academicJournals

Vol. 8(40), pp. 1018-1024, 29 October, 2014
DOI: 10.5897/AJPP2013.3628
Article Number: A92B5B748468
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Development and validation of simple and rapid high performance liquid chromatographic method for routine analysis of human insulin in formulations

Mohammad Javed Ansari*, Shahid Jamil, Mohammad Khalid Anwer, Ramdhan Al-shdefat and Bahaeldin Elsyed Ali

Department of Pharmaceutics, College of Pharmacy, Salman Bin Abdulaziz University, Al-kharj, Saudi Arabia.

15 April, 2013; Accepted 17 October, 2014

A simple, specific, precise and accurate reversed phase liquid chromatographic (RP-LC) method has been developed for determination of insulin in dosage form. The chromatographic separation was achieved on a Symmetry® RP-C18, (150 × 4.6 mm, 5 µm) column at a detector wavelength of 214 nm and a flow rate of 1.0 ml/min. Mobile phase comprised of 55 volume of 1 mmol sodium sulphate in high performance liquid chromatographic (HPLC) water pH 3.2, adjusted by phosphoric acid and 45 volume of acetonitrile. The retention time of insulin was 4.3 min. The linear regression analysis data for the calibration plots showed good linear relationship in the concentration range between 1 and 45 µg/ml. The value of correlation coefficient, slope and intercept were, 0.9997, 67755 and 10773, respectively. Standard deviations of the slope and intercept for the calibration curves were 1824 and 17908, respectively. Limit of detection (LOD) and limit of quantitation (LOQ) values were determined to be 0.10 and 0.25 µg/ml, respectively. The method was validated for accuracy, precision and ruggedness. Mean recovery was 100.46%, while intra- and inter-day relative standard deviations were 0.395 and 0.289%, respectively. The proposed RP-LC method can be applied for the quality control or routine analysis of bulk insulin as well as commercially available formulations of insulin.

Key words: Human insulin, isocratic, simple, rapid, high performance liquid chromatography (HPLC), pharmaceutical formulations.

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 5800 (Chien, 1996). Insulin, like other proteins, is not a stable entity but is liable to modification by chemical reactions with molecules in its vicinity. Thus during storage and use of pharmaceutical preparation, insulin is degraded by hydrolytic reactions or is transformed by formation of intermolecular covalent bonds with other insulin molecules, leading to higher

*Corresponding author. E-mail: javedpharma@gmail.com. Tel: +966115886041. Fax: +966115886001.

molecular weight transformation products (Olivia et al.,1996).

Several analytical techniques have been published for human insulin estimation such as radioimmuno assays, bioassays, electrophoresis, spectroscopy and reverse phase high performance liquid chromatography (RP-HPLC) with their own advantages and disadvantages. For instance, immunoassay methods are cheaper and faster, but can be less sensitive due to cross reactivity, matrix effects, presence of isomers, impurities or metabolites.

RP-HPLC is probably the most used analytical method for separation and determination of peptides and proteins in an extensive range of applications (Aguilar, 2004). The United States Pharmacopeia (USP) describes a RP-HPLC method for the determination of recombinant human insulin with insulin peak detected at 214 nm (USP 30th, 2007, retention time 29 min).

RP-HPLC for analysis of insulin in formulations has been reported, but was not simple, sensitive and rapid. These methods employed complex (Chen et al., 2002; Vuppugalla et al., 2003; Sarmento et al., 2006) long run time (Asahara et al., 1991; Chen et al., 2002; Vuppugalla et al., 2003; Moussa et al., 2010) high flow rate (Vuppugalla et al., 2003) that could be impractical for routine analysis of multiple samples. Use of a special column or column temperature controllers, which are not popular in most of the analytical laboratories, is another limitation (Farid et al., 1989; Yomota et al., 1996).

Recently, a rapid and sensitive RP-HPLC method for analysis of human insulin injection has been developed and reported (Rajan et al., 2006) but that utilized addition of an ion-pairing reagent triethylamine (TEA) as a competing base. Although it may reduce the tailing, this additional component to mobile phase may also alter the HPLC column in a way that is not easily reversed. Furthermore, strong amines, such as TEA, are significantly difficult to wash off the column.

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 assay. The method uses a simple mobile phase composition without addition of ion-pairing agent such as triethylamine and the rapid run time of 8 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 purchased from Sigma Aldrich. HPLC grade acetonitrile and sodium sulfate, Na_2SO4 was purchased from Merck. Water was Milli Q grade and all other chemical and solvents used were analytical grade. Insulin human injection of recombinant DNA origin was obtained from Eli Lilly and Company, USA.

Chromatographic system and conditions

The HPLC system consisted of Waters 600 controller pump, auto-sampler (Waters 717 plus) fitted with a 20 µl loop and Waters 486 tunable absorbance detector. The output signal was monitored and processed using a Breeze Software. The chromatographic column used was a 150 × 4.6 mm, Symetry^R RP-C18 with 5 µm particles. Mobile phase consisted of 55 volume of 1 mmol sodium sulphate in water, pH 3.2 adjusted by phosphoric acid, and 45 volume of acetonitrile. The mobile phase was filtered through nylon membrane of 0.45-mm pore size. The column was maintained at ambient temperature and 10 µl of test or quality control samples or standard were injected then eluent was monitored with a UV detector at 214 nm with flow rate of 1 ml/min and run time of 8 min.

Preparation of the standard and test samples

A standard stock solution was prepared by accurately weighting and dissolving human insulin in 0.01 M HCl solution at a concentration of 100 µg/ml and kept stored at 4°C. The standard working solutions from 1 to 45 µg/ml concentrations were made by serial dilution. Similarly, three quality control (QC) samples at the concentration of 7.5, 20 and 40 µg/ml were prepared from the stock solution. The injection formulation, containing 3.5 mg/ml human insulin, was diluted with 0.01 M HCl to achieve appropriate concentrations.

Method validation

The suggested analytical method was validated according to international guidelines (ICH, 1997) with respect to certain parameters such as, accuracy, precision, linearity, detection and quantitation limits and robustness.

System suitability

The system suitability was assessed by six replicate analyses of human insulin at a concentration of 5 µg/ml. The acceptance criterion was ±2% for the percent relative standard deviation (% RSD) for the peak area and retention times for human insulin.

Linearity

The linearity of the method was established by spiking a series of standard solutions containing 1 to 45 µg/ml of recombinant human insulin and injecting 10 µl of each standard onto the HPLC column. Calibration plots were constructed by plotting the peak area responses against their respective concentrations. Linear regression was applied and slope (a), intercept (b), correlation coefficient (r) and standard error (E) were determined.

Detection and quantitation limits (sensitivity)

Limits of detection (LOD) and limit of quantitation (LOQ) were estimated through dilution method using signal-to-noise ratio (S/N) approach by injecting a 10 µl sample. LOD and LOQ were considered as the lowest concentrations level resulting in a peak height of at least three times (S/N≈3) and ten times (S/N≈10) the baseline noise, respectively with precision (% RSD) and accuracy (% bias) within ±10%.

Accuracy

The accuracy of the method was determined by calculating the

percent recovery of human insulin. Sample solutions spiked with the recombinant human insulin at three different concentration levels (7.5, 20 and 40 µg/ml) were analyzed in triplicate.

Precision

Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying the QC samples in triplicate at three concentration levels during the same day. Intermediate precision was assessed by getting complete repeatability assays done by another analyst on second day and evaluating the overall results (day 1 and day 2 repeatability).

Robustness

Robustness of the method was determined by introducing small variations in the experimental conditions such as composition of mobile phase (buffer: ACN; 60:40, 55:45 and 50:50) pH of mobile phase (3.2 ± 0.1 unit), mobile phase flow rate (1 ± 0.1 unit) and detection wavelength (214 ± 5 units) followed by calculation of the responses, retention time and RSD.

Solution stability

The stability of the insulin standard preparation was tested by analyzing freshly prepared insulin solutions (7.5, 20 and 40 µg/ml) immediately after preparation as well as after 12, 24 and 72 h at room temperature. The percent ratios of concentrations determined in each case to known added concentrations were calculated.

RESULTS AND DISCUSSION

The method is based on separation of insulin from other excipients for quantifying the insulin in finished product. Several parameters such as composition, pH and flow rate of mobile phase along with detection wavelength were evaluated for their effect on location and shape of peak of the insulin while developing the method. Human insulin injections, like other multiple dose injection formulations, contain preservative(s). Many preservatives exhibit high ultraviolet absorption, making it necessary to have analytical techniques capable of separating the protein from the preservative. Ideally, it would be desirable to have quantitative separation methods capable of resolving the preservative(s) and degradation product with no interfering interaction with the analyte.

Selection of column

A common cause of peak tailing in reversed phase HPLC is the ion-exchange interaction that takes place between a positively charged analyte and a negatively charged silanol on the surface of silica stationary phase support particles. Addition of triethylamine to the mobile phase as a competing base, reduces the availability of stationary phase silanols and hence interaction of the analyte with the silanols. However, this additional component to the mobile phase may alter the HPLC column in a way that is not easily reversed because strong amines, such as triethylamine, are significantly difficult to wash off the column. In order to remove the triethylamine, it is necessary to wash the system with 5 to 10% acetic acid as the flushing solvent. This means that a column modified by triethylamine is not suitable for applications that do not use triethylamine in the mobile phase. Moreover, if triethylamine is added to an unbuffered or lightly buffered mobile phase, it can cause the pH of the solvent to dramatically rise, perhaps to the detriment of the column, possibly affecting the separation and ultimately shortening column life. The best fix is to use a column that minimizes the need for triethylamine. Peak tailing is observed most often when using HPLC columns packed with stationary phases that have significant silanol activity. Phases made with high purity silica (less acidic silica) generally can be expected to provide better peak shape for basic compounds. Therefore, HPLC columns with high purity silica such as Waters Sunfire (C18, 250 × 4.6 mm, 5 µl) and Waters symmetry (C18, 250 × 4.6 mm, 5 µl) were evaluated for the method development and later was selected for the method.

Selection of mobile phase

Considering the final optimized method, a mobile phase consisting of 55 volume of 1 mmol sodium sulphate, pH 3.2 adjusted by phosphoric acid, and 45 volume of acetonitrile were used. The retention time for human insulin was 4.3 min (Figures 1 and 2). It should be noted that the retention time varied considerably unless the relative content of acetonitrile in the mobile phase was carefully kept constant, but slight change in the pH did not affect the retention time of insulin.

Validation

System suitability

The percent RSD of peak area and retention time for human insulin were within 2% indicating the suitability of the system (Table 1). Retention times of insulin in test samples and marketed formulation were found to be similar (Figures 1 and 2). The efficiency of the column as expressed by the number of theoretical plates for the six replicate injections was 2893±48.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

Standard solutions containing 1 to 45 µg/ml of human insulin were prepared and 10 µl was injected into the HPLC column (Figure 1). Calibration plots were constructed for human insulin standard solutions by plotting the

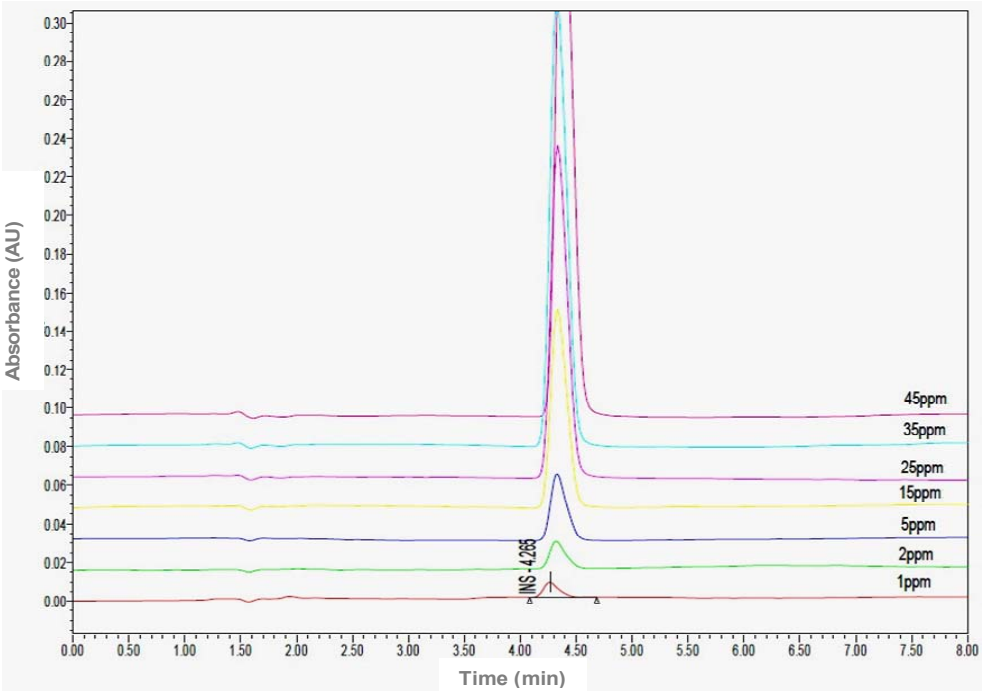

Figure 1. Chromatograms of Calibrators (10 µl of standard solutions containing 1 to 45 µg/ml of human insulin). Chromatograms of insulin Calibration standards on Symmetry® RP-C18 (150 × 4.6 mm, 5µm). Regression equation; Y= 67755x + 10773, and correlation coefficient R^2=0.9997

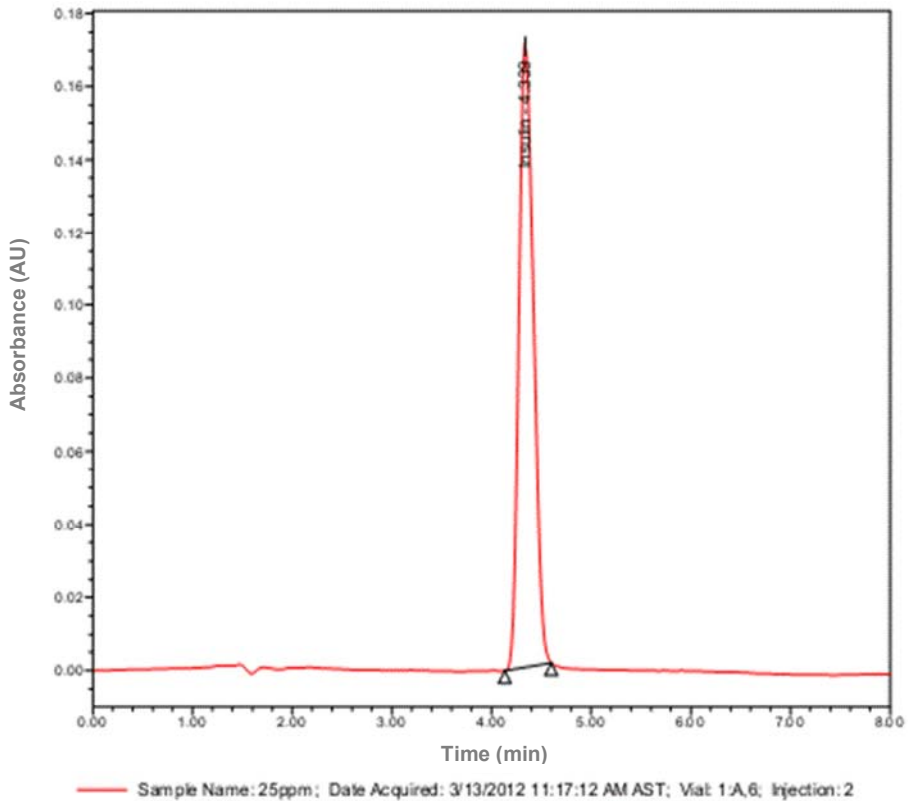

Figure 2. Representative chromatogram of Humulin injection (25 µg/ml).

Table 1. System suitability of the method.

Injection	AUP	H	RT	N
1	348920	34582	4.33	2849.775
2	350618	34556	4.335	2886.507
3	350741	34535	4.331	2911.752
4	346238	34257	4.33	2941.451
5	352377	34303	4.335	2826.684
6	350121	34064	4.333	2945.529
Mean	349835.8	34382.83	4.332333	2893.616
SD	2085.815	208.1686	0.002338	48.50655
RSD	0.596227	0.605443	0.053968	1.67633

AUP: Area under peak, H: Peak height, RT: retention time of insulin, N: number of theoretical plates calculated as $N=5.545$ (width at 50% peak height/peak area)^2 SD: Standard deviation, RSD: Relative standard deviation.

Table 2. Accuracy of method for determination of human insulin.

Added amount (µg/ml)	AUP	Calculated conc. (µg/ml)	Mean conc. (SD)	RSD (%)	Recovery (%)
7.5	519990 519132 512585	7.6366 7.6240 7.5278	7.5961 (0.0594)	0.783	101.28
20	1360500 1361770 1365371	19.9803 19.9989 20.0519	20.0104 (0.0371)	0.185	100.05
40	2724909 2719460 2731256	40.0181 39.9380 40.1113	40.0225 (0.0867)	0.217	100.06
Overall recovery					100.46

AUP: Area under peak, SD: Standard deviation, RSD: Relative standard deviation, Accuracy: % Recovery (found/added) ×100.

concentration of compounds versus peak area response. The calibration curve constructed was evaluated by its correlation coefficient. The calibration equation, $y = 67755x + 10773$ ($r = 0.9997$), demonstrated the linearity of the method. Standard deviations of the slope and intercept for the calibration curves were 1824 and 17908, respectively. LOD and LOQ values were determined to be 0.10 and 0.25 µg/ml, respectively.

Accuracy

The accuracy of the method was determined by calculating the percent recovery of human insulin. Sample solutions spiked with the recombinant human insulin at three different concentration levels (7.5, 20 and 40 µg/ml) were analyzed in triplicate. The results presented in Table 2 show that the percent recovery ranges between 100.05 and 101.28% indicating the acceptable accuracy of the method.

Precision

Precision of the assay was determined at two levels, namely, repeatability (intra-day) and intermediate precision (inter-day) by analyzing sample solutions spiked with the recombinant human insulin at three different concentration levels (7.5, 20 and 40 µg/ml) in triplicate. The results presented in Table 3 show that the percent RSD ranges between 0.185 and 0.783 indicating the acceptable precision of the method (Yılmaz and Kadıoglu, 2010).

Table 3. Precision of method for determination of human insulin.

Added amount (µg/ml)	Intra-day precision (repeatability-day 1)			Intra-day precision (repeatability-day 2)			Inter-day/Intermediate precision
	AUP	Mean area (SD)	RSD (%)	AUP	Mean area (SD)	RSD (%)	RSD (%)
7.5	519990 519132 512585	517235.67 (4050.38)	0.783	516990 516090 515689	516256.33 (666.26)	0.129	0.456
20	1360500 1361770 1365371	1362547 (2526.75)	0.185	1385560 1379530 1382400	1382496.67 (3016.16)	0.218	0.201
40	2724909 2719460 2731256	2725208.33 (5903.69)	0.217	2756266 2750306 2761656	2756076 (5677.38)	0.206	0.211
Overall intra-day precision			0.395	Overall Intermediate precision			0.289

AUP: Area under peak, SD: standard deviation of three replicate determinations, RSD: relative standard deviation.

Table 4. Robustness of method

Parameter observed		Mean AUP (SD)	Mean RT (SD)	RSD (%)
Composition of mobile phase	60:40	1642357 (3564)	6.61 (0.0547)	0.83
	55:45	1854628 (2201)	4.32 (0.0635)	1.47
	50:50	1546245 (3024)	3.52 (0.0842)	2.39
pH of mobile phase	3.1	1645236 (1852)	4.21 (0.0404)	0.96
	3.2	1859293 (1965)	4.33 (0.0345)	0.79
	3.3	1745628 (2031)	4.56 (0.0642)	1.40
Flow rate	0.9 ml/min	1739475 (1586)	4.36 (0.0404)	0.92
	1 ml/min	1789716 (1678)	4.33 (0.0358)	0.82
	1.1 ml/min	1742542 (1489)	4.22 (0.0542)	1.28
Wave length	209	1742657 (1547)	4.29 (0.0416)	0.96
	214	1764359 (1469)	4.33 (0.0395)	0.91
	219	1739546 (1652)	4.36 (0.0514)	1.17

Mean AUP: Mean area under peak of triplicate samples; SD: standard deviation, mean RT: mean retention time of triplicate samples; RSD: relative standard deviation.

Robustness

Robustness of the method was determined by introducing small variations in the experimental conditions such as pH of mobile phase (3.2 ± 0.1 unit), mobile phase flow rate (1 ± 0.1 unit) and detection wavelength (214 ± 5 units) followed by calculation of the responses, retention time and RSD. The results are presented in Table 4. It should be noted that the retention time varied considerably with respect to composition of mobile phase, however, slight change in the pH, flow rate, and detection wavelength did not affect the retention time of insulin.

Solution stability

The stability of the insulin standard preparation was tested by analyzing freshly prepared insulin solution (7.5, 20 and 40 µg/ml) immediately after preparation as well as after 12, 24 and 72 h at room temperature. The percent ratios of concentrations determined in each case to known added concentrations were calculated and presented in Table 5. About 3 to 4% and 7 to 11% decrease in concentration of samples were observed after 24 and 72 h storage at room temperature respectively, however, it was negligible after 12 h. Therefore, it is recommended

Table 5. Stability of human insulin in solution.

Added amount (µg/ml)	Stability at room temperature (25ºC) % Recovery (SD)			
	0 h	12 h	24 h	72 h
7.5	99.63 (0.24)	98.45 (0.56)	96.28 (0.79)	93.45 (0.89)
20	100.13 (0.39)	99.07 (0.55)	97.05 (0.19)	93.75 (0.26)
40	99.28 (0.45)	98.52 (0.46)	96.16 (0.22)	89.42 (0.69)

% Recovery: (Found/Added) × 100; SD: Standard deviation of triplicate readings.

to use freshly prepared samples.

Conclusion

A rapid, simple and isocratic HPLC method has been developed and validated for the determination of human insulin in formulations. The advantages of this method are, relatively short insulin retention time (4.3 min), simplicity of mobile phase without addition of any modifiers, isocratic mode and non-dependency on requirements such as column temperature controller. Sensitivity, wide range of linearity with considerable accuracy and precision of analysis and reproducibility are further benefits. Because of its specificity, good reproducibility, accuracy, inexpensiveness, relative rapidity and sufficient sensitiveness, the method may be successfully used in evaluation of formulations as well as bulk insulin.

ACKNOWLEDGEMENT

The authors acknowledge the generous financial support from the Science and Technology Unit, Scientific Research Deanship, Salman bin Abdulaziz University, Ministry of Higher Education, Saudi Arabia.

Conflict of interest

There is no conflict of interest as regard this study.

REFERENCES

Aguilar MI (2004). HPLC of peptides and proteins: basic theory and methodology. Methods Mol. Biol. 251:3

Oliva A, Farina JB, Llabres M (1996). Influence of temperature and shaking on stability of insulin preparations: Degradation kinetics. Int. J. Pharm. 143: 163-170.

Asahara K, Yamada H, Yoshida S (1991). Stability of human insulin in solutions containing sodium bisulfate. Chem. Pharm. Bull. 39: 2662-2666.

Moussa BA, Farouk F, Azzazy HME (2010). A Validated RP-HPLC Method for the Determination of Recombinant Human Insulin in Bulk and Pharmaceutical Dosage Form. E-J. Chem. 7(S1):S449-S457.

Chen XJ, Zhu JB, Wang GJ, Zhou MX, Xu YN (2002). HPLC determination of insulin and its related substances in insulin powder for inhalation. Yao Xue Xue Bao 37:131-133.

Chien YW (1996). Human insulin: Basic sciences to therapeutic uses. Drug. Dev. Ind. Pharm. 22: 753-789.

Farid NA, Atkins LM, Becker GW, Dinner A, Heiney RE, Miner DJ, Riggin RM. (1989). Liquid chromatographic control of the identity, purity and "potency" of biomolecules used as drugs. J. Pharm. Biomed. Anal. 7:185-188.

International Conference on Harmonization, (ICH) (1997). Q2B, Validation of Analytical Procedures, Methodology, May 1997.

Rajan DS, Gowda KV, Mandal U, Ganesan M, Bose A, Sarkar AK, Pal TK (2006). Development of RP-HPLC for analysis of human insulin. Ind. J. Pharm Sci. 68: 662-665.

The United States Pharmacopoeia/National Formulary (2007). USP 30th/ NF 25, New York, Twinbrooke Parkway. P 1375.

Yılmaz B, Kadıoglu Y (2010). Development and validation of HPLC method for determination of human insulin in pharmaceutical preparation. IJPSRR 2:40-43.

Yomota C, Yoshii Y, Takahata T, Okada S. (1996). Separation of B-3 monodesamidoinsulin from human insulin by high-performance liquid chromatography under alkaline conditions. J. Chromatogr. A. 721:89-96.