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A Validated RP-HPLC Method for the Determination of Recombinant Human Insulin in Bulk and Pharmaceutical Dosage Form

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Abstract: A modified RP-HPLC method was developed for the quantitative determination of recombinant human insulin in bulk and pharmaceutical dosage form with reduced retention time. Study of the effects of the column temperature, pH of the mobile phase and presence of vial additives (phenol and *m*-cresol), or impurities (A-21 Disamido) on the accuracy of the assay were assessed. Separation was achieved using a Hypersil BDS C-18 column and the mobile phase was composed of solution A (aqueous solution of 28.3 anhydrous Na_2SO_4g/L, pH 2.3) and solution B (28.5 g anhydrous Na_2SO_4 g/L in 50:50 mixture of water and acetonitrile, pH 2.3) in a ratio 48:52 (v/v) at 45–50 ºC. The column temperature was 40 ºC, the flow rate was 1 mL/min and detection was performed at 216 nm. The procedures were validated according to international conference on harmonization (ICH) guidelines. Recovery study was done applying standard addition technique for further validation of the procedure. The retention time of recombinant human insulin was 19.7 min as compared to 29 min obtained by the reference method. Analytical conditions fluctuations or presence of vial additives or impurities did not show any significant effect on the accuracy of the method. The prepared standard insulin solution in 0.01 N HCl was found to be stable for 5 days. Statistical comparison showed no significant difference between the described method and reference method regarding the accuracy and precision. The modified method can be applied for routine quality control applications for determination of recombinant human insulin.

Keywords: RP-HPLC, Recombinant human insulin, Quantitative, Validation, Effect of additives, Impurities.

Introduction

Insulin is composed of two peptide chains, referred to as A chain (21 amino acids) and B chain (30 amino acids) with molecular weight of 5.8 kDa. Two disulfide bridges link these chains together and another intra-peptide disulfide bond exists in the A chain[1,2]. Human insulin produced by recombinant DNA technology is the first commercial health care product derived from this technology. A weakened form of *Escherrichia coli* is used as a vector for the synthesis of recombinant human insulin. The protein is produced in the bacterial cell as two chains A and B. The two chains are mixed and reconnected in a reaction that forms the disulfide cross bridges, resulting in pure recombinant human insulin. Insulin is administered subcutaneously for the treatment of type I diabetes mellitus when pancreatic *β*-cells daily production of insulin is insufficient. Several methods have been reported for the determination of insulin in the pharmaceuticals and plasma including voltametric[3], spectroscopic[4], electrochemical[5] and chromatographic methods[6,7].

RP-HPLC is probably the most used analytical method for separation and determination of peptides and proteins in an extensive range of applications[8]. The United States Pharmacopeia (USP) describes a RP-HPLC method for the determination of recombinant human insulin with insulin peak detected at 214 nm and appearing[9] after 29 min. The aim of the present study is to provide a RP-HPLC method suitable for industrial application with reduced analysis time that can withstand fluctuations in analytical conditions, presence of vial additives (phenol and *m*-cresol) or impurities (A-21 disamido) without significant effect on its accuracy.

Experimental

Chromatographic separation was performed on Shimatzu 2010 HPLC system (Germany) equipped with an auto-sampler, a Hypersil BDS C-18 (30 x 4.6 mm, 3µm) column and a UV detector. A Kontes Ultra-Ware® filtration system with 5 liter reservoir part no. 31862(USA) was used.

Pure standards

Recombinant human insulin USP reference standard and bulk crystals were obtained from the egyptian company for biological products and vaccines (VACSERA, Egypt).

Pharmaceutical dosage forms of insulin

Commercially available insulin-VACSERA R®40 IU was obtained from public pharmacy.

Chemicals and reagents

All chemicals and reagents used throughout this work were of analytical grade and the solvents were of HPLC grade. Water for injection (WFI) was obtained from VACSERA (Egypt). Hydrochloric acid, *o*-phosphoric acid, sodium sulphate, acetonitrile, phenol and *m*-cresol were obtained from Fisher (USA).

Standard solutions

Recombinant human insulin standard solutions

Into a series of 50 mL volumetric flasks, different concentrations of recombinant human insulin USP reference standards (20, 40, 80, 90, 100, 10 and 120 IU/mL) were prepared by dissolving 35.3 mg, 70.6 mg, 141.2 mg, 158.8 mg, 176.5 mg, 194.2 mg and 211.8 mg of the recombinant human insulin USP reference standard crystals, respectively, in 0.01N HCl.

Phenol and m-cresol standard solution

In a 50 mL volumetric flask, 65 mg of phenol and 150 mg of *m*-cresol were dissolved in acetonitrile. This was the manufacturer reported concentration in the final dosage form.

Preparation of A-21 disamido (degradation product of insulin)

In a suitable clean capped vial, 1 mg of insulin crystals was dissolved in 1 mL 0.01 N HCl and incubated at 35 ºC for 4 days[9].

Insulin, phenol and m-cresol mixture

In suitable clean capped vials, 5 mL from the 80, 100 and 120 IU/mL insulin standard solutions were separately mixed with 5 mL of the prepared phenol and *m*-cresol standard solution.

Chromatographic conditions

Chromatographic separation was performed using a Hypersil BDS C-18 (30x4.6 mm, 3 µm) analytical column. The flow rate was maintained at 1 mL/min and the detection was performed at 216 nm. The column temperature was kept at 40 °C. The mobile phase consists of solution A and B of pH 2.3 in a ratio of 48:52 (v/v) at 45-50 ºC.

Preparation of mobile phase

Solution A was prepared by dissolving 56.8 g of anhydrous Na_2SO_4 in WFI then the pH was adjusted to 2.3 with *o*-phosphoric acid and the volume was completed to 2 L with WFI and filtered. Solution B was prepared by dissolving 28.5 g of Na_2SO_4 in 400 mL WFI, the pH was adjusted to 2.3 with *o*-phosphoric acid, volume was completed with WFI to 1 L then 1 L of acetonitrile was added. The two solutions (A & B) were kept on hot-plate magnetic stirrer at a temperature 45-50 ºC with continuous stirring and filtered before being introduced into the HPLC system.

Determination of method specificity

Identification

20 µL of 0.01 N HCl, 100 IU/mL insulin standard, A-21 disamido preparation, phenol & *m*-cresol were separately injected into the HPLC system and chromatographed under the previously mentioned chromatographic conditions. The retention time for each was recorded.

Assay

The effect of vial additives was tested by carrying out the experiment on the prepared insulin, phenol and *m*-cresol mixtures. Area under the peak (AUP) corresponding to insulin concentration was recorded for each mixture and insulin recovery percent was calculated using the regression equation as described below (linearity).

Linearity

A series of different concentrations of recombinant human insulin ranging from 80-120 IU/mL were injected into the HPLC system and analyzed using the specified chromatographic conditions. The retention times and the AUPs were recorded from the resulting chromatograms. A calibration curve plotted for the AUP versus insulin concentration and the respective regression equation was computed.

Accuracy

Different aliquots of insulin standard solutions (80, 100 and 120 IU/mL) were injected into the HPLC system and the same procedures used for establishing linearity were followed. AUP for each insulin solution was recorded and the recovery percent of insulin was calculated using the regression equation.

Precision

Inter-assay precision (Repeatability)

Insulin standard solutions (80, 100 and 120 IU/mL) were analyzed three times each intra-daily. The AUPs were recorded and the relative standard deviation (RSD) was calculated.

Intermediate precision

Intermediate precision expresses within-laboratory variations including different days, analysts, equipments, *etc*. According to the International Conference on Harmonization (ICH) guidelines it is not necessary to study these effects individually; the use of an experimental design (matrix) is encouraged[10]. The previous procedures were repeated inter-daily (on different days) using different insulin standards concentrations (80, 100 and 120 IU/mL) three times each for each concentration using freshly prepared mobile phase prepared by another analyst. The AUPs were recorded and RSD was calculated.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated based on the standard deviation of the analytical response represented by AUP and the slope of the calibration curve. The following equations LOD = 3.3 σ/S, LOQ = 10 σ/S were used, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Robustness

To determine the robustness of the method, small variations in the experimental conditions were introduced and the AUP was recorded followed by calculation of the RSD. In the above mentioned experiments, the pH of the mobile phase was 2.3 and the temperature of the column was 40 ºC. The effect of small variations in pH of the mobile phase and the temperature of the column on the method accuracy were separately tested on the 100 IU/mL insulin standard solution. The pH was varied between 2.2 and 2.4 in 0.1 unit increments using *o*-phosphoric acid, while the column temperature was varied between 39 ºC and 41 ºC in 1 ºC increments. The procedure was repeated accordingly, the AUP was recorded and RSD was calculated.

Insulin solution stability

The stability of the insulin standard preparation was tested by analyzing freshly prepared 100 IU/mL insulin solution using the modified RP-HPLC method. The prepared solution was stored at 2 - 8 °C and analyzed every 24 h for 5 successive days. The AUPs were recorded and RSD was calculated.

Application of the method to commercial products

Testing purity of bulk recombinant human insulin from commercial suppliers

Recombinant human insulin solutions of concentrations (80-120) IU/mL were prepared according to their labeled potency as per the supplier's certificate of analysis. The prepared concentrations were analyzed using the modified RP-HPLC, AUPs were recorded and recovery percent of pure recombinant human insulin was calculated using the regression equation.

Application on vial dosage form

40 µL of 6N HCl were added to a commercial 40 IU/mL insulin vial to obtain a clear solution which was injected into the HPLC system and analyzed. Recovery study was performed by applying standard addition for further validation of the procedure.

Results and Discussion

Figure 1 showed that recombinant human insulin had a peak with retention time of 19.7 min. The insulin peak was resolved from the peaks of the additives (phenol & *m*-cresol; 6.5 and 9.8 min, respectively) and degradation product (A-21 disamido; 25.8 min) (Table 1). The additives had no effect on average recovery percent of recombinant human insulin which was 99.76±0.15 (Table 2), hence the proposed method was found to be of sufficient specificity for assay of recombinant insulin.

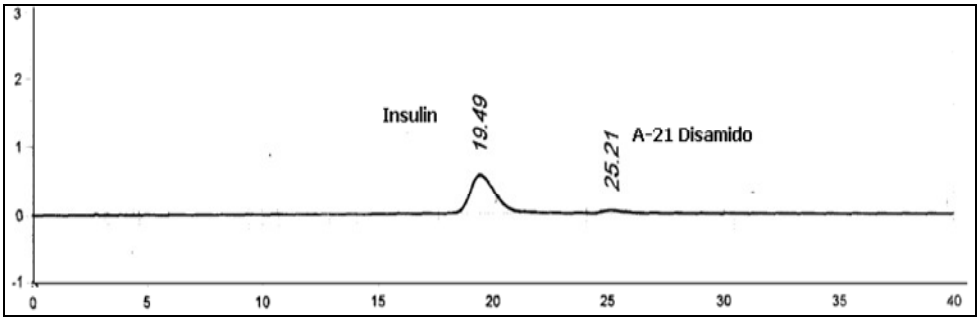

Figure 1. RP-HPLC chromatogram of pure recombinant human insulin (40 IU/mL)

Table 1. Specificity of the insulin RP-HPLC assay

Sample name	Average retention time, min	RSD
Blank (0.01 N HCl)	3.533	0.11
Standard insulin	19.7	0.28
A21-Disamido	25.892	0.8
Phenol	6.526	0.15
m-cresol	9.861	1.21

Table 2. Specificity results in presence of vial additives

Concentration of insulin standard, IU/mL	Mean of AUP of insulin	Calculated concentration of insulin, IU/mL	Recovery %
40	30071002	39.98	99.9
50	37280943	49.9	99.8
60	44478965	59.8	99.6
	Average recovery %		99.77
	SD		0.15
	RSD		0.15

The relationship between AUP and concentration of recombinant human insulin was linear over the concentration range 20-120 IU/mL and the correlation coefficient was 0.9993. Parameters of the regression equation are shown in Table 3. There was good correlation between AUP and recombinant human insulin concentration.

The AUPs corresponding to insulin concentrations of 80, 90, 100 and 120 IU were used to calculate the recovery percent of insulin using the regression equation. The recovery percent was 99.85±1.39 (Table 4). The results demonstrate that the modified method was suitable for quantitative determination of recombinant insulin.

Table 3. Parameters of regression equation relating insulin concentration to analytical response (AUP)

Parameter	Value
Linearity range	20-120 IU/mL
Detection limit, IU/mL	4.17
Quantitation limit, IU/mL	13.9
Slope of regression equation	727073
Standard error (SE) of slope	8777
Confidence limit (CL) of slope	704512.19 - to 749634
Intercept	1002375.65
SE of intercept	763683.7
CL of intercept	-960735.5 to 2965487
Correlation coefficient	0.9993
SE	794751.8

Table 4. Accuracy of the modified RP-HPLC method for determination of recombinant human insulin

Concentration of insulin standard, IU/mL	AUP	RSD	Mean of AUP of insulin	Calculated conc. insulin, IU/mL	Recovery %
80	60197960 59935564 60292500	0.307	60142004	81.34	101.67
90	66717900 66795644 66508343	0.227	66673962.33	90.33	100.37
100	73546600 72764200 73881451	0.78	72764200	98.70	98.70
120	87294288 87156272 87053168	0.1	87167909.33	118.15	98.46
Average recovery %					99.8
SD					1.51
RSD					1.51

RSDs of analysis for the inter-assay precision 0.3, 0.78 and 0.1; and those for the intermediate precision were 0.2, 0.2 and 0.1 for the three chosen concentrations (80, 100 & 120 IU/mL) (Table 5).

LOD and LOQ were calculated based on standard deviation of the analytical response represented by AUP and slope of the calibration curve and were 4.17 and 13.9 IU/mL, respectively.

In the robustness study, the effect on AUP was studied and RSDs of the response were 1.8 and 0.6 for pH of the mobile phase and column temperature variations, respectively. Small fluctuations in analytical conditions had no significant effect on method performance (Table 6).

Table 5. Precision of the modified RP-HPLC assay for determination of insulin determination

Sample concentration	AUP for day 1	Average	RSD	AUP for day 2	Average	RSD	Overall RSD
80 IU/mL	60197960 59935564 60292500	60142008.0	0.30	56546831 56291687 56402177	56413565.00	0.2	4.5
100 IU/mL	73546606 72764200 73881451	73397419.0	0.78	78988190 78782067 78621052	78797103.00	0.2	4.9
120 IU/mL	87294288 87156272 87053168	87167909.3	0.10	87433128 87622793 87502037	87519319.33	0.1	0.2

Table 6. Effect of pH of the mobile phase and column temperature variation on the AUP of insulin (Robustness)

Parameter	Mean AUP	RSD
Mobile phase pH		
2.4	72193456	
2.3	73397417	1.8
2.2	71920313	
Column temperature, ºC		
39	72474471	
40	73397417	0.6
41	72694456	

In the stability study, the prepared insulin standards were assayed once for 5 consecutive days. The RSD was 0.8 indicating that the standard solution is stable for 5 days (Table 7). This allows the use of the same recombinant insulin standard solution for 5 successive days when stored at temperature 2-8 °C.

Table 7. Stability of standard insulin preparation (100 IU/mL)

Day	Mean AUP	Overall RSD
1	72193456	
2	73397417	
3	71920313	0.8
4	72130189	
5	72131716	

The described method was used to quantify insulin in bulk crystals from commercial supplier to determine its purity. Recovery was calculated using the regression equation and results are shown in Table 8.

The method was found applicable to pharmaceutical dosage form with average recovery percent of 100.32 (Figure 2). Recovery study was performed on insulin vial form by applying standard addition technique where known amounts of standards were added to pre-analyzed vial samples. The added insulin standard solution was recovered with average accuracy percent of 101.44±0.896 (Table 9). Statistical comparison between the described method and the USP reference method showed no significant difference with respect to the accuracy and precision of the method (Table 10).

Table 8. Application of the modified RP-HPLC method for the determination of insulin in bulk crystals from commercial supplier

Concentration of insulin standard IU/mL	AUP	RSD	Mean of AUP of insulin	Calculated conc. of insulin (IU/mL)	Recovery %
80	60097960 60035564 60192500	0.13	60108675	81.3	101.60
100	73466000 72694200 71971558	1.02	72710586	98.63	98.63
120	88294199 86154872 87450000	1.23	87299690	118.69	98.90
Average recovery%					99.71
SD					1.64
RSD%					1.64

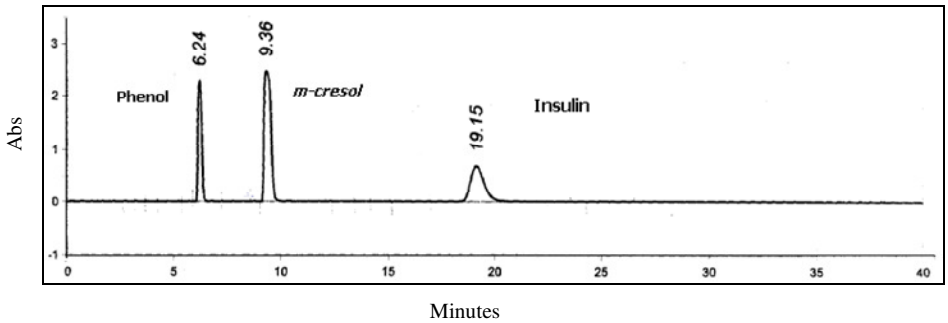

Figure 2. RP-HPLC chromatogram of insulin in vial form. Peaks of phenol, *m*-cresol and insulin appear at 6.24, 9.36 and 19.15 min, respectively.

Table 9. Recovery study of insulin from vial dosage form by applying standard addition technique using the modified RP-HPLC method

Dosage form	Claimed taken IU/mL	AUP	Found insulin (IU/mL)	Recovery %.	Total insulin IU/mL	AUP	Found insulin IU/mL	Rec. %	Added nsulin IU/mL	Found insulin IU/mL	Recovery%
Insulin VACSERA R® 40 IU/mL	20	15585084.32	20.06	100.3	60	45369120	61.02	101.7	40	40.96	102.4
					65	48809760.1	65.75	101.1	45	45.69	101.6
					70	52257195.1	70.49	100.7	50	50.44	100.9
					75	55697735.2	75.23	100.3	55	55.17	100.3
					80	60172749.7	81.38	101.7	60	61.32	102.2
Average								101.1			101.47
SD								0.62			0.88
RSD								0.61			0.87

Table 10. Statistical comparison between the modified and reference RP-HPLC methods for determination of recombinant insulin

Recombinant human insulin	Proposed RP-HPLC method	Reference method [6]
Accuracy	99.85±1.39	99.87±1.6
N	4	4
SD	1.39	1.6
RSD	1.39	1.6
Variance	1.93	2.56
F-test	1.32(9.28)*	
t-test	0.019(3.18)*	

* $p = 0.05$

Conclusion

The objectives of this study were to reduce the analysis time of recombinant insulin using RP-HPLC method and investigate the effect of analytical condition fluctuations and presence of vial additives or impurities on the accuracy of modified method. This is in order to make the RP-HPLC method more amenable for routine quality control procedures of bulk crystals, in-process quality control and finished product analysis.

The retention time of recombinant insulin was 19.7 min as compared to 29 min obtained by the reference method. Analytical conditions fluctuations or presence of vial additives or impurities did not show any significant effect on the accuracy of the modified method. The insulin standard was found to be stable for 5 days and validation experiments were satisfactory for all parameters tested.

The modified RP-HPLC method for determination of recombinant human insulin can be applied for routine quality control procedures with no interference from vial additives or impurities and can withstand normal fluctuation in the analysis conditions that may arise during extended operation of the HPLC system.

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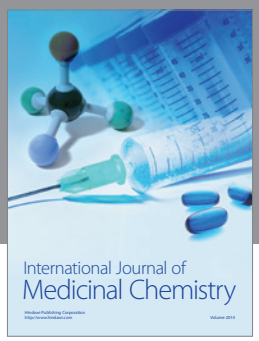

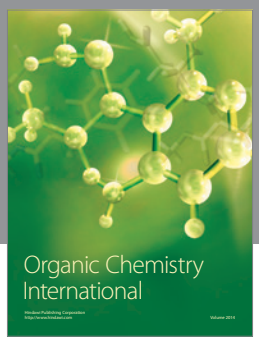

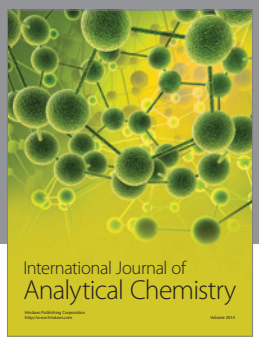

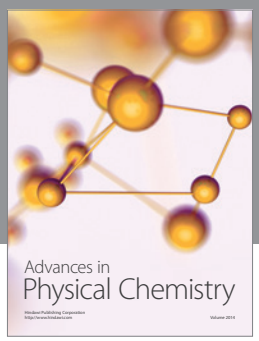

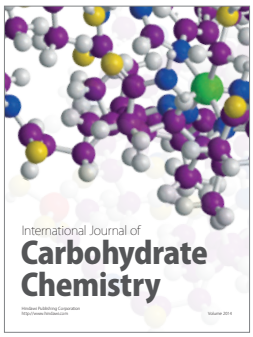

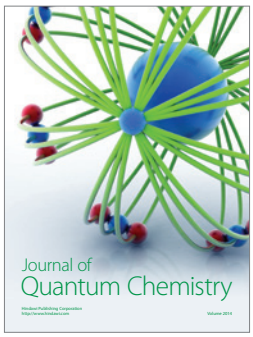

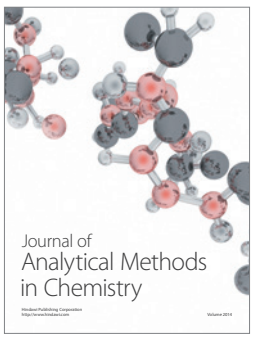

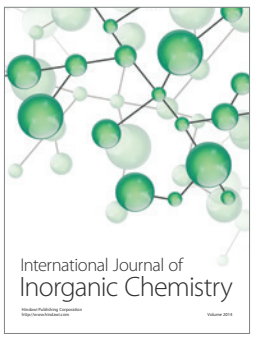

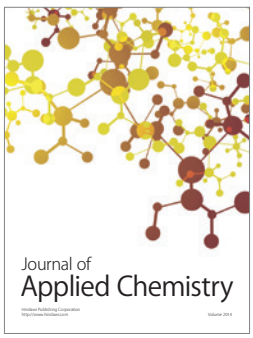

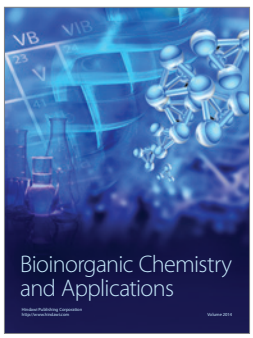

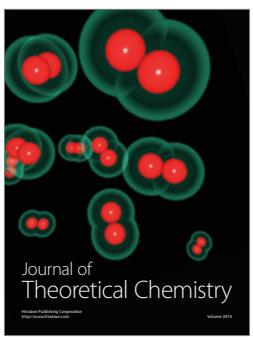

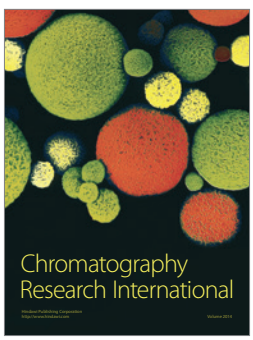

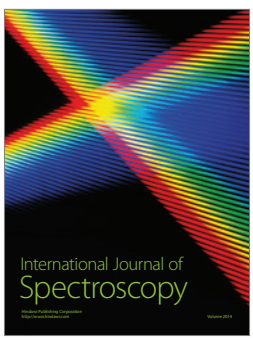

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