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Research Article

A Rapid, Isocratic HPLC Method for Determination of Insulin and Its Degradation Product

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This paper aimed to develop a simple, sensitive, and rapid chromatographic procedure for the simultaneous analysis of human insulin and its main decomposition product using isocratic RP-HPLC/UV. A column type RP-C18 (100 × 4.6 mm, 3 μm particle size, and pore size 130 Å) was used. *o*-Nitrophenol was used as internal standard. The eluent consists of 62% KH_2PO_4 buffer (0.1 M), 26% ACN, and 12% MeOH. The final pH was adjusted to 3.1. The eluent was pumped at a flow rate of 1.0 mL/min and the effluent was monitored using DAD detector at 214 nm. The method produces a linear response over the concentration range of 0.0106 to 0.6810 mg/mL with detection limit of 0.0029 mg/mL. Considering the specifications of this method, the system was found to be suitable for rapid, direct routine analysis and stability studies of insulin.

1. Introduction

Since insulin was discovered in 1921 by Frederick Banting and Charles Best, it has become one of the most thoroughly studied molecules in scientific history. Insulin is a pancreatic hormone that treats diabetes by controlling the amount of sugar in the blood. This peptide hormone is composed of 51 amino acids distributed between two peptides chains, one comprising 21 amino acids (chain A) and the other 30 amino acids (chain B). The two chains are joined together by two disulfide bonds between two cysteine residues, where a disulfide bond is the linkage bond between two sulfurs [1].

Like most proteins, insulin is not stable in aqueous solutions. Its degradation mainly goes through hydrolytic reactions (deamidation) or polymerization mechanisms. Deamidation at asparagine amino acid in chain A of insulin (A21) is considered as the main degradation product of insulin at low pH values, while in neutral or alkaline medium, deamidation at asparagine B3 occurs [2]. Polymerization may also occur in neutral and alkaline medium through transamidation reactions [3]. As a diabetes medicine, the purity of recombinant insulin must be more than 98%, with the insulin-related compound A21 desamido insulin being less than 2% [4, 5]. Most commercial insulin preparations are containing low concentrations of phenol and/or *m*-cresol as preservatives from bacterial contaminations.

Separation of insulin and its related compounds is a difficult mission due to the insignificant differences in chemical structures. Many immune and nonimmune methods have been reported for determination of human insulin. Radioimmunoassay [6–9], enzyme immunoassay [10–13], luminescent immunoassay [14], capillary electrophoresis [15–17], and high performance liquid chromatography (HPLC) [18–24] had been widely used for human insulin detection in vivo and in vitro.

In the determination of insulin in commercial products using RP-HPLC, chromatographic procedure should effectively separate insulin from its major degradation products, such as A21 desamido insulin, and eliminate the interference from the vial additives such as phenol or *m*-cresol. Although many researchers reported the determination of insulin content using HPLC as a powerful technique for purity determinations of proteins [18–24], most of the assays failed to achieve adequate resolution between insulin and its desamido form due to poor chromatographic conditions [5, 24]. Most of these methods tried to control the degree of insulin ionization

by using a mobile phase with low pH and high salinity, which affect the column pressure and led to response fluctuations. Other methods applied the use of gradient mobile phase but were considered time consuming and not applicable for routine analysis because of high retention time (20–45 min). Other limitations of the published methods are applying an ion-pairing reagent, special columns or column temperature controller which is not popular in most of the analytical laboratories.

This paper aimed to develop a rapid and effective chromatographic procedure for simultaneous analysis of human insulin and its main decomposition product, using isocratic RP-HPLC method which also applied the addition of internal standard. The developed chromatographic method was successfully validated in terms of selectivity, linearity, precision, accuracy, solution stability, LOD and LOQ.

2. Experimental

2.1. Materials and Methods

2.1.1. Chemicals and Reagents. The chemicals needed with their purity grade and the sources of purchasing are as follows: methanol-HPLC grade from TEDIA (Fairfield, USA), acetonitrile-HPLC grade from Merck (Darmstadt, Germany), hydrochloric acid 32% from Panreac (Barcelona/Spain), *o*-phosphoric acid 85% from Panreac (Barcelona/Spain), phenol-extra pure from Across (Geel, Belgium), *m*-cresol-pure from Across (Geel, Belgium), *o*-nitrophenol 99% from Across (Geel, Belgium), potassium dihydrogen phosphate-analytical grade from Merck (Darmstadt, Germany), and human insulin from Biocon (Navarangpura/India).

2.1.2. Instrumentation. The HPLC system consisted of a Thermo Spectra Physics delivery pump (P2000 pump, TSP) equipped with a 6-valve sample injection port (7725i, Rheodyne) fitted with a 20 μL sample loop, a diode array detector model (2800 Knauer), and integration software (EuroChrom, Knauer).

2.1.3. Method Development. Insulin, as all large molecules, does not easily gives sharp chromatography peak, but usually gives a broad peak with tailing. Another problem is to get good chromatographic resolution between insulin and insulin-like molecules (degraded products) in acceptable retention time. Finally it is important to use internal standard in the insulin determination method because of fluctuated response of insulin. The method was developed for the determination of insulin in the presence of main degraded product (A21 desamido insulin) and common vial additives like phenol and *m*-cresol and also in the presence of internal standard with suitable run time and commonly available instruments.

During the development, the researchers persist to apply isocratic HPLC system with safe mobile phase (not highly saline, pH more than 2.5, and acceptable flow rate). By trying different columns (RP-C2, RP-C8, and RP-C18) from different manufacturers with different lengths (25.0, 15.0, 12.5, and 10.0 cm) and constant internal diameter of 4.6 mm and using different mobile phases prepared from different solvents as methanol (MeOH), acetonitrile (ACN), and aqueous buffer solutions at different pHs (<2.5), the following chromatographic conditions were adopted:

column: Hypersil RP-C18, 100 × 4.6 mm, 3 μm particle size (pore size 130 Å) from Thermo Scientific (New Hampshire, USA);

detector: Diode Array Detector model 2800 Knauer (Berlin/Germany);

wavelength: 214 nm;

sample injection loop: 20 μL;

flow rate: 1.0 mL/min;

elution mode: isocratic;

mobile phase: 62% KH_2PO_4 buffer (0.1 M), 26% ACN, and 12% MeOH; the final pH was adjusted to 3.1.

2.1.4. Preparation of Internal Standard (IS) Stock Solution (I). 0.2000 g of *o*-nitrophenol was dissolved in 1.0 L of 0.01 M HCl to make a solution of 0.2000 mg/mL internal standard (IS).

2.1.5. Preparation of Internal Standard (IS) Stock Solution (II). 20.0 mL of IS stock solution (I) was further diluted by addition of 80.0 mL 0.01 M HCl to give a concentration of 0.0400 mg/mL.

2.1.6. Preparation of Insulin Stock Solution (I). 0.1362 g of Insulin was dissolved in 100 mL of 0.01 M HCl solution; this will give an insulin solution with the concentration of 1.362 mg/mL. The solution was kept in refrigerator (<5°C) for not more than two days [24].

2.1.7. Preparation of Insulin Stock Solution (II). 12.5 mL of insulin stock solution (I) was diluted to 100 mL with 0.01 M HCl to give a solution of insulin with a concentration of 0.17025 mg/mL. The solution was kept in refrigerator (<5°C) for not more than three days.

2.1.8. Preparation of A-21 Desamido Insulin. 10 mL aliquot of insulin stock solution (I) was transferred to a clean test tube and incubated at 35°C for 4 days [25].

2.1.9. Phenol and m-Cresol Mixture. 0.600 g of each phenol and *m*-cresol were dissolved in 25.0 mL deionized water and few drops of methanol. 200 μL of this mixture with 25 mL of IS stock solution (I) was diluted to 100 mL with 0.01 M HCl to give a final concentration of 0.0480 mg/mL for each phenol and *m*-cresol and 0.0400 mg/mL for IS.

2.1.10. Preparation of Standard Insulin Solutions for the Calibration Curve. For the preparation of an insulin calibration curve, eight standard solutions were prepared in the concentration range between 0.0106 and 0.6810 mg/mL.

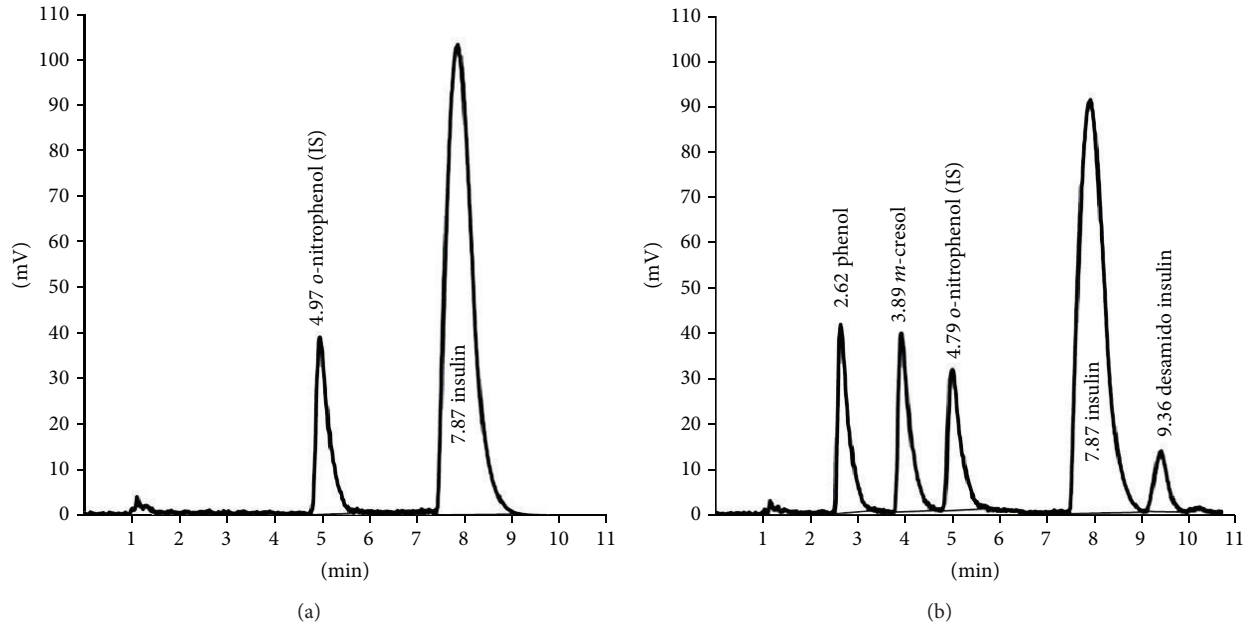

Figure 1: (a) Insulin standard in addition of IS (*o*-nitrophenol). (b) Degraded insulin in addition of IS (*o*-nitrophenol) and vial additives (phenol and *m*-cresol).

Table 1: Insulin calibration curve data.

Con. (mg/mL)	Cal. 1 AR*	Cal. 2 AR	Cal. 3 AR	Cal. 4 AR	Average	SD	RSD %
0.6810	11.8730	11.8194	11.8894	11.9334	11.8788	0.0471	0.40
0.3405	5.8769	5.7887	5.8790	5.8062	5.8377	0.0471	0.81
0.1703	3.0726	3.0054	3.0551	3.0715	3.0512	0.0315	1.03
0.0851	1.4171	1.4513	1.4614	1.4350	1.4412	0.0194	1.35
0.0426	0.7468	0.7283	0.7324	0.7504	0.7395	0.0108	1.46
0.0213	0.3682	0.3681	0.3601	0.3688	0.3663	0.0041	1.13
0.0106	0.1872	0.1802	0.1792	0.1811	0.1819	0.0036	1.96
Slope	17.4223	17.3179	17.4471	17.4714	17.4147	0.0676	0.39
Intercept	−0.0003	−0.0088	−0.0030	−0.0091	−0.0053	0.0044	
R^2	0.99983	0.99985	0.99989	0.99969	0.99982	0.00009	0.01

*AR: area ratio.

2.1.11. Preparation of Quality Control (QC) Insulin Solutions. The insulin stock solution (II) with the concentration of 0.17025 mg/mL was used to prepare quality control samples with the concentrations of 0.17025, 0.06810, and 0.01703 mg/mL to check the method precision, accuracy, and stability.

2.2. Method Validation Experiments

2.2.1. Selectivity and Interference of Additives. Selectivity of the method was studied by processing blank solutions (0.01 M HCl) and mixture solution of insulin, A21 desamido insulin, IS, and vial additives (phenol and *m*-cresol). 2.0 mL of phenol and *m*-cresol mixture was diluted with 2.0 mL of A21 desamido solution (prepared as previously described); this mixture was processed and compared with a solution of freshly prepared insulin (standard 1). Chromatograms are depicted in Figure 1.

2.2.2. Linear Range. The linear range is important to clarify the concentration range where the relation between the concentrations and signals is linear. From the development experiments, it was shown that the method is linear up to insulin concentration of 0.6810 mg/mL in 0.01 M HCl. In order to validate this assumption, four sets of calibration standard solutions containing seven concentrations ranging from 0.0106 to 0.6810 mg/mL were freshly prepared as shown in Table 1. These solutions were injected in duplicate, and accumulated chromatograms of these standard solutions are represented in Figure 2. The relative peak areas (peak area of insulin/peak area of IS) were plotted against the nominal concentrations of insulin solutions and linearity was tested

Table 2: Data of precision experiment ($n = 6$, each preparation was injected twice).

Conc.	0.06810 mg/mL					0.01703 mg/mL				
	Ins. area	IS area	AR	Ave.	Conc.	Ins. area	IS area	AR	Ave.	Conc.
	9.9868	8.1734	1.2219	1.2260	0.07070	2.6968	8.8942	0.3032	0.3057	0.01786
	9.9792	8.1126	1.2301			2.7007	8.7643	0.3082		
	11.7923	9.7909	1.2044	1.2004	0.06923	2.7409	9.0975	0.3013	0.3025	0.01767
	11.6048	9.7004	1.1963			2.7348	9.0055	0.3037		
	11.6483	9.5581	1.2187	1.2212	0.07043	2.8628	9.7853	0.2926	0.2926	0.01711
	11.6895	9.5531	1.2236			2.8648	9.7891	0.2926		
	10.9214	9.0979	1.2004	1.2117	0.06988	3.1066	10.1297	0.3067	0.3081	0.01800
	11.0028	8.9965	1.2230			3.1642	10.2212	0.3096		
	11.2385	9.2503	1.2149	1.2206	0.07039	3.1953	10.6644	0.2996	0.2989	0.01747
	11.3289	9.2381	1.2263			3.1761	10.6510	0.2982		
	10.8315	8.9431	1.2116	1.2003	0.06923	2.9253	9.6708	0.3025	0.3048	0.01780
	10.6340	8.9408	1.1894			2.9792	9.7013	0.3071		
Average					0.06998					0.01765
SD					0.00064					0.00032
RSD %					0.91					1.82

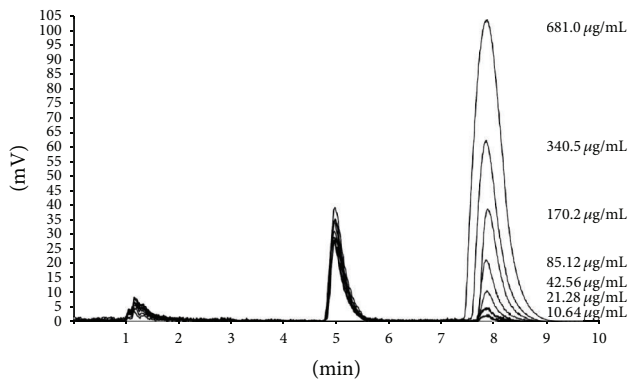

Figure 2: Chromatograms for calibration insulin standard solutions.

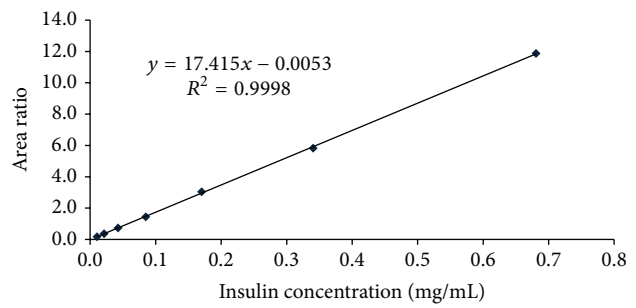

Figure 3: Calibration curve of insulin solution using *o*-nitrophenol as internal standard ($n = 4$).

by calculating R^2 (correlation coefficient) value, which should be >0.99; Figure 3 shows the average constructed calibration curve for four sets of calibration standards.

2.2.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ). LOD and LOQ were calculated based on the signal-to-noise ratio. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD and signal-to-noise ratio of ten is used for estimating LOQ [26].

2.2.4. Precision (Repeatability). Six different preparations of insulin solutions of the concentrations 0.06810 and 0.01703 mg/mL in the presence of 0.0200 mg/mL IS were prepared in 0.01 M HCl. Each solution was injected twice. The average area ratio for each solution was calculated and used to calculate the concentration of these solutions using the linear equation. These back calculated concentrations were subjected to precision calculations (standard deviation and RSD%). All these data are shown in Table 2.

2.2.5. Accuracy. Three different solutions were used to present the accuracy of the analytical method. Each of these solutions was injected three times and the average was calculated. The back calculated concentration for these solutions was evaluated from the slope and intercept of the calibration curve. The average, standard deviation, relative standard deviation (RSD%), and relative error were calculated for each concentration and shown in Table 3. These values were used to illustrate the accuracy of the method.

2.2.6. Stability. The stability was evaluated for aqueous insulin solution during analysis time and storage period. Insulin was analyzed immediately after preparation as well as after 12, 24, and 48 h in room temperature for the concentrations of 0.01703 mg/mL and 0.17025 mg/mL. Stability was calculated as the percent ratio of concentrations determined after each storage time with respect to the fresh assay. Results are presented in Table 4.

3. Results and Discussion

The aim of this work was focused on developing a simple chromatographic method for insulin determination without

Table 3: Data of accuracy experiments.

Conc. mg/mL	Insulin area	IS area	AR*	Calc. conc.	Average calc. conc.	Standard deviation	RSD %	Re. error %
0.17025	28.45996	9.47664	3.00317	0.17275	0.17158	0.00158	0.92	0.78
	27.98159	9.34673	2.99373	0.17221				
	25.80710	8.74393	2.95143	0.16978				
0.06810	10.95933	9.13004	1.20036	0.06923	0.06906	0.00045	0.65	1.41
	11.27432	9.36966	1.20328	0.06940				
	11.44450	9.62872	1.18858	0.06855				
0.01703	2.85957	9.81390	0.29138	0.01703	0.01672	0.00029	1.73	−1.79
	2.74505	9.63243	0.28498	0.01667				
	3.02176	10.7402	0.28135	0.01646				

*AR: area ratio.

Table 4: Stability of insulin solutions at room temperature ($n = 6$).

Concentration (mg/mL)	Fresh sample	12 h sample	24 h sample	48 h sample
0.17025	98.99 ± 1.12%	96.31 ± 1.36%	93.87 ± 0.37%	87.33 ± 1.31%
0.01703	100.14 ± 0.53%	95.45 ± 0.98%	86.66 ± 1.82%	81.73 ± 1.27%

the use of sophisticated and rare gradient elution and without the employing of harsh mobile phase (low pH and highly salinity) or uncommon chemicals and columns. On the same time, the aimed method should be suitable for routine analysis of insulin in the presence of its major degraded product and vial additives. This was done by fine-tuning of mobile phase composition and using small particle size for stationary phase.

The use of internal standard (IS) in insulin determinations in pharmaceutical preparations is not common [18–23]. The developed method applied the use of internal standard to overcome any changes that might happen with chromatographic conditions, especially which related to the mobile phase fine composition. Several chemicals were tested as internal standard, among these *o*-nitrophenols, has been chosen as the most fitting because of its proper retention time. Figure 1 illustrates chromatograms for insulin in the presence of internal standard and insulin in the presence of its degraded product and vial additives.

The method approved its rapidity where the retention time of insulin is 7.9 minutes and that for insulin degraded product is 9.36 minutes where the repeatability is less than 0.52%. The selectivity of the method was established by comparing the chromatograms of standard insulin with degraded insulin as shown in Figure 1(b). The chromatograms proved that no interferences occurred with the retention time (t_R) of insulin peak. The method also shows good resolution (R_s) of 1.56 between insulin and A21 desamido insulin (main degradation product).

Linearity test of the method is demonstrated by the standard solutions chromatograms presented in Figure 2 and calibration curve is depicted in Figure 3. Linearity was confirmed by correlation coefficient of 0.9998 over the range of 0.0106–0.6810 mg/mL; data are listed in Table 1.

LOD and LOQ were assessed for the developed method and found to be 2.93 μg/mL for LOD and 9.78 μg/mL for LOQ.

The precision (repeatability) was tested at two different levels of insulin concentrations. Data presented in Table 2 revealed acceptable values for method precision. Relative standard deviations (RSD%) were 1.82 for the low concentration level (0.01703 mg/mL) and 0.91 for the middle concentration level (0.06810 mg/mL).

The accuracy of the method was studied at three different concentration levels and the relative error was ranging from −1.79 to 1.41%, as shown in Table 3.

Stability experiments were performed at two concentration levels (0.17025 and 0.01703 mg/mL) to determine bench top stability (after 12, 24, and 48 h) at room temperature. Stability was calculated as the percent ratio of the determined concentration after each storage time with respect to the fresh assay. The stability results are summarized in Table 4.

The results show that insulin is stable in acidic medium (pH 2) at room temperature for not more than 12 hours, which is enough time for analysis. The samples should be stored in refrigerator and it is recommended to prepare fresh calibrators for each day of analysis.

4. Conclusions

The new developed isocratic RP-HPLC analytical method for the determination of insulin is simple, sensitive, selective, precise, accurate, and rapid with a short run time of 7.87 min compared to the USP method of 90 min. The method employed a C18 column with ultraviolet detection at 214 nm. This method was tested for the study of insulin and its desamido degradation product and in the presence of phenol and *m*-cresol which present in low concentration in the commercial insulin preparations as preservatives with good separation between their peaks. The method proved its validity and therefore can be used for the routine analysis of human insulin. *o*-Nitrophenol was successfully used as internal standard which improves the accuracy and linearity of the calibration curve.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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