degassed by sonication before use. A previous DAD (190–600 nm) scanning was done to select the optimal absorbance wavelength. The sensitivity of the detector was set at 0.01 AUFS. The detection was performed at 206 nm and injection volume was 20 μL. Before injecting solutions, the column was equilibrated for at least 15 min with the mobile phase flowing through the system.

Material and Methods

Preparation of stock and standard solutions

The stock solution of insulin was prepared in 0.01M HCl at a concentration of 100 μg/mL. Standard solutions of insulin

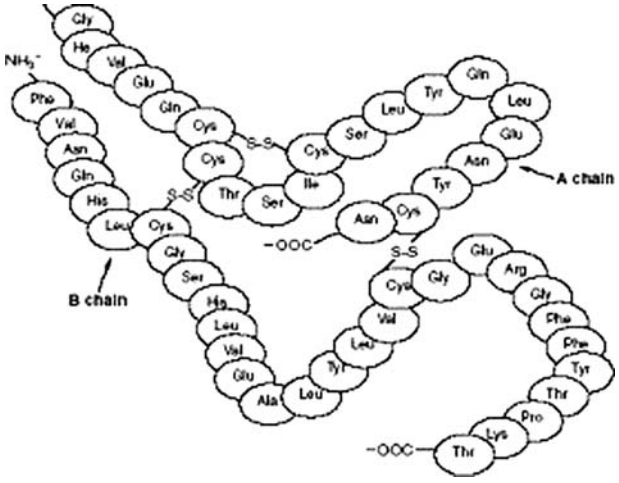

Figure 1. The sequence of human insulin

(0.15, 0.30, 0.75, 1.5, 3.0, 5.0, 10, 15 and 25 μg/mL) were prepared by diluting with 0.01M HCl from stock solution. Also, quality control (QC) samples were prepared from the stock solution at concentrations of 0.3, 7.5 and 20 μg/mL.

Extraction procedure

A 0.2-mL blank plasma was transferred to a 12-mL centrifuge tube with 1 mL phosphate buffer (25 mM, pH 7.4) and the solutions were briefly vortexed. Then, 1 mL of dichloromethane was added, vortexed for 1 min and centrifuged at 2,000 × g for 3 min. The organic phase was transferred to a 5-mL tube and 0.15 mL of 0.05M HCI was added and vortexed for 1 min. The supernatant was transferred into a glass centrifuge tube and evaporated to dryness at room temperature under a stream of nitrogen. The residue was reconstituted with 1 mL of 0.01M HCl. Then, the samples were filtered through a 0.45-μm membrane filter using a syringe filter holder. An aliquot of 20 μL was placed into the automatic sample injector of the HPLC system for analysis.

Results

System suitability

The system suitability was assessed by six replicate analyses of insulin at a concentration of 10 μg/mL. The acceptance criterion was ± 2% for the percent relative standard derivation (% RSD) for the peak area and retention times for human insulin. The % RSD of peak area and retention time for human insulin are within 2%, indicating the suitability of the system (Table I). The efficiency of the column, as expressed by number of theoretical plates for the six replicate injections, was 2,987 ± 1.13% (mean ± % RSD) and the tailing factor was 1.08 ± 0.2% (mean ± % RSD).

Specificity

The specificity of the method was verified by investigating the peak interference from the endogenous plasma substances. The chromatogram of the plasma spiked with insulin was compared to that of the blank plasma sample. No interference peak was found near the retention times of insulin. The retention

Table I
System Suitability Study of Method (10 μg/mL)

	Retention time (min)*	Peak area
	13.8	786,546
SD	0.0295	6,371.2
% RSD	0.21	0.81

*Based on six analyses.

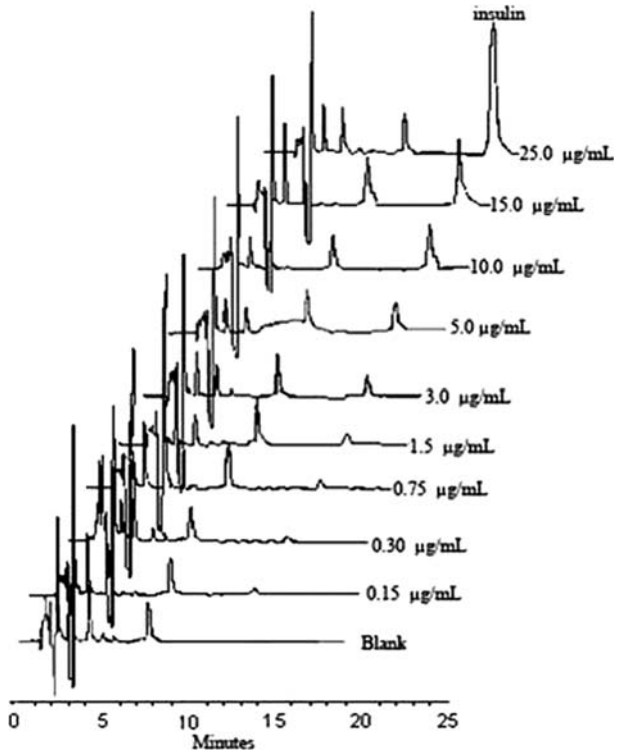

Figure 2. HPLC-DAD chromatograms of insulin in human plasma

time of insulin was approximately 13.8 min with good peak shape (Figures 2 and 3).

Linearity

A calibration curve (insulin peak area versus insulin concentration) in human plasma was constructed by spiking nine different concentrations of insulin. The chromatographic responses were found to be linear over an analytical range of 0.15–25 μg/mL. The equation of the calibration curve obtained from nine points was y = 88379x − 13793 with a correlation coefficient (R = 0.9961). The linear regression equation was calculated by the least squares method using Microsoft Excel and summarized in Table II.

Precision and accuracy

The precision of the analytic method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analyzing spiked blank human plasma six times per day at three different concentrations that were plasma QC samples (0.3, 7.5 and 20 μg/mL). The intermediate

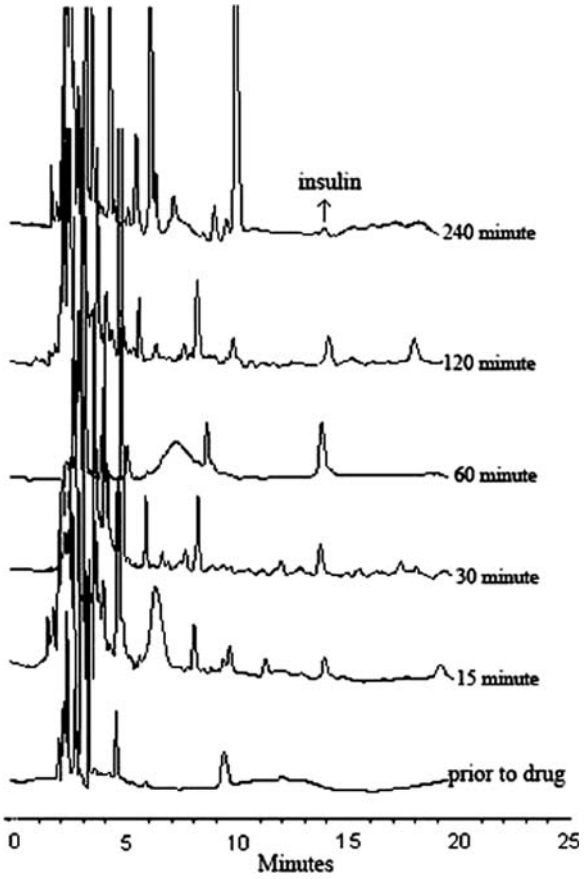

Figure 3. A typical chromatogram of a plasma sample collected from the insulin-dependent diabetes mellitus patient

Table II
Linearity of Insulin in Human Plasma

Parameters	HPLC–DAD
Linearity (μg/mL)	0.15–25
Regression equation*	y = 88379x – 13793
SD of slope	1,529.6
SD of intercept	7,613.8
Correlation coefficient	0.9961
SD of correlation coefficient	4.57×10^{-3}
LOD (μg/mL)	0.10
LOQ (μg/mL)	0.15

*Based on six calibration curves, y: peak area, x: insulin concentration (μg/mL).

precision was evaluated by analyzing the same plasma samples once daily for three days. The accuracy of this analytic method was determined by a method calculated as percent of mean deviation from known concentration [(concentration found − known concentration) × 100/known concentration]. The results for insulin in human plasma are shown in Table III. Precision and accuracy studies in human plasma showed acceptable RSD values; the relative errors were ≤8.52% with high accuracy for both intra-day and inter-day ($n = 6$) studies (≤13.7%).

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) values of the method were determined by injecting progressively low concentrations of the standard solution under the chromatographic conditions. The lowest concentrations assayed at which the signal-to-noise ratio was at least 10:1 was regarded as the LOQ. The LOD was defined as a signal-to-noise ratio of 3:1. The LOD and LOQ values of the HPLC–DAD method were determined to be 0.10 and 0.15 μg/mL, respectively (Table II).

Table III
Precision and Accuracy of Insulin in Human Plasma

	Intra-Day			Inter-Day		
Added (μg/mL)	Found ± SD*	Precision % RSD†	Accuracy‡	Found ± SD*	Precision % RSD†	Accuracy‡
Plasma§						
0.3	0.28 ± 0.016	5.71	−6.67	0.27 ± 0.023	8.52	−10.0
7.5	6.84 ± 0.430	6.29	−8.80	6.57 ± 0.455	6.93	−12.4
20	17.32 ± 0.793	4.58	−13.4	17.26 ± 0.981	5.68	−13.7

*Standard deviation of six replicate determinations.
†Average of six replicate determinations.
‡Accuracy: (% relative error) (found−added) / added × 100.
§Plasma volume (0.2 mL).

Table IV
Recovery of Insulin in Human Plasma

Added (μg/mL)	Found (Mean ± SD*)	% Recovery	% RSD†
0.15	0.14 ± 0.010	93.3	7.14
0.30	0.27 ± 0.015	90.0	5.56
0.75	0.66 ± 0.029	88.0	4.39
1.5	1.35 ± 0.088	90.0	6.52
3.0	2.75 ± 0.198	91.6	7.20
5.0	4.38 ± 0.249	87.6	5.68
10	9.12 ± 0.573	91.2	6.28
15	12.9 ± 0.594	86.0	4.60
25	22.1 ± 1.257	88.4	5.69

*Standard deviation of six replicate determinations.
†Average of six replicate determinations.

Recovery

The extraction recovery of insulin in human plasma was determined at all levels of the calibration graph by comparing the data obtained by the direct injection of standard aqueous solutions to those obtained after the whole extraction procedure. The extraction recoveries of insulin from human plasma were between 86.0 and 93.3%, as shown in Table IV.

Stability

Stability studies indicated that the samples were stable when kept at room temperature, 4°C and −20°C refrigeration temperature for 24 h (short-term) and refrigerated at 4°C and −20°C for 72 h (long-term). The results of these stability studies are given in Table V and the percent ratios are within the acceptance range of 90–110%.

Application of the method

Before the study, the clinical protocol was approved by the Ethics Committee of Faculty of Medicine, Ataturk University. All volunteer patients gave written informed consent to

Table V
Stability of Insulin in Human Plasma ($n = 6$)

Concentration (μg/mL)	Room temperature 12 h	Room temperature 24 h	Refrigeration 4°C, 12 h	Refrigeration 4°C, 24 h	Frozen −20°C, 24 h	Frozen −20°C, 72 h
2.5	95.9 ± 2.35	96.2 ± 4.54	97.3 ± 4.23	95.2 ± 4.26	98.1 ± 2.32	94.6 ± 4.21
15	92.4 ± 1.58	94.5 ± 2.98	96.5 ± 3.68	96.1 ± 3.96	97.2 ± 1.97	93.4 ± 3.73

participate in the study according to the principles of the Declaration of Helsinki. The study was performed on eight insulin-dependent diabetes mellitus patients (four females and four males). Their ages ranged from 21 to 60 (mean ± SD, 37.3 ± 12.8) and their weights ranged from 61 to 75 kg (mean ± SD, 69.0 ± 6.14). All the patients were informed about the purpose, protocol and risk of the study. Blood samples (2 mL) were collected at different times (5, 15, 30, 45, 60, 90, 120, 180 and 240 min) after subcutaneous injection of 25 IU of Actrapid HM and analyzed immediately.

The maximum plasma concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were directly determined from the plasma concentration versus time curves. The area under the curve from 0 to t (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve from 0 h to infinity ($AUC_{0-\infty}$) was estimated by summing the area from 0 to t (AUC_{0-t}) and t to infinity ($AUC_{t-\infty}$), where $AUC_{t-\infty} = C_t/K_{el}$, with C_t defined as the last measured plasma concentration at time t, and K_{el} the slope of the terminal portion of the ln(plasma concentration) versus time curve. The elimination half-life ($t_{1/2}$) was calculated using the pharmacokinetic relationship $t_{1/2} = \ln(2)/k_{el}$ (20).

A typical chromatogram of a plasma sample collected from the insulin-dependent diabetes mellitus patients is shown in Figure 3. The mean plasma concentration–time profile is represented in Figure 4. The pharmakinetic parameters of insulin in plasma samples of insulin-dependent diabetes mellitus patients are reported in Table VI.

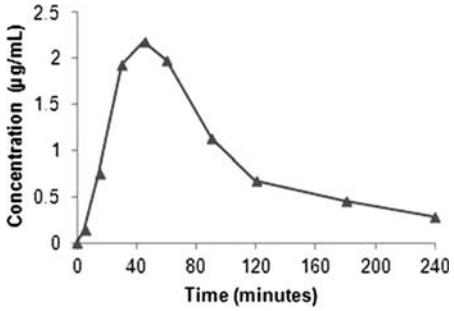

Figure 4. Mean plasma concentration–time profile of insulin-dependent diabetes mellitus patients (n = 8)

Table VI
Pharmacokinetic Parameters of Insulin after Subcutaneous Injection

Parameters	(Mean ± SD)	% RSD
Maximum plasma concentration, C_{max} (μg/mL)	2.39 ± 1.072	44.9
Mean residence time, MRT_{iv} (h)	3.28 ± 1.232	37.6
Time required for maximum plasma concentration (T_{max}) (min)	56.3 ± 15.52	27.6
Area under curve, $AUC_{(0-4h)}$ (μg/mL h)	42.3 ± 13.44	31.8
Half-life, $t_{1/2}$ (h)	1.57 ± 0.571	36.4
Clearance, CL (L/h)	0.06 ± 0.013	21.7
Elimination rate constant (K_{el}) (min^{-1})	0.009 ± 0.001	11.1
Plasma half life ($T_{1/2}$) (min)	73.8 ± 20.90	28.3
Volume of distribution, V_d (L)	0.22 ± 0.049	22.3

Discussion

Today, HPLC is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples.

The composition of the mobile phase plays an important role in the chromatographic separation of analytes. In our preliminary experiments, the mobile phase containing buffer solution with different pH values in combination with different volume fractions of organic modifier was tested. As reported by Khaksa *et al.* (11) and Moslemi *et al.* (12), a mobile phase with low pH and high salinity is required to produce good linearity. The experiments were performed with different strengths of buffers 0.05, 0.1, 0.15 and 0.2M at pH 2.4. No significant effect was found on the retention time of insulin, but a better peak shape was found for insulin at 0.2M Na_2SO_4 buffer with pH 2.4. The effect of pH of the mobile phase was observed over the range of 2.0–3.5 using 0.2M Na_2SO_4 as buffer salt. An increase in the peak height was proportional to an increase in the concentration of insulin at pH between 2.3 and 2.5. However, at a pH above 2.5, the peak height of insulin at low concentration decreased beyond the proportional relationship. A mobile phase pH of 2.4 with 0.2M Na_2SO_4 buffer was chosen because it provided good linearity. Looking at the different chromatographic parameters during the method development, the final recommended mobile phase consisted of acetonitrile–0.2M Na_2SO_4 buffer (pH 2.4) of 25:75. The best separation and sensitivity of the method were obtained at 206 nm and mobile phase flow rate of 1.2 mL/min. The results were similar to the findings of Khaksa *et al.* (11). A typical chromatogram at the optimized condition gave sharp and symmetric peaks with retention time of 13.8 min for insulin (Figure 2). The retention time of insulin was much shorter than that studied by Moslemi *et al.* (12), Asahara *et al.* (21) and Sato *et al.* (22).

When this method was applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has the following advantages over the reported methods (9, 11). The LOQ of the reported methods was 0.70 and 2.62 μg/mL, whereas the present method LOQ was 0.15 μg/mL.

Insulin was extracted from plasma with a solid-phase extraction procedure by Visser *et al.* (22). This method can also extract insulin in a single extraction procedure. The mean recovery is better for plasma than those of the studies reported by Ravi *et al.* (9) and Visser *et al.* (23).

Also, the primary pharmacokinetic parameters of insulin were calculated and summarized in Table VI. Maximum plasma concentration of insulin (C_{max}) and time to reach this value

(t_{max}) were determined to be 2.39 μg/mL and 56.3 minute. Elimination half-life ($t_{1/2}$) and area under curve (AUC_{0-4h}) of the drug were calculated as 1.57 h and 42.3 μg/mL h, respectively.

Conclusion

In the present work, a simple and reliable HPLC method was developed for determination of insulin in human plasma. The method was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters. Additional advantages of this method include small sample volume (0.2 mL) and good extraction recovery from plasma. No significant interferences and matrix effect caused by endogenous compounds were observed. Therefore, the method can be a very useful alternative for performing pharmacokinetic studies in determination of insulin for clinical use.

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