Research Article

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Determination of Insulin in saline by RP-HPLC combined with UV

UV kombine RP-HPLC ile İzotonik Serum Fizyolojikte İnsülin Tayini

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ABSTRACT

In the treatment of type I diabetes mellitus, insulin is widely used intravenously. This study aims to develop an innovative, fast and simple analysis technique for insulin determination in 0.9% NaCl solution samples. Insulin was analyzed by HPLC with a UV detector. The method has been validated according to FDA guidelines and satisfactory results have been observed. Samples were manually injected into the HPLC system using ACE 5 µm C18 100 Å LC column (250 x 4.6 mm). The mobile phase was a mixture of acetonitrile: phosphate buffer (pH 3.0, 10 mM) (50:50 v/v). The injection volume is 20 µL. UV detection was carried out at 240 nm. Based on validation studies, the method was found as specific, linear, precise, accurate, and sensitive.

Keywords: Regular insulin, HPLC, saline solution, validation, UV.

ÖZET

Tip I diabetes mellitus tedavisinde insülin yaygın olarak intravenöz olarak kullanılmaktadır. Bu çalışmanın amacı, % 0.9'luk NaCl solüsyon örneklerinde insülin tayini için yenilikçi, hızlı ve basit bir analiz tekniği geliştirmektir. İnsülin, UV detektörlü HPLC ile analiz edilmiştir. Yöntem, FDA yönergelerine göre onaylanmış ve tatmin edici sonuçlar gözlenmiştir. Örnekler, ACE 5 µm C18 100 Å LC kolonu (250 x 4.6 mm) kullanılarak HPLC sistemine manuel olarak enjekte edilmiştir. Mobil faz, bir asetonitril: fosfat tamponu (pH 3.0, 10 mM) (50:50 v / v) karışımıdır. Enjeksiyon hacmi 20 µL'dir. UV saptaması 240 nm'de gerçekleştirilmiştir. Yöntemin, özgün, doğrusal, kesin, doğru ve duyarlı bir yöntem olduğu validasyon çalışmalarıyla ortaya konulmuştur.

Anahtar Kelimeler: Reguler insulin, HPLC, izotonik serum fizyolojik, validasyon, UV.

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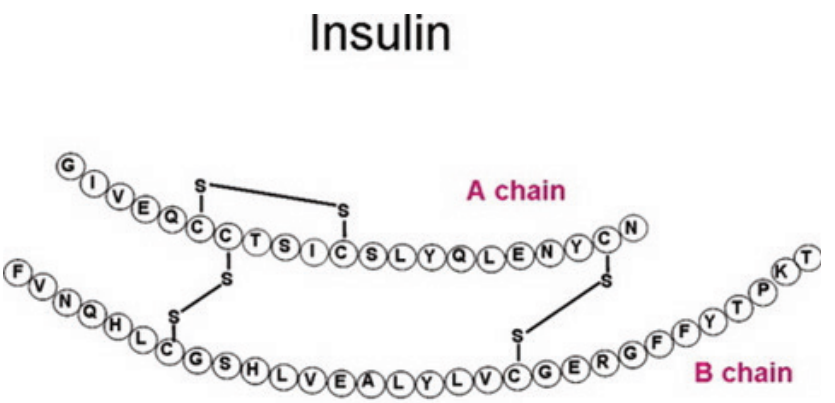
1. Introduction

After the food is digested, it is broken down into sugar by the enzymes in our body. Sugar (glucose) is transported by blood flow to all parts of the body. Sugar, the main food source of our body, must enter the body cells (muscle, fat and liver cells) from the blood in order to provide energy. Insulin is a hormone secreted by the beta cells of an organ called the pancreas, located under and behind the stomach in our body. It allows the sugar in the blood to leave the blood and enter the cell. Thus, the level of sugar in the blood does not increase. In a non-diabetic person, after each food intake, the pancreas produces insulin to transform the nutrients taken into energy. This means that all people are dependent on insulin. In those with diabetes, the pancreas does not produce enough insulin or the insulin produced is not used by target cells (muscle, fat and liver cells). In this case, we need to provide insulin, which is vital for our body, from outside. It should not be forgotten that insulin is a life-saving drug and a healthier life will be lived with the injection. Insulin is the first protein sequenced by Frederick Sanger in 1955 [1, 2]. The molecular weight of insulin is 5.8 kDa. In its structure, there are two peptide chains containing the A chain (21 amino acids) and the B chain (30 amino acids) connected by two disulfide bridges (Figure 1) [3]. Human insulin was produced by recombinant DNA technology where an attenuated *Escherichia coli* bacterium is used as a vector to produce A and B chains. By connecting these chains with cross bridges, recombinant human insulin is obtained. In patients having type I diabetes mellitus, the pancreas cannot produce enough insulin to manage the glucose level. Recombinant human insulin, i.e., regular insulin, is used in the treatment of type I diabetes mellitus [4, 5]. The insulin formulation can be administered by subcutaneous, intramuscular, and intravenous injection.

HPLC is a very powerful tool for the analysis of peptides and proteins. Due to the recognition of this fact and the emergence of favorable chromatographic conditions, there has been an increase in the use of HPLC for peptide and protein analyzes. Analysis of insulins has traditionally been done by biological tests [6]. Today, it is widely accepted that suitably developed chromatographic analysis is superior to biological analysis for many applications [7, 8]. In literature, insulin concentrations in plasma, pharmaceutical products, and saline were determined using high-performance liquid chromatography (HPLC) combined with UV detection and mass spectrometry (MS) [4, 9-14]. As expected for MS, the sensitivity of the developed methods was more satisfactory than the ones for UPLC-UV. However, as a basic concept, advanced analytical techniques like MS may cost higher financial management sources and they may not be available for routine applications on regular laboratories.



	A8	A9	A10	B30
Human	Thr	Ser	Ile	Thr
Porcine	Thr	Ser	Ile	Ala
Bovine	Ala	Ser	Val	Ala
Sheep	Ala	Gly	Val	Ala

Figure 1: Structure of insulin

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Table 1. HPLC analysis of insulin

References	Publication Year	Column	Mobile Phase	Detection method	Sample Matrix	Analyses time
[4]	2019	Agilent RRHD 300 Å SB-C18 (50×2.1 mm, 1.8 µm	from ACN with 0.2% TFA:water with 0.2% TFA (22:78,v/v) to ACN with 0.2% TFA:water with 0.2 TFA (95:5,v/v)	MS/MS	Pharmaceutical Dosage Form	Insulin is within 3 minutes
[9]	2012	Ace C18 (250 × 4.6 mm, 5 µm)	Acetonitrile: 0.2 M sodium sulfate buffer pH was adjusted to 2.4 by using ortho-phosphoric acid (25:75 v/v)	Diode Array Detector at 206 nm	Human plasma	Insulin is within 14 minutes
[10]	2010	Phenomenex C18 (250 × 4.6 mm, 5 µm)	Acetonitrile: 0.2 M sodium sulfate buffer pH was adjusted to 2.4 by using ortho-phosphoric acid (25:75 v/v)	Diode Array Detector at 206 nm	Pharmaceutial preparation	Insulin is within 14 minutes
[11]	2010	Hypersil BDS C-18	A (aqueous solution of 28.3 anhydrous Na_2SO_4g/L, pH 2.3) and solution B (28.5 g anhydrous Na_2SO_4g/L in 50:50 mixture of water and acetonitrile, pH 2.3) in a ratio 48:52 (v/v) at 45– 50 ºC	UV detection at 216 nm	Bulk and Pharmaceutical Dosage Form	Insulin is within 20 minutes
[12]	2006	Waters Columns µbondapak C18 (3.9x30 mm, 10 µm)	Acetonitrile:phosphate buffer (30:70 v/v)	UV-VIS detection at 2280 nm	Pharmaceutical Dosage Form	Insulin is within 20 minutes
[13]	2005	XTerra RP18 C18 (250 × 4.6 mm, 5 µm)	from ACN:water with 0.1% TFA (30:70,v/v) to ACN:water with 0.1 TFA (40:60,v/v)	UV detection at 214 nm	Insulin-loaded nanoparticulates composed of polyelectrolytes	Insulin is within 6 minutes
[14]	2004	JupiterC1S, C18 (250 × 4.6 mm, 5 µm)	ACN with 0.1% TFA:water with 0.1% TFA (31:69,v/v)	UV detection at 280 nm	Normal Saline Infusion in polyvinyl chloride (PVC) plastic	No informetion

To present the already published methodologies, Table 1 provides a summary. As it is seen on table, there is no method for analysis of insulin in a polypropylene bag containing 0.9% NaCl (known as saline) and the analysis time for the reported methods reaches almost 20 minutes. Therefore, this present study aimed to develop and validate a method using a simple, fast, precise, accurate, and specific reverse phase HPLC-DAD test.

2. Materials and Methods

2.1. Chemicals

Regular insulin was obtained from the Humulin R U-100 injection formulation from Lilly pharmaceutical company. Analytical grade acetonitrile and sodium phosphate monobasic were purchased from Merck (Darmstadt, Germany). All the solutions were prepared by using Milli-Q water. Turkey. 0.9% isotonic solutions, polypropylene bag, a pharmaceutical warehouse (Polifarma, Turkey) was purchased from local pharmacies. The pH meter (OHAUS starter5000) was used to adjust the pH of the buffer solutions.

2.2. Apparatus

Analysis of insulin from an isotonic solution containing 0.9% NaCl, that is, saline, was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC system (Agilent 1220 Infinity II) consisting of a gradient pump, a degasser, a manual injection system, and a UV detector was used. IKA Vortex Genius (IKA, Germany) was used to vortex the samples.

2.3. Preparing mobile phase buffer of HPLC analysis

To prepare 10.0 mM phosphate buffer, 1.56 g of sodium dihydrogen phosphate (NaH_2PO_4) was dissolved in approximately 800 mL of water. The pH of the solution was adjusted to pH 3.0 with o-phosphoric acid. Then the volume was filled up to 1000 mL with water.

2.4. Chromatographic conditions

Separations were carried on a Phenomenex 5 µm C18 100 Å LC Column (250 x 4.6 mm). The flow rate was 1.0 mL min^{-1} while using isocratic elution with the mobile phase [acetonitrile: phosphate buffer (pH 3.0, 10 mM) (50:50 v/v)] mixture. The injection volume was 20 µL and UV detection was performed at 214 nm.

2.5. Standard stock solutions

Humulin RU-100 contains 100 units (IU) per mL. Also, 1 IU of insulin is known to be equivalent to 24 mg of insulin. The standard stock solutions of insulin (5 and 50 IU) were prepared. All of the standard solutions were kept at 4 ° C during analysis and were prepared freshly each day. Appropriate dilutions were applied using micropipettes to prepare calibration standards and sample solutions.

2.6. Preparing Calibration Standards

Eppendorf Research® plus micropipettes (adjustable for maximum 10 µL, 100 µL, and 1000 µL volumes) were used to prepare the calibration standards. Six different sets of calibration standards containing between 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, and 5 IU of insulin were prepared in the microcentrifuge tubes by saline. The solutions having a concentration below 1 IU for insulin were prepared by using the diluted standard stock solutions (5 IU of insulin). All of the standard stock solutions were prepared in microcentrifuge tubes and the volume was filled up to 1000 µL with the mobile phase. Finally, 48 solutions were prepared for calibration curves having eight points including six sets (n=6).

These calibration standards were injected into the HPLC system by manual injection. Peak areas of insulin were integrated automatically by ChemStaion software. Peak areas were plotted against the concentrations of insulin by using MS Excel. Standard errors of slope and intercept were evaluated for the linearity of the proposed method. Mean values of slope and intercept were used to find the main calibration equation for the analysis of insulin spiked saline samples.

2.7. Recovery studies

Two different sample groups were prepared. The first group was saline samples and the second group was aqueous solutions of insulin. Both of these groups were prepared three times and contained 0.25, 1.0, and 3.0 IU insulin. After the solutions were prepared with saline or water, 500 µL of the mobile phase of HPLC was added to each solution to fill the final volume to 1000 µL. The triplicate peak areas for the two groups were compared for each concentration level.

2.8. Analytical Method Validation

The developed method was validated according to the FDA guidelines and the calibration curves, sensitivity, precision, accuracy, and selectivity of the method were evaluated [15] .

3. Results and Discussion

Regular insulin is one of the well-known and widely used in the treatment of type I diabetes mellitus. Generally, patients hospitalized in the intensive care unit are given 0.5-2 units per mL per hour via infusion pumps. For this, insulin infusion is given intravenously by preparing the bag in 0.9% sodium chloride. It is recommended to be stored in the refrigerator at 2-8ºC before opening the manuals. After opening, it is recommended to be stored below 30 ° C and used within 28 days. Insulin is stable when diluted in normal saline for clinical use. However, it is thought that there is a loss of insulin in saline bags, and the insulin solutions in the bag are renewed [5, 14, 16-19]. Therefore, it is important to quickly and simply analyze insulin in saline samples for subsequent studies. With this study, it is planned to develop a new approach to insulin analysis in saline samples in polypropylene bags.

3.1. Optimization of the Chromatographic Conditions

The previously reported studies were overviewed and it was decided to use phosphate buffer (pH: 3.0) and acetonitrile mixture as the mobile phase. A short C18 column [Phenomenex LC Column (250 x 4.6 mm, 5 µm, 100 Å)] was used. Since the aim was to analyze

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insulin from saline samples, a mixture of various acetonitrile and phosphate buffer solutions was injected into the insulin saline samples. It was observed that the water-soluble interference in the saline solution was easily eluted with the dead volume, and from baseline. The optimum mobile phase conditions were found to be acetonitrile: phosphate buffer (pH 3.0, 10 mM) (50:50 v/v) where the total analysis time was shorter than 6.0 minutes. The chromatograms of blank, spiked sample, and standard solutions were given in Figure 2. Since the stability of insulin is already known on previous studies [14], the injection stability of insulin was investigated in the scope of this study. The prepared calibration standards were dissolved in the mobile phase and stored at ambient temperature (24 - 28 ºC). Then, these solutions were injected after 24 hours. The peak areas were compared with the previous injections. This process was applied three times for 1 IU of the insulin calibration standard. The results of these stability studies are given in Table 2 and the percent ratios are within the acceptance range of 90–110%. Also, the system compatibility of the developed HPLC method is given in Table 2. These results show that the system suitability of the developed method meets the requirements and that the prepared solutions can be injected within 24 hours when kept in a laboratory condition.

3.2. Method Validation

3.2.1. Linearity and sensitivity of the developed method

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. The calibration curves were constructed within the range between 0.1 and 5.0

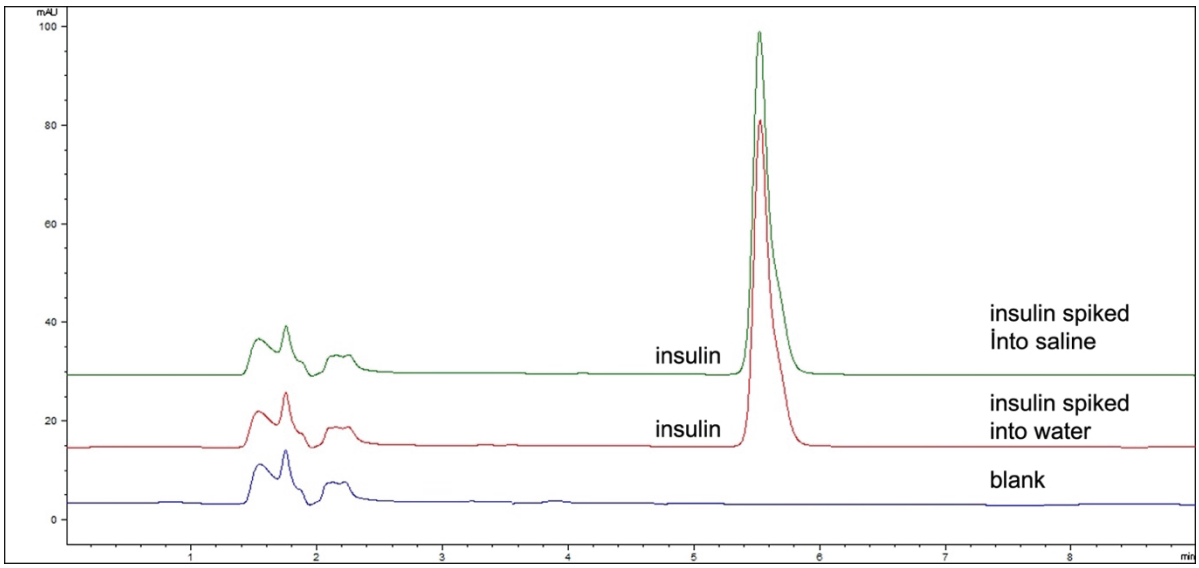

Figure 2: The overlaid chromatograms of blank saline sample, insulin spiked into the water and spiked into saline samples under experimental conditions. Insulin concentration is 1 IU mL^{-1}

Table 2. System suitability parameters of insulin for the proposed method (n = 6)

	Found	Requirements
Injection repeatability (RSD of t_r)	0.21 %	< 1%
Capacity Factor (k')	5.985	1 < k' < 10
Efficiency (N)	4507	> 2000
Tailing Factor	1.29	< 1.5
Injection stability (after 24 hours at ambient temperature)	102.06 %	±1.15

IU where the method is linear and it is possible to analyze insulin in saline solutions up to 24 h. The signal-to-noise ratios of 3:1 and 10:1 were taken as limit of detection (LOD) and limit of quantification (LOQ), respectively [20]. The linearity and sensitivity of the developed method were summarized in Table 3. The chromatograms of calibration curves are given in Figure 3.

3.2.2. Accuracy and precision of the developed method

Three different concentrations of insulin (0.25, 1.0, and 3.0 IU) within the linear range were analyzed three consecutive days (inter-day studies) and three times within the same day (intra-day studies). The procedure was repeated six times for three different concentration levels within the linear range. The relative standard deviation (RSD) and the bias of intra- and inter-day studies were calculated by us-

Table 3. Linearity of the developed method (n=6)

Regression equation*	y = 713.852x + 36.661
Standard error of intercept	0.6221
Standard error of slope	0.3815
Regression coefficient (R^2)	0.9993
Range (µg mL^{-1})	0.1 - 5
Number of data points	8
LOD (µg mL^{-1})	0.0064
LOQ (µg mL^{-1})	0.0213

* Based on six calibration curves where y: peak area of insulin and x: concentration of insulin as IU, LOQ: Limit of quantification, LOD: Limit of detection

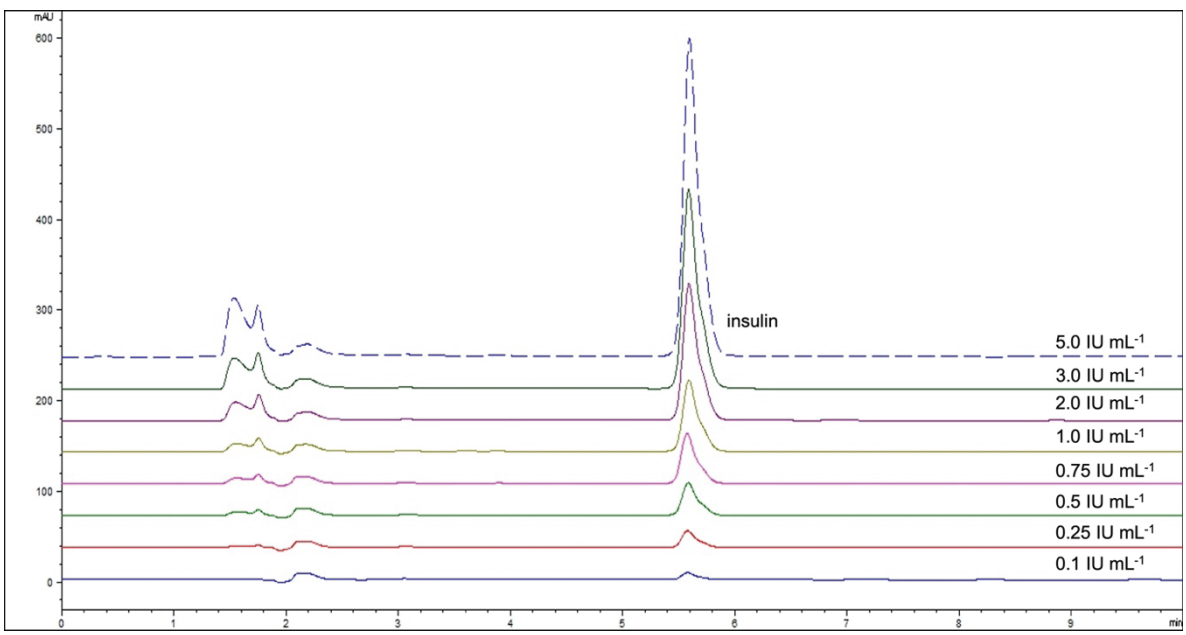

Figure 3: The overlaid chromatograms of calibration curve samples (0. 25 – 5 IU of insulin) under experimental conditions: Mobile phase: acetonitrile:phosphate buffer (pH 3.0, 10 mM) (50:50 v/v), flow rate: 1 mL min-1, injection volume: 20 µL, detection wavelength: 214 nm.

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ing regression equations. The biases of intra- and inter-day studies were between -0.316 and 1.641. The relative standard deviations of intra- and inter-day studies were between 0.295 and 1.457 (Table 4). These results indicate that the developed method was accurate and precise.

3.2.3. Recovery of the developed method

The recovery of the developed method was investigated for three different concentration levels (0.25, 1.0, and 3.0 IU) of saline samples as described in the experimental section. The procedure was repeated three times for three different concentration levels within the linear range. The recoveries were 100.16 % (SD: 0.16), 100.14 % (SD: 0.05) and 99.90 % (SD:0.69) (SD: Standard deviation, n=3) for the plasma samples containing 0.25, 1.0 and 3.0 IU of insulin, respectively (Table 5). The results show that it is successful for the recovery of insulin from saline samples.

3.2.4. Selectivity of the developed method

The selectivity of an assay is a measure of the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. Therefore, the chromatograms obtained from blank, spiked insulin into saline, and spiked insulin into the water as described in the recovery section were compared (Figure 2). The interference peaks coming from matrix components were tracked to show if they interfere with the peak of insulin. From the water-soluble matrix components and dead volume, insulin was separated in 5.58 minutes.

3.3. Analysis of spiked plasma samples

Insulin spiked saline samples (0.25, 1.0, and 3.0 IU insulin) were prepared as described in the experimental section. These solutions were analyzed by the developed HPLC method and the results were evaluated by using regression equation (Table 3) obtained from calibration standards. The analysis results were given in Table 5. As can be seen from the results, the developed RP-HPLC method can be used successfully for NS analysis in saline samples.

Table 4. Precision and Accuracy of Insulin in Human Plasma Analysis of spiked saline samples (n=6)

Added insulin into the blank saline samples	**0.25 IU**		**1.0 IU**		**3.0 IU**	
	intraday	**interday**	**intraday**	**interday**	**intraday**	**interday**
Found insulin in the blank saline samples*	0.249 ± 0.001 IU	0.249 ± 0.001 IU	1.016 ± 0.003 IU	1.013 ± 0.002 IU	3.049 ± 0.007 IU	3.040 ± 0.003 IU
Bias of the analysis**	-0.316	-0.223	1.637	1.030	1.641	1.338
Relative standard deviation of the results	1.342	1.457	0.653	0.567	0.559	0.295

*Found : mean ± standard error (n=6)
**Bias : [(Found - Added) / Added] x 100

Table 5. Analysis of spiked saline samples (n=3)

Added insulin into the blank saline samples	0.25 IU	1.0 IU	3.0 IU
Found insulin in the blank saline samples*	0.249 ± 0.001 IU	1.013 ± 0.003 IU	3.045 ± 0.007 IU
Bias of the analysis**	-0.270	1.334	1.489
Relative standard deviation of the results	1.336	0.662	0.454
Recovery (%)	100.16 ± 0.16	100.14 ± 0.05	99.90 ± 0.69

*Found : mean ± standard error (n=3)
**Bias : [(Found - Added) / Added] x 100

4. Conclusion

Pharmaceutical analysis is one of the main components on pharmaceutical development processes. Analytical scientists and analyst in pharmaceutical companies perform quality control of pharmaceuticals as a regular application. Compared to electrochemistry, capillary electrophoresis, and UV-Spectroscopy methods, HPLC is one of the most versatile technique as a result of its reproducibility and capability of preventing the analyte peak coming from matrix components [21]. In this study, a fast and specific method was developed for determination of regular insulin. With this study, since the analysis time of insulin is shortened, it will be easier and faster to apply compared to other methods. In addition, insulin analysis was performed for the first time using polypropylene saline bags, which are widely used today and have less interaction with drugs. The proposed and validated method could be used in the analysis of human insulin in saline bags on quality control applications.

Acknowledgement

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