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RESEARCH ARTICLE

Determination of Insulin in Rabbit Plasma Using High-Performance Liquid Chromatography

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Summary

The aim of the present study was to develop and validate a procedure based on high-performance liquid chromatography (HPLC) for determination of insulin in rabbit plasma. Separation of insulin was achieved on an Ace C18 column (5 µm, 250×4.6 mm i.d.) using diode array detection (DAD) with λ=206 nm. The mobile phase consisted of 0.2 M sulfate buffer (pH 2.4)-acetonitrile (75:25, v/v). The analysis was performed in less than 15 min with a flow rate of 1.2 mL/min. Excellent linearity was found between 0.15 and 10 µg/mL. Intra- and inter-day precision values for insulin in plasma were less than 10.2, and accuracy (relative error) was better than 12.4%. The recoveries for all samples were >86.7%. The limits of detection (LOD) and quantification (LOQ) of insulin were 0.10 and 0.15 µg/mL, respectively. The described HPLC method has adequate sensitivity and specificity to study pharmacokinetics of insulin in rabbits, and could be adapted also to clinical pharmacokinetic study.

Keywords: *Insulin, HPLC, Liquid-liquid extraction, Rabbit, Pharmacokinetic study*

Yüksek Performanslı Sıvı Kromatografisi Kullanarak Tavşan Plazmasında İnsülinin Tayini

Özet

Bu çalışmanın amacı tavşan plazmasında insülinin tespit edilmesi için yüksek basınçlı sıvı kromatografisine (YBSK) dayalı bir yöntem geliştirmek ve geçerlilik testi yapmaktır. İnsülinin ayırımı dalga boyu λ=206 nm olan diyot array dedektör (DAD) kullanılarak Ace C18 kolon (5 µm, 250×4.6 mm i.d.) ile yapılmıştır. Hareketli faz 0.2 M sülfat tamponu (pH 2.4)-asetonitril (75:25, h/h) den oluşmuştur. Analiz 1.2 mL/dk akış hızı ile 15 dakikadan daha az sürede yapılmıştır. 0.15 ve 10 µg/mL arasında çok iyi doğrusallık bulunmuştur. Plazmada insülin için gün içi ve günler arası kesinlik değerleri %10.2'den küçüktü ve doğruluk (bağıl hata) %12.4'den daha iyiydi. Bütün örnekler için geri kazanım >%87.3 idi. İnsülinin belirlenebilen (LOD) ve ölçülebilen (LOQ) en düşük değeri sırasıyla 0.10 and 0.15 µg/mL'dir. Tanımlanan YBSK yöntemi tavşanlarda insülinin farmakokinetiğini incelemek için yeterli duyarlılığa ve özgüllüğe sahiptir ve klinik farmakokinetik çalışmalara da uyarlanabilir.

Anahtar sözcükler: *İnsülin, YBSK, Sıvı-sıvı ekstraksiyon, Tavşan, Farmakokinetik çalışma*

INTRODUCTION

Insulin is the most important regulatory hormone in the control of glucose homeostasis consisting of 51 amino acids shared between two intramolecular chains and with a molecular weight of 5.800 g/mol [1]. It is used for the treatment of insulin dependent diabetes mellitus.

Several immune and non-immune methods have been developed for the separation and quantitation of insulin in different biological fluids. Immune methods [2,3], enzyme immunoassay [4], luminescent immunoassay [5] and non-immune methods, especially high performance liquid chromatography (HPLC) [6-12] and capillary electrophoresis [13-16] have been widely used.

The disadvantages of the enzymatic methods are high cost, short shelf life of kits, the inability to distinguish between endogenous and exogenous insulin and levels of circulating pro-insulin and true levels of circulating insulin. All these disadvantages make these methods ineffective estimating of endogenous β-cell insülin [9]. As we known,

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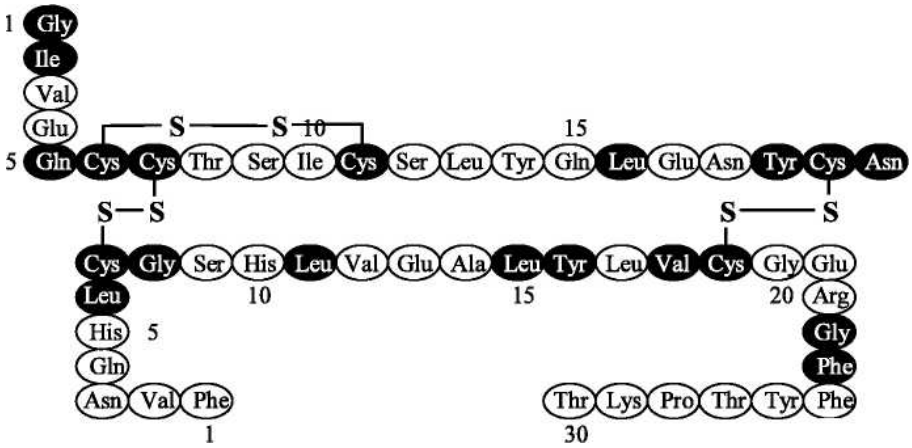

Fig 1. Chemical structure of insulin

Şekil 1. İnsülinin kimyasal yapısı

non-immune method for insulin detection had insufficient sensitivity compared with immune method because of the interferences from the sample matrix. However, HPLC could be a rapid and accuracy method, if proper pretreatment procedure was applied for reverse-phase HPLC, a typical analytical method for quantifying peptide and protein in liquid drug [11].

In addition, no method is reported till date for determination of insulin by HPLC-DAD in rabbit plasma. Therefore, we report a HPLC-DAD method for determination of insulin in rabbit plasma. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to literatüre [17,18]. Also, the advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time.

MATERIAL and METHODS

HPLC System and Chromatographic Conditions

Chromatographic analysis was carried out on a HPLC system equipped with Thermoquest Spectra System P 1500 isocratic pump, Spectra System UV 6.000 LP photodiode array detection, SCM 1000 vacuum membrane degasser and Chromquest software. The analysis was run at flow rate of 1.2 mL/min with asetonitril/0.2 M 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 prepared freshly everyday. 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 in order 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 mL. Prior to injecting solutions, the column was equilibrated for at least 15 min with the mobile phase flowing through the system. The analytical column was an Ace reversed-phase C_{18} column with particle size of 5 mm. The column temperature was 40°C.

Chemicals and Reagents

Recombinant human insulin *(Fig. 1)* [12] was provided by Eli Lilly & Co. (Indianapolis, USA). Water was Milli-Q grade and all other chemicals and solvents used were of analytical grade. Actrapid HM (100 U/mL) was obtained from Department of Endocrinology and Metabolism, Faculty of Medicine, Ataturk University. HPLC grade acetonitrile was purchased from Merck.

Preparation of Stock and Standard Solutions

The stock solution of insulin was prepared in 0.01 M HCl at concentration of 100 µg/mL. Standard solutions of insulin (0.15, 0.30, 0.75, 1.50, 3.0, 5.0 and 10 µg/mL) were prepared by diluting with 0.01 M HCl from stock solution. Also, quality control (QC) samples were prepared from stock solution at concentrations of 0.5, 2.5 and 7.5 µg/mL.

Extraction Procedure

A 0.5 mL blank plasma of New Zealand white rabbit was transferred to a 12 mL centrifuge tube together with 1 mL phosphate buffer (25 mM, pH 7.4) and the solutions were briefly vortexed. Then, 1 mL of dichloromethane was added and 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 0.05 M 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 0.01 M HCl. Then, the samples were filtered through a 0.45 mm membrane filter using syringe filter holder. An aliquot of 20 µL was injected into the automatic sample injector of HPLC system for analysis.

Rabbits

The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for

Medical Experimental Research and Application Centre of Ataturk University (2006/1). The rabbits are male which is 4.7-5.1 kg weight. Three rabbits were housed with free access to food and water, except for the final 2 h before experiment.

After a single subcutaneous injection of 25 U of insulin (Actrapid HM), 1.5 mL of blood samples were collected from the marginal ear vein at 0, 15, 30, 60, 90, 120, 180, 240 and 360 min time-points into EDTA collection tubes. The blood was immediately centrifuged 6.000 × *g* for 10 min at ambient temperature. The plasma was separated and analyzed for insulin concentrations as described above.

Pharmacokinetic Analysis

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 $t_{1/2} = \ln(2)/k_{el}$ [19].

RESULTS

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 *(Fig. 2a,b)*. There was no interference peak near the retention times of insulin.

Calibration curve (insulin peak area versus insulin concentration) in rabbit plasma was constructed by spiking seven different concentrations of insulin. The chromatographic responses were found to be linear over an analytical range of 0.15-10 µg/mL. The equation of the calibration curve obtained from seven points was y = 75954x+11316 with a correlation coefficient. The linear regression equation was calculated by the least squares method using Microsoft Excel® program and summarized in *Table 1*.

The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.5, 2.5 and 7.5 µg/mL. The intra- and inter-day accuracy and precision results are shown in *Table 2*. The intra- and inter-day precisions of the QC samples were satisfactory with RSD less than 10.2% and accuracy with RE within ± 12.4%.

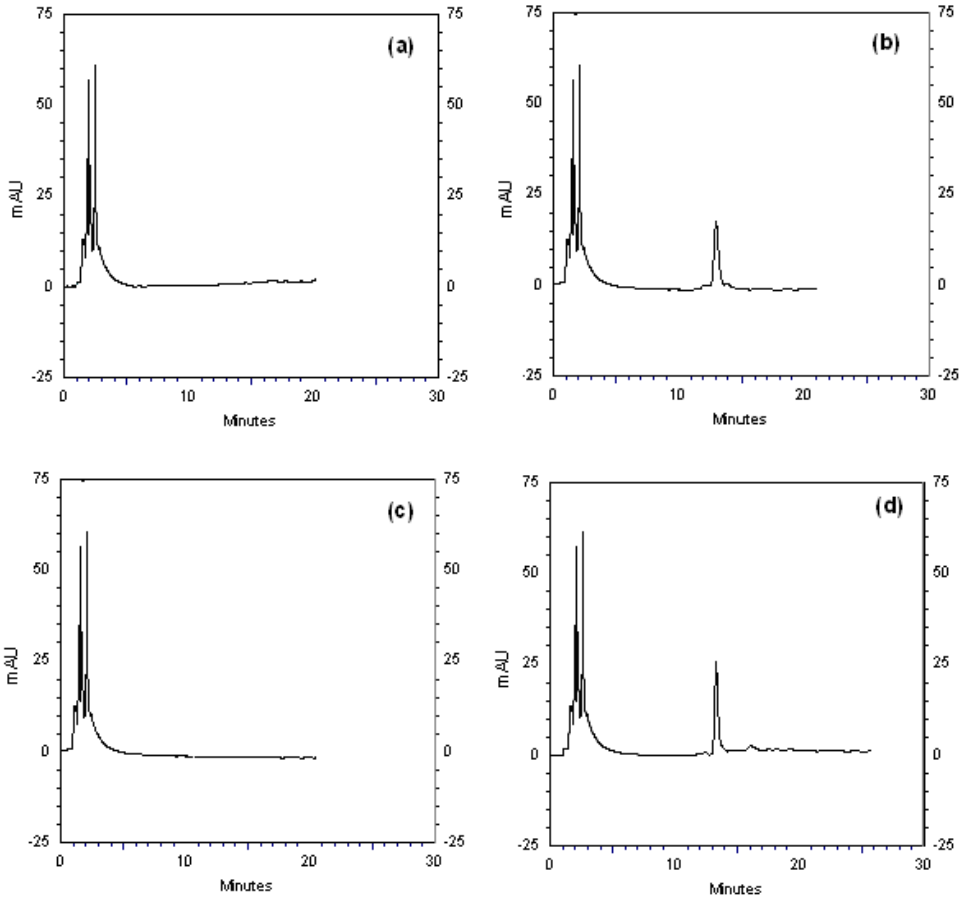

Fig 2. Chromatograms of (a) blank rabbit plasma, (b) rabbit plasma spiked with 3 µg/mL insulin, (c) plasma sample prior to insulin administration from a rabbit, (d) plasma sample obtained a rabbit 2 h after subcutaneous administration 25 U of insulin

Şekil 2. (a) Boş tavşan plazması, (b) insulin (3 µg/mL) eklenen tavşan plazması, (c) tavşana insülin verilmesinden önceki plazma örneği, (d) 25 U insülinin subkutan verilmesinden sonraki 2. saatte elde edilen plazma örneğinin kromatogramları

The sensitivity was evaluated by the limit of quantification (LOQ), the lowest concentration of the plasma spiked with insulin in the calibration curve. The LOQ was defined as the concentration producing a precision less than 20% and accuracy between 80% and 120% of the theoretical concentrations. The LOQ was determined to be 0.15 µg/mL.

Recovery was performed to verify the effectiveness of the extraction step and the accuracy of the proposed method. The recovery of insulin from rabbit plasma samples was satisfactorily ranged from 86.7 to 93.3% (RSD was less than 7.14) at all the concentration levels *(Table 3)*.

For the determination of the stability of insulin in rabbit plasma at room temperature, 4 and -20°C refrigeration temperature, low (2.5 µg/mL) and high (10 µg/mL) insulin concentrations were kept for 24 h and 3 days. Then, the stability measurements were carried out. Rabbit plasma samples were found to be stable after 3 days with no significant change in concentration when stored at 4 and -20°C. The results of stability studies were given in *Table 4*.

The plasma samples obtained three rabbits were assayed with the validated method described above. The peaks of insulin were completely separated from endogenous peaks with similar retention times to those of the samples used for the validation studies *(Fig. 2c,d)*. The mean plasma concentration-time curve was shown in *Fig. 3.* The mean values of pharmacokinetic parameters estimated by the computer program WinNonlin with non-compartmental method were shown in *Table 5*.

Table 1. *Linearity of insulin in rabbit plasma*
Tablo 1. *Tavşan plazmasında insülinin doğrusallığı*

Linearity (µg/mL)	**0.15-10**
Regression equation[a]	y= 75954x+11316
Standard deviation of slope	1621
Standard deviation of intercept	1632
Correlation coefficient	0.9941
Standard deviation of correlation coefficient	$1.83x10^{-3}$
Limit of detection (µg/mL)	0.10
Limit of quantification (µg/mL)	0.15

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

Table 3. *Recovery of insulin in plasma (n=6)*
Tablo 3. *Plazmada insülinin geri kazanımı (n=6)*

Added (µg/mL)	**Found (Mean±SD[a])**	**%Recovery**	**%RSD**
0.15	0.14±0.010	93.3	7.14
0.30	0.27±0.015	90.0	5.56
0.75	0.65±0.029	86.7	4.46
1.5	1.34±0.088	89.3	6.57
3.0	2.74±0.198	91.3	7.22
5.0	4.38±0.249	87.6	5.68
10	9.12±0.573	91.2	6.28

SD[a]: *Standard deviation of six replicate determinations,*
RSD: *Relative standard deviation*

Table 2. *Intra-day and inter-day precision and accuracy of insulin in plasma (n=6)*
Tablo 2. *Plazmada insülinin gün-içi ve günler-arası kesinlik ve doğruluğu (n=6)*

Added (µg/mL)	**Intra-day**			**Inter-day**		
	Found (Mean±SD[a])	**Precision %RSD**	**Accuracy[b]**	**Found (Mean±SD[a])**	**Precision %RSD**	**Accuracy[b]**
Plasma[c]						
0.5	0.45±0.025	5.56	-10.0	0.44±0.045	10.2	-12.0
2.5	2.19±0.125	5.71	-12.4	2.22±0.187	8.42	-11.2
7.5	6.84±0.430	6.29	-8.80	6.57±0.455	6.93	-12.4

SD[a]: Standard deviation of six replicate determinations, ***RSD:*** Relative standard deviation
Accuracy[b]: (% relative error) (found-added)/addedx100, [c]Plasma volume (0.5 mL)

Table 4. *Stability of insulin in plasma*
Tablo 4. *Plazmada insülinin stabilitesi*

Concentration (µg/mL)	**Room Temperature**		**Refrigeratory**		**Frozen**	
	12 h	**24 h**	**4°C, 24 h**	**4°C, 72 h**	**-20°C, 24 h**	**-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
10	93.4±3.47	91.2±4.79	95.7±2.71	92.7±4.94	91.1±6.32	92.4±5.47

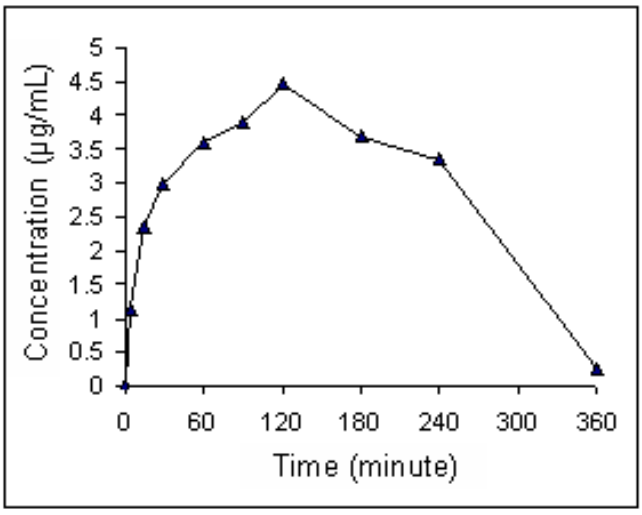

Fig 3. Mean plasma insulin concentration-time profile for three rabbits after a single subcutaneous injection of 25 Unite insulin

Şekil 3. Üç tavşan için 25 Unite insülinin tek subkutan verilmesinden sonra ortalama plazma insülin derişim-zaman profili

a mobile phase with low pH and high salinity was required to produce good linearity. The experiments were performed with different strength of buffers 0.05, 0.1, 0.15 and 0.2 M at pH 2.4. There is no significant effect on the retention time of insulin, but the better peak shape was found for insulin at 0.2 M 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 0.2 M Na_2SO_4 as buffer salt. The 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 0.2 M Na_2SO_4 buffer was chosen since it provides good linearity. Looking at the different chromatographic parameters during the method development, the finally recommended mobile phase consisted of asetonitril/0.2 M Na_2SO_4 buffer (pH 2.4) of 25:75. The best separation and sensitivity of the

Table 5. *Mean pharmacokinetic parameters of insulin for three rabbits after subcutaneous injection of 25 U insulin*
Tablo 5. *Üç tavşan için 25 U insulin subkutan verilmesinden sonra insülinin ortalama farmakokinetik parametreleri*

Parameter	Mean±SD	%RSD
Maximum plasma concentration C_{max} (µg/mL)	4.47±0.432	9.66
Area under curve at infinite time $AUC_{(0\rightarrow\infty)}$ (µg/mL h)	389.6±102.4	26.3
Plasma half life, $T_{1/2}$ (h)	3.65±0.407	11.2
Total clearance rate, CL (L/h)	0.072±0.006	8.33
Time required for maximum plasma concentration T_{max} (min)	120±17.32	14.4
Mean residence time, MRT (h)	4.60±0.246	5.34
Volume of distribution, V_d (L)	0.098±0.009	9.18

DISCUSSION

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

Reversed-phase column (C_{18}) can be used for the separation of non-ionic as well as ion forming non-polar to medium polar substances while normal phase chromatography can be used for the separation of non-ionic and/or non-polar substances. Majority of the ionizable pharmaceutical compounds can be very well separated on C_{18} column [20]. Thus, insulin can be satisfactorily separated by reversed phase chromatography.

The composition of 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] method was 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]. Typical chromatogram at the optimized condition gave sharp and symmetric peak with retention time of 13.8 min for insulin *(Fig. 2)*.

When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has the following advantages over the reported method [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.

The developed method was applied to quantify insulin concentration in pharmacokinetic study on three rabbits. HPLC chromatogram of rabbit plasma is shown in *Fig. 2*, which shows (a) typical chromatograms of blank rabbit plasma, (b) rabbit plasma spiked with 3 µg/mL insulin, (c) plasma sample prior to insulin administration from a rabbit and (d) plasma sample from a rabbit 2 h after subcutaneous administration 25 U of insulin.

Representative mean plasma concentrations versus time profiles following a single subcutaneous injection of insulin to three rabbits are presented in *Fig. 3*. Various pharmacokinetic parameters have been summarized in *Table 5*. The pharmacokinetic of insulin in rats was also reported by Ravi et al.[9]. Rats were treated with insulin 25 U/kg. The mean AUC_{0-t} value for insulin in rat plasma was 434.38±35.13 µg/mL h. Our AUC result (389.6±102.4 µg/mL h) for insulin in rabbit plasma was similar to the findings previously reported [9].

In conclusion, a new rapid, simple and sensitive reversed phase HPLC method has been developed and optimized. Also, the method was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters for determination of insulin in rabbit plasma. Method was found to be linear over an analytical range of 0.15-10 µg/mL. The mean recovery of insulin was found to be 89.9%. Therefore, the method can also apply for the determination of insulin pharmacokinetic in humans after partial validation.

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