DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR DETERMINATION OF HUMAN INSULIN IN PHARMACEUTICAL PREPARATION

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ABSTRACT

A simple and reliable high-performance liquid chromatography (HPLC) method with diode array detection was developed and validated for determination of human insulin in pharmaceutical preparation. The HPLC separation was achieved on a Phenomenex reversed-phase column (250 mm×4.6 mm, 5 μm) using a mobile phase of acetonitrile and 0.2 M sodium sulfate buffer (pH 2.4) solution (25:75, v/v) at a flow rate of 1.2 ml min^{-1}. The diode array detector (DAD) was operated at 206 nm and column temperature was adjusted at 40 ^{0}C. The method was validated for specificity, linearity, precision, accuracy and limit of quantitation. Also, the method was applied for the quality control of commercial Actrapid HM formulation.

Keywords: Human insulin, HPLC, Pharmaceutical preparation, Validation

INTRODUCTION

Insulin (Figure 1) is the most important regulatory hormone in the control of glucose homeostasis consisting of 51 amino acids shared between two intermolecular chains and with a molecular weight of 5800 g mol^{-1} [1]. Insulin, a pancreatic peptide hormone, is used for the treatment of insulin dependent diabetes mellitus.

Several immune and non-immune methods have been reported for determination of human insulin. Radioimmunoassay [2-5], enzyme immunoassay [6-9], luminescent immunoassay [10], capillary electrophoresis [11-14] and high performance liquid chromatography (HPLC) [15-19] had been widely used for human insulin detection in vivo and in vitro media.

Recently HPLC has been applied to determine the concentration of human insulin in various preparations [20]. Although HPLC has been utilized in these studies, there have been several problems. For example, retention times of 25 min [21] and 40 min [22] for the compound of interest may be considered excessive when it is necessary to analyze multiple samples.

Therefore, the purpose of this investigation was to develop and validate a method using a simple, rapid, sensitive, precise, accurate and specific reversed phase HPLC-DAD assay. The method uses a simple mobile phase composition and the rapid run time of 15 min. Hence, this method can be used for the analysis of large number of samples.

MATERIALS AND METHODS

Chemicals and reagents

Recombinant human insulin was provided by Eli Lilly & Co (USA). Water was Milli-Q grade and all other chemicals and solvents used were of analytical grade. Buffer solution (sodium sulfate, Na_2SO_4) was prepared with deionized water. Actrapid HM was obtained from Department of Endocrinology and Metabolism, Faculty of Medicine, Ataturk University. HPLC grade acetonitrile was purchased from Merck.

Chromatographic system and conditions

The HPLC system consisted of a Thermoquest Spectra System P 1500 isocratic pump coupled with a Spectra System UV 6000 LP photodiode array detection system, a Spectra System AS 3000 autosampler, a SCM 1000 vacuum membrane degasser, a SN 4000 system controller. The detector was set to scan from 200 to 500 nm and had a discrete channel set at 206 nm, which was the wavelength used for quantification. Separation was operated on a Phenomenex reversed-phase C_{18} column with particle size of 5 μm (250mm×4.6 mm i.d.). The mobile phase consisted of acetonitrile and 0.2 M sodium sulfate buffer (pH 2.4) 25:75 (v/v). The mobile phase was filtered through nylon membrane of 0.45-mm pore size. The flow-rate was 1.2 ml min^{-1}, column temperature was set at 40 ^{0}C and 20 μl of samples was injected to the HPLC system.

Preparation of the standard and quality control solutions

A standard stock solution containing human insulin was prepared in 0.01 M HCl at a concentration of 100 μg ml^{-1} and kept stored at 4 ^{0}C. The standard solutions from 0.75 to 50 μg ml^{-1} (0.75, 1.25, 2.5, 5, 10, 15, 25, 35, 50 μg ml^{-1}) in 0.01 M HCl were made by a serial dilution. A calibration graph was constructed in the range of 0.75 to 50 μg ml^{-1} for human insulin (n = 6). Three quality control (QC) samples at the concentrations of 7.5, 20 and 40 μg ml^{-1} were prepared from the stock solution.

Method validation

System suitability

The system suitability was assessed by six replicate analyses of human insulin at a concentration of 25 μg ml^{-1}. The acceptance criterion was ±2% for the percent relative standard deviation (% RSD) for the peak area and retention times for human insulin.

Figure 1: The sequence of human insulin

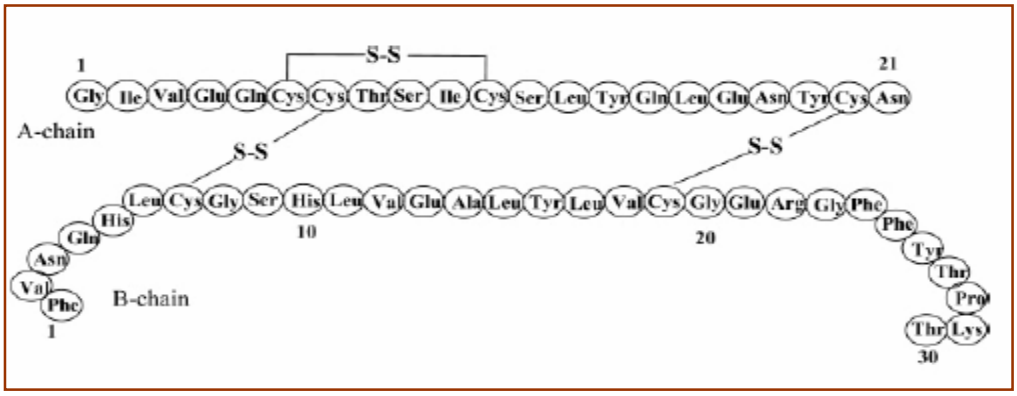

Table 2: Precision and accuracy of method for determination of human insulin

Added (µg ml^{-1})	Intra-day			Inter-day		
	Found±SD (µg ml^{-1})	Accuracy	Precision RSD%[a]	Found±SD (µg ml^{-1})	Accuracy	Precision RSD%[a]
7.5	7.68±0.079	2.40	1.03	7.71±0.129	2.80	1.67
20	20.32±0.239	1.60	1.18	20.40±0.435	2.00	2.13
40	39.29±0.692	-1.78	1.76	41.28±1.061	3.20	2.57

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation, [a]Average of six replicate determinations, Accuracy: (%relative error) (found-added)/addedx100

Table 3. Stability of human insulin in solution

Stability (%)	Room temperature stability (Recovery % ± SD)		Refrigeratory stability, +4 °C (Recovery % ± SD)		Frozen stability, - 20 °C (Recovery % ± SD)	
Added (µg ml^{-1})	24 h	72 h	24 h	72 h	24 h	72 h
1.25	97.8±1.35	92.3±6.19	99.3±2.36	96.3±5.16	98.2±4.24	97.2±4.34
15	98.6±0.46	93.7±2.32	99.5±1.87	97.5±4.65	96.5±5.16	98.2±3.46
40	101.1±0.35	88.3±3.42	100.8±2.96	97.4±3.93	99.1±4.08	98.4±2.89

Linearity

Calibration plots were constructed for human insulin standard solutions by plotting the concentration of compounds versus peak area response. Standard solutions containing 0.75-50 µg ml^{-1} of human insulin were prepared and 20 µl was injected into the HPLC column (Figure 2). The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope (Sb) and intercept (Sa) on the ordinate.

Accuracy and precision

Accuracy of the assay method was determined for both intra-day and inter-day variations using the six times analysis of the QC samples. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days).

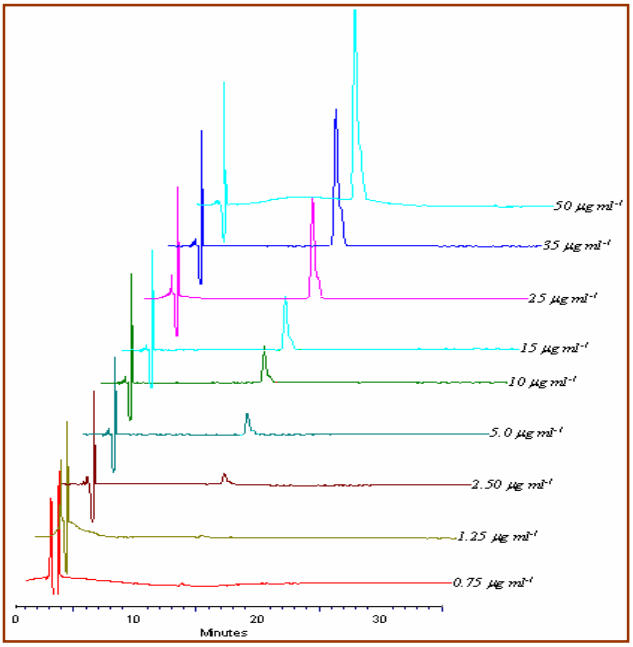

Figure 2: HPLC-DAD chromatograms of human insulin

Detection and quantitation limits (sensitivity)

Limits of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (% RSD) and accuracy (% bias) within ±10%.

RESULTS AND DISCUSSION

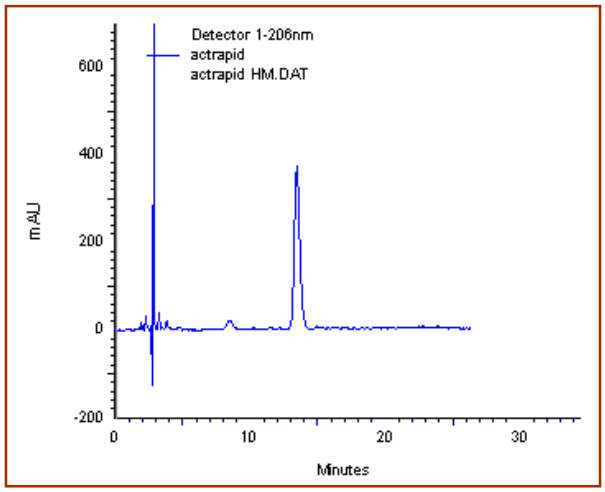

Figure 3: HPLC-DAD chromatogram of Actrapid HM (50 µg ml^{-1})

System suitability

The % RSD of peak area and retention time ($t_{R)}$ for human insulin are within 2% indicating the suitability of the system (Table 1). The efficiency of the column as expressed by number of theoretical plates for the six replicate injections was 3003±1% and the tailing factor was 1.08±0.2%.

Table 1: System suitability study of human insulin

Concentration (25 µg ml^{-1})	Retention time (t_R) (min)	Peak area
Mean (n=6)	13.8	4312173
SD	0.0306	14033.6
% RSD	0.22	0.33

Linearity

The calibration curve constructed was evaluated by its correlation coefficient. The peak area of the human insulin was linear in the range of 0.75-50 µg ml^{-1}. The calibration equation from six replicate experiments, y = 201781x-277239 (r = 0.9946), demonstrated the linearity of the method. Standard deviations of the slope and intercept for the calibration curves were 13324 and 18364, respectively.

Accuracy and precision

Accuracy and precision calculated for the QC samples during the intra- and inter-day run are given in Table 2. The RSD values for intra-day precision was ≤1.76% and for inter-day precision was ≤2.57% . The bias values for intra-day accuracy was ≤2.40% and for inter-day accuracy was ≤3.20%. These values are summarised in Table 2.

Sensitivity

LOD and LOQ were determined by an empirical method that consisted of analyzing a series of standard solutions containing decreased amounts of human insulin. LOQ is defined as the lowest concentration on the calibration curve at which both accuracy and precision should be within % 10. LOD and LOQ values of HPLC-DAD method were determined to be 0.10 and 0.25 µg ml^{-1}, respectively.

Stability

Stability studies indicated that the samples were stable when kept at room temperature, 4 ^{0}C and -20 ^{0}C refrigeration temperature for 24 h (short-term) and refrigerated at 4 and -20 ^{0}C for 72 h (long-term). The results of these stability studies are given in Table 3, where the percent ratios are within the acceptance range of 90-110%.

Recovery

To determine the accuracy of the proposed method and to study the interference of formulation additives, the recovery was checked as three different concentration levels (3, 25, 45 µg ml^{-1}) and analytical recovery experiments were performed by adding known amount of pure drugs to pre-analyzed samples of commercial dosage form. The percent analytical recovery values were calculated by comparing concentration obtained from the spiked samples with actual added concentrations. These values are also listed in Table 4.

Table 4: Recovery of human insulin in pharmaceutical preparation

Commercial preparation	Actrapid (5 µg ml^{-1})		
Added (µg ml^{-1})	Found±SD (µg ml^{-1})	Recovery (%)	RSD [a] (%)
3	2.98±0.064	99.3	2.15
25	25.40±0.602	101.6	2.37
45	45.95±0.909	102.1	1.98

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

Assay sample preparation

Actrapid HM injectable formulation containing 100 U ml^{-1} human insulin was diluted with 0.01 M HCI to achieve an appropriate concentration (50 µg ml^{-1}) (Figure 3).

CONCLUSION

A rapid, specific and isocratic HPLC method has been developed for the determination of human insulin using a diode array detector. The method was validated for linearity, accuracy, precision, sensitivity and stability. Therefore, this method can be used for the routine QC analysis of human insulin in pharmaceutical preparations.

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