SYNTHESIS AND CHARACTERIZATION OF NEW N-TERMINAL B-CHAIN MODIFIED HUMAN INSULIN ANALOGUES

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Abstract

New human insulin analogues were synthesized by fragment condensation reactions of $N^{\alpha A1}$, $N^{\varepsilon B29}$ Msc protected native human insulin of different B-chain lengths with the dipeptide Boc-Asn-Thr-OH. Edman degradation was used to obtain shortened B-chain insulin intermediates. Purification of the products was achieved using gel filtration, ion exchange chromatography and RP-MPLC chromatography. Analysis of synthesized analogues accomplished by RP-HPLC chromatography and amino acid analysis. The modified insulin analogues with the amino acids Asparagine in the first position and Threonine in the second position of the N-terminal B-chain of insulin would be expected to show an enhanced T→R* transition compared with that of wild-type structure.

Keywords: Insulin, Peptide Synthesis, T-R Transition.

Introduction

The hormone named Insulin is a two-chain molecule consisting of a 21 amino acid A-chain and a 30 amino acid B-chain linked by two disulfide bonds [1]. In metal-free solutions, insulin monomers self associate to dimers and higher aggregates [2]. However, in the presence of various divalent metal, physiologically zinc ions (at 0.33 eq./monomer), insulin associates to a discrete 2Zn hexamer [2].

Insulin hexamers in solution undergo a conformational reorganization termed the T->R transformation in which the N-terminal B-chain (residues B1-B8) changes from an extended conformation (T-state) to a helical one (R-state). The T- and R-states are related by a dynamic equilibrium T6→ T3R3→R6 which is pronouncedly T-sided [3, 4, 5].

The cooperative T->R transformation is inducible by two classes of profoundly different agents. These are inorganic anions like thiocyanate ion acting on the metal ions, on the one hand, and phenolic compounds occupying pockets of the R-state on the other hand.

The insulin hexamers behave as a dimer of two cooperative trimers which are linked by a negative cooperativity [6, 7, 8]. The binding of SCN^- ions only transforms one trimer and so the transition does not exceed the T3R3-state, whereas titration with phenol achieves complete transformation which, however, proceeds in two consecutive steps: T6→ T3R3→R6 [5, 6, 7, 8, 9, 10].

To study this structural transformation in future work modifications at the N-terminus of the B-chain of insulin were to be performed in this work. These Modifications will be concerned the positions B1 and B2. From our previous study the amino acid Asparagine found that it has high tendency to promote the helix formation in N-terminus position of B-chain [11, 12]. In this work the amino acid Threonine was suggested to be studied adjacent to Asparagin in the second position with different B-chain lengths. These modifications together were meant to stabilize the R-state or to make the T->R transition easier.

After Chou and Fasman empirical work threonine found to be indifferent with regard to its helix propensity [13]. Numerous of published works have taken other factors into account like solvent effect, position in a given sequence to predict definite suggestions for helix formation [14, 15]. Threonine is also selected because of its hydrophobic side chain (Methyl group) and the hydrophilic hydroxyl group that could introduce required structural features for helix formation.

Materials and Methods

Biosynthetic human insulin was a generous gift from Hoechst Marion Deutschland GmbH, Frankfurt, Germany.

All chemicals are commercially available and were at least of p. a. grade. Boc- and F-moc amino acids and 2-chloro-tritylchloride resin were purchased from Novabiochem, Bad Soden (Germany). Thin layer chromatography (TLC) on silica-coated aluminum plates (Merck AG, Darmstadt, Germany). SP-Fractogel (Merck AG, Darmastadt, Germany. Sephadex G25F (Pharmacia, Sweden), DEAE-Fracrogel-S (Merck AG).

Sephadex G25F (Pharmacia, Sweden) was used for gel permeation chromatography. Ion exchange chromatography at pH 2.7 was carried out on SP-Fractogel with 1.5 M acetic acid in 2-propanol/water (2/3- v/v). Ion exchange chromatography at pH 7.8 was carried out on DEAE-Fractogel with 0.02 M Tris/HCl in 2-propanol/water (2/3- v/v). Linear sodium chloride gradients were applied for elution.

Synthesis of Boc-Asn-Thr-OH *[16 , 17, 18]*
0.9 mmol F-moc-Thr-OH and 3.6 mmol DIPEA dissolved in 6 ml DMF was shaken with 1.0 g for 4 h. Then the F-moc group was removed by incubating for 3 min. in 20 % piperidine dissolved in DMF. 1.3 mmol Boc-Asparagine, 1.3 mmol TBTU, and 1.3 mmol DIPEA were dissolved in 4 ml DMF and then added to the resin and shaken for 1 h. A mixture of TFE/acetic acid/DCM (1/1/8, v/v/v) was then added to the resin. The resin was filtered off and the solution was then mixed with 200 ml diethylether under cooling. The precipitated Boc-Asn-thr-OH was centrifuged and dried on air. The crude product was purified by RP-MPLC in 0.1 % aqueous TFA on RP18 silica gel with a 2-propanol gradient at 5-10 bar.

Synthesis of insulin analogues
(Exemplarily [Asn^{B1},Thr^{B2}]-insulin)

1. N$^{\alpha A1}$, N$^{\varepsilon B29}$-Bis(Msc)-insulin

To a solution of 1.00 g (172.2 µmol) zinc-free insulin dissolved in 70 ml Dimethylsulfoxide (DMSO) and 0.7 ml Triethylamine, a solution of 91.4 mg (344.4 µmol) Msc-ONSu in 14 ml DMSO is added drop-wise at room temperature [19]. After 20 min the reaction was stopped by the addition of 2 ml glacial acetic acid. The solution was then eluted over sephadex G-25 f using 10 % acetic acid. The result was then evaporated and purified by ion exchange chromatography and desalted.

2. N$^{\alpha A1}$, N$^{\varepsilon B29}$-Bis(Msc)-des(phe^{B1})-insulin [20, 21]

700 mg (112,4 µmol) of N$^{\alpha A1}$, N$^{\varepsilon B29}$ Bis(Msc)insulin obtained from step 1 was dissolved in 60 ml 90% aqueous solution of pyridine. To the resulting solution 450 µl (189,00 µmol) phenylisothiocyanate was added drop-wise and stirred for 3 h under darkness. The solvent was then removed under vacuum, washed three times with diethylether and dried. The resulting precipitate was dissolved in 14 ml trifluoroacetic acid and stirred for 45 min. at room temperature. The product was then precipitated by addition of 70 ml diethylether and centrifuge. The protein was washed three times with ether and dried. The protein was purified by G25f chromatography and lyophilized.

3. N$^{\alpha A1}$, N$^{\varepsilon B29}$-Bis(Msc) - des(phe^{B1}, Val^{B2})-insulin

The above procedure in step 2 was repeated to achieve Edman degradation of B2Valine (Scheme (1), route A).

4. N$^{\alpha A1}$, N$^{\varepsilon B29}$-Bis(Msc)-[Boc-Asn^{B1}, Thr^{B2}]-insulin [22, 23]

A solution of 20 mg (65.75 µmol) Boc-Asn-Thr-OH, 10.00 mg (65.87 µmol), HOBt, and 13.5 mg (65.96 µmol) DCC in 1.0 ml DMF was stirred for 3 h at 20 °C. 600 µl of this solution was added to a solution of the protected insulin resulted from step 3, 2 or 1 dissolved in 2.5 ml DMF and 20 ml (0.18 µmol) NMM and stirred for 60 min at 10 °C. The reaction was then stopped by addition of 1 ml glacial acetic acid. The crude insulin derivative was then desalted by G25f chromatography and lyophilized.

5. N$^{\alpha A1}$,N$^{\varepsilon B29}$-Bis(Msc)-[Asn^{B1}, Thr^{B2}]-insulin

The lyophilized insulin derivative from step 4 was dissolved in 7 ml TFA and stirred for 45 min. at Room temperature. The solvent was then removed and the residue was desalted by G25f chromatography and lyophilized.

6. [Asn^{B1},Thr^{B2}]-insulin

The lyophilized insulin derivative from step 5 was dissolved in 20 ml 10% aqueous piperidine and stirred for 3 h at 0 °C. The reaction was stopped with 1 ml acetic acid,

purified by G25f chromatography and lyophilized.

The crude products were purified by ion exchange chromatography on DEAE Fractogel-S with 20 mM Tris/HCl in 2-propanol/Water (2/3 v/v pH 7.8, desalted by G25f chromatography and lyophilized.

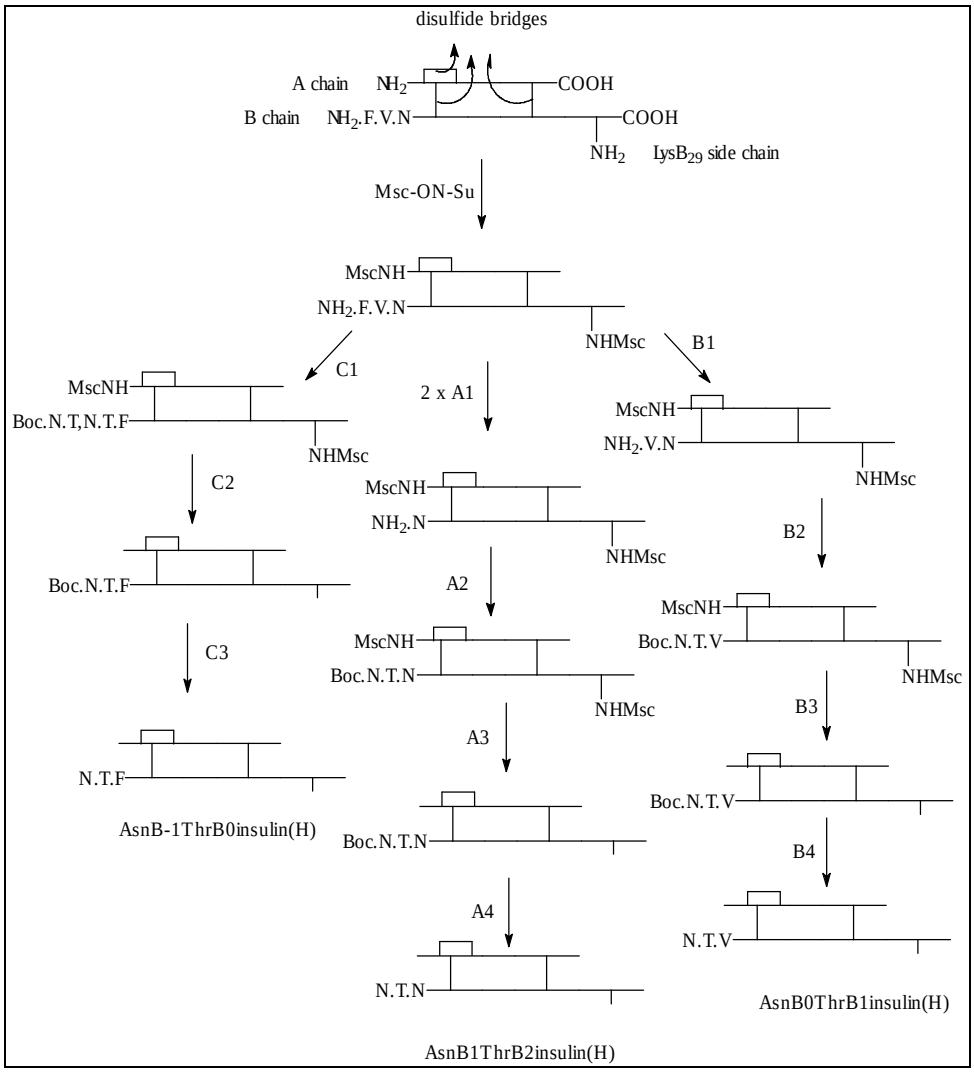
Analytical Methods

An automatic Alpha Plus II analyzer (Pharmacia) LKB, Freiburg, Germany) with resin BTC 5118 was used for amino acid analysis.

RP-HPLC was performed on a LC41D/CD instrument (Bruker-Franzen Analytic GmbH, Bremen, Germany) using a Nucleosile 5C18 colomn (0.4 cm x 25 cm) (C&S chromatography Service GmbH, Langerwehe, Germany) and a gradient of acetonitrile in 25 mM aqueous triethylammoniumphosphate (TEAP) buffer (pH 2.25).

Results and Discussion

The scheme 1 illustrates synthetic routes used in this work. To ensure regioselectivity within the synthesis of insulin analogues the amino functions of glycine A1 and Lysine B29 side chains were selectively blocked with the protecting group (Msc) according to Geiger [19]. This reaction proceeds in a basic medium and therefore reaction was stopped by acidification with glacial acetic acid. By-products, mono(Msc)- and tris (Msc)-insulin and unreacted insulin, have been removed by ion exchange chromatography on SP-Fractogel at pH 2.7 owing to the charge difference (Fig. (1)).



Scheme (1): Reaction steps to the synthesis of insulin analogues; reaction conditions: A1, B1= phenylisothiocyanate/pyridine; A2, C1, B2= Boc-Asn-Thr-OH/ DCC/HOBt/10 °C; C2, A3, B3= piperidine/ 0 °C; C3, A4, B4= Triflouroacetic acid, N= Asn; T= Thr; V= Valine; F= Phenylalanine.

After desalting of the obtained Bis(Msc)-insulin, the N-terminal B-chain was shortened down to the substitution site by Edman degradation (route A and B in Scheme (1)). These reactions occur completely without any competing reactions, therefore the resulting insulin derivatives with N-terminal shortened B-chain were introduced to the next coupling reactions after desalting.

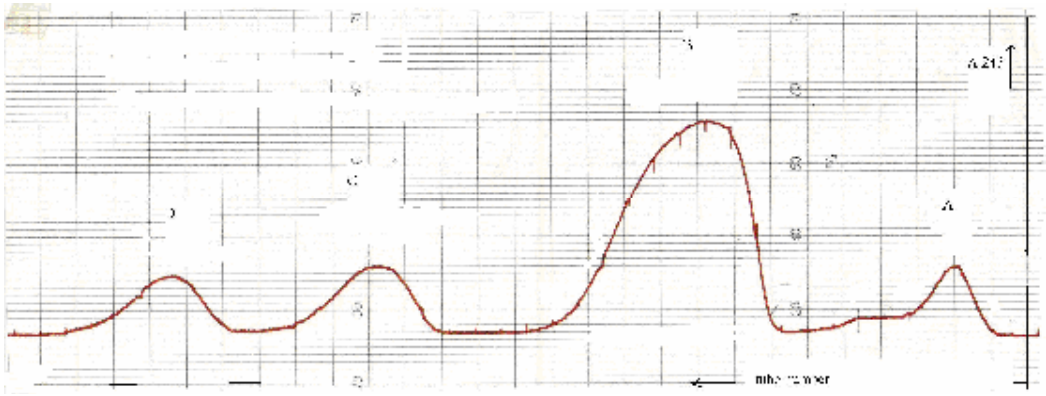


Fig.(1): Ion exchange chromatography: Tris(msc)insulin= A, Bis(msc)-insulin= B, Mono(msc)insulin= c, Insulin= D; Absorption at λ = 245nm , pH= 2.7, stationary phase= SP-Fractogel , mobile phase= 40 % 2-propanol in 1.5 M acetic acid, Gradient: 800 ml x 2 from 0.0 M to 0.15 M sodium chloride.

Asn-Thr-OH was synthesized using the technique of solid phase synthesis. To couple F-moc-Threonine to 2-chlorotritylchloride resin it was activated with DIPEA. After connecting on resin the F-moc group was removed by incubating in 20 % piperidine dissolved in DMF. Boc-Asparagine was coupled with the resin by activation with TBTU/DIPEA. The dipeptide Boc-Asn-Thr-OH was cleaved off by TFE/acetic acid/DCM (1/1/8, v/v/v) from the resin. The protection of N-terminal function of the dipeptide was essential, in order to ensure the following coupling reaction with the amino function of B-chain will take place on its carbonyl function. The dipeptide was purified by RP-MPLC in 0.1 % aqueous TFA on RP18 silica gel with a 2-propanol gradient at 5-10 bar. Reaction progress was followed by TLC.

$N^{\alpha A1}$, $N^{\varepsilon B29}$-Bis(Msc)-insulin, des(B1)- and des(B1,B2)-insulin, respectively were coupled to Boc-Asn-Thr-OH by fragment condensation. The DCC /HOBt method was applied for activation and coupling which has been proved as very effective. By-products in the coupling reactions could enormously be reduced by using mild temperature and short incubation time (<10 C°; < one hour). Fig.(2) shows the separation of the product Boc-(Bis)Msc-insulin analogue from the unreacted Bis(Msc)-insulin using ion exchange chromatography.

The final reaction of the synthesis was the removal of the protecting groups. The Boc group was removed by TFA at room temperature. After desalting Msc groups were cleaved off by 10 % aqueous piperidine at 0 °C. The products were optimal purified by ion exchange chromatography at pH 7.8 using a suitable gradient of sodium chloride due to the charge difference of the obtained insulin analogue and the by-product A21-desamido-insulin that has an additional negative charge (Fig. (3)). The retention time of the analogues and by-products are very close to each other, therefore separation via RP-MPLC only possible with high loss of yield. RP-HPLC method was always used to control all above reaction courses. The purity of the Analogues was confirmed by amino acid analysis and RP-HPLC chromatography (Table (1)).

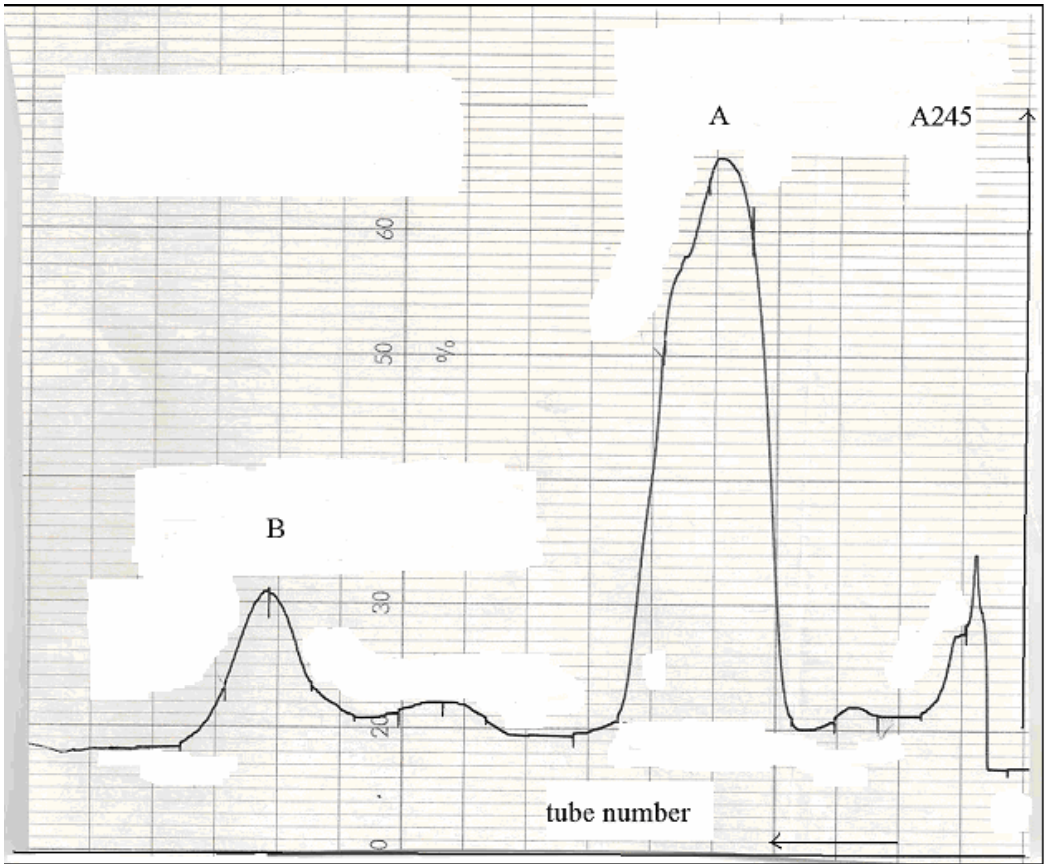


Fig.(2) : Ion exchange chromatography; $N^{\alpha A1}$, $N^{\varepsilon B29}$-Bis(Msc)-des(phe^{B1},Val^{B2})-insulin= A; $N^{\alpha A1}$, $N^{\varepsilon B29}$-Bis(Msc)-[Boc-Asn^{B1},Thr^{B2}]-insulin= B; Absorption at λ= 245 nm, pH= 2.7; stationary phase= SP-Fractogel; mobile phase= 40 % 2-propanol in 1.5 m acetic acid; Gradient: 400 ml x 2 from 0 M to 0.15 M sodium chloride.

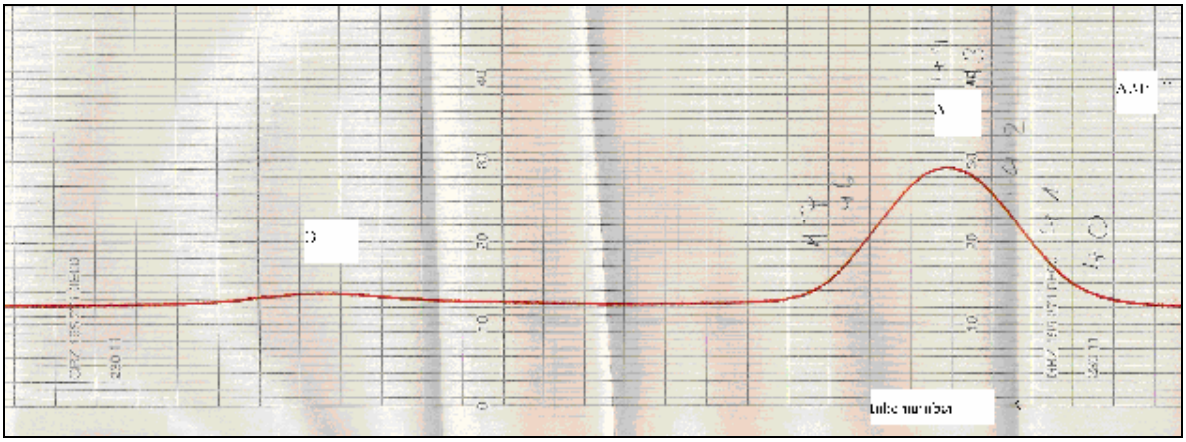


Fig.(3): Ion exchange chromatography; [Asn^{B1}, Thr^{B2}]-insulin= A; A21-desamidoinsulin= B; Absorption at λ= 245 nm; pH= 7.8; stationary phase= DEAE-Fractogel; mobile phase= 0.02 M Tris/HCl in 2/3; 2-propanol/Water; Gradient: 400 ml x 2 from 0 M to 0.15 M sodium chloride.

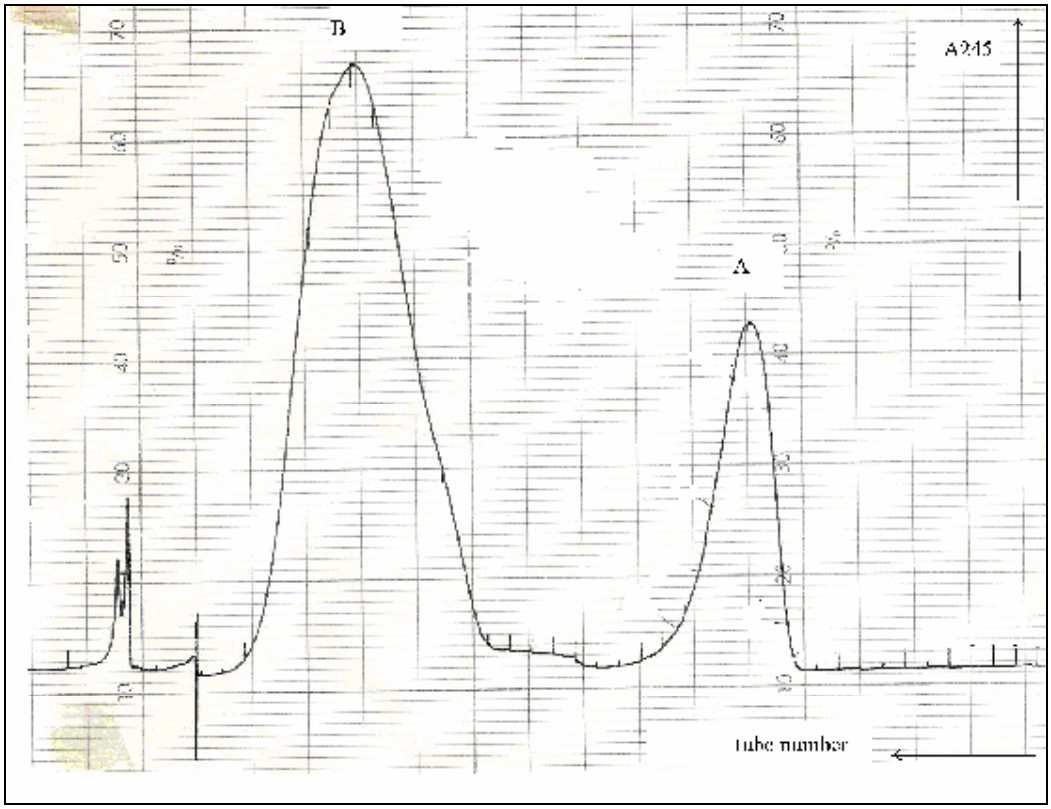


Fig.(4) : Desalting via Gel filtration on G25 F Sephadex after last step; A= [Asn^{B1}, Thr^{B2}]-insulin, B = salts, eluting agent= 0.05 M ammonium bicarbonate.

Table (1)
Purity data of synthesized insulin analogues; numbers in brackets refer to theoretical values; wild-type Insulin represents the reference of insulin analogues; Yield with respect of Bis(Msc)-insulin.

					Amino acid analysis			
No.	***Insulin analogue***	***Mol mass g/mol***	***RP-HPLC purity***	***Yield %***	***Phe***	***Val***	***Asn + Asp***	***Thr***
0	Insulin, wild type	5769.23	-	-	3	4	3	3
1	Asn^{B-1},Thr^{B0}-insulin	6020.43	99%	40.9	3.04 (3)	4.14 (4)	4.04 (4)	3.46 (4)
2	Asn^{B0},[Thr^{B1}]insulin	5855.23	98%	42.9	2.00 (2)	4.00 (4)	3.94 (4)	3.63 (4)
3	[Asn^{B1},Thr^{B2}]insulin	5738.03	99%	43.0	2.01 (2)	3.21 (3)	4.00 (4)	3.69 (4)

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خلاصة

حضرت المشتقات الجديدة للانسولين البشري Asn^{B-1},Thr^{B0}-insulin, $Asn^{B0},[Thr^{B1}]$insulin, $[Asn^{B1},Thr^{B2}]$insulin بواسطة تفاعلات التكاثف للانسولين الطبيعي المحمي في موقعي $N^{\varepsilon B29}$,$N^{\alpha A1}$ مختلفة اطوال السلسلة ب مع الببتايد الثنائى المحمي في الموقع الامين الطرفي Boc-Asn-Thr-OH. استخدم تفاعل ادمان لغرض تقصير السلسلة ب للانسولين٠ تمت تنقية المركبات الوسطية والنهائية باستخدام طرق كروماتوكرافيا السائل المعكوس و المبادل الايوني والترشيح الهلامي. تم تشخيص المركبات الناتجة بواسطة كروماتوغرافيا السائل المعكوس وتحليل الأحماض الامينية٠ يتوقع المشتقات المحضرة بوجود الأحماض الجديدة الاسبارجين في الموقع الأول والثريونين في الموقع الثاني من الطرف الاميني للسلسلة ب للأنسولين لها القدره على بناء التركيب الثنائي الهلكس مقارنة بالأنسولين الطبيعي.

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† ***Abbreviations***

Asn= asparagine; Boc= *tert*-butyloxycarbonyl; DCC= dicyclohexylcarbodiimide; DIC= N,N-diisopropylcarbodiimide; DEAE= diethyl-aminoethyl; DIPEA= diisopropylethylamine; DMF= dimethylformamide; DMSO= di-methylsulphoxide; Fmoc= 9-flourenyl-methoxycarbonyl; HOBt=1-hydrixybenzo-triazol; HONSu= N-hydroxysuccinimide; Msc=methylsulfonylethoxycarbonyl; NMM= N-methylmorpholine; SP= sulfopropyl; TBTU= 2-(1H-benzothiazol-1yl)-1,1,1,3-tetra-methyl-uroniumtetraflouroborate; TFA= tri-flouroaceticacid; TFE= triflouroethanol; Thr= Threonine; TRIS= tris(hydroxymethyl)-aminomethane.

* T=tense; R=relaxed: this description is adopted from T/R terminology of hemoglobin.