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A rapid and sensitive method for simultaneous determination of insulin and A21-desamido insulin by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatography (RP-HPLC) method with UV-detection at room temperature has been developed for the analysis of insulin and its main degradation product, A21-desamido insulin. Octadecylsilica was used as stationary phase and a mixture of water and acetonitrile containing tetrametylammonium hydroxide as eluent. The method produced linear response over the concentration range of 10–100 μg/ml, with an average accuracy of 97.35 ± 1.36% as well as average intra- and inter-day variations of 1.29 and 5.24%, respectively. The limits of detection and quantitation of the method were 0.25 and 0.75 μg/ml, respectively. Considering the analysis specifications, the system is suitable for direct analysis of routine formulations and stability studies. By this method human insulin can be separated from its principal degradation product, A21-desamido insulin. Also there is no need for any particular requirement and it is easily available in most of laboratories.

Keywords: Insulin; Reversed-phase high-performance liquid chromatography; A21-desamido insulin

1. Introduction

Following the discovery of insulin in 1921 [1], due to lack of knowledge of its chemical structure, biological assays developed to assess the potency of the hormone, were based on the measurement of the hypoglycaemic response in rabbits and convulsive response in mice.

The characterization of a protein drug is a complex undertaking, requiring the use of a wide range of methods to establish such properties of the drug substance as structural integrity, consistency, activity, purity and safety. The complexity of protein molecules means that there are many potential degradation pathways, each with its individual dependences on such parameters as pH, ionic strength, and temperature. Each protein may represent a unique combination of such pathways and dependences. It is, therefore, critical that a broad spectrum of methods have to be used

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to evaluate the effect of processing and storage to assure optimal maintenance of safety and efficacy of the drug [2].

Insulins consist of two peptide chains, the A-chain containing 21 amino acid residues and the B-chain containing 30 amino acid residues, connected by two disulphide bridges (Fig. 1). Their molecular weights are about 6000. Stability experiments on purified crystalline insulin have shown that it deteriorates at normal refrigeration temperature (5 °C) and storage at −20 °C is necessary to ensure stability. Purified insulin degrades by two mechanisms: deamidation and polymerization. Higher temperature accelerates both reactions. Below approximately 10 °C the main mechanism of insulin breakdown is deamidation, but above this temperature polymerization predominates. Deamidation occurs due to progressive loss of $-NH_2$ groups from the glutamine residues at positions 5 and 15, the asparagine residues at positions 18 and 21 in the A-chain of insulin and asparagine residue at position 3 and the glutamine residue at position 4 in the B-chain of insulin. These moieties have no effect on the potency and immunogenicity of the finished product. So deamidated insulin shows almost the same potency as insulin itself [3].

In fact due to complexity of proteins, no single analytical method can detect all possible chemical, physical, and immunological changes in the protein structure. Thus, several analytical techniques such as electrophoresis, spectroscopy, chromatography, thermal analysis, immunoassays, and bioassays may be required to completely characterize a protein and examine its degradation profile [4].

Among these methods, HPLC is a very powerful technique for purity determinations of proteins. The introduction of HPLC to the analysis of peptides and proteins some 25 years ago revolutionised the biological sciences by enabling the rapid and sensitive analysis of peptide and protein structure in a way that was inconceivable before [5].

Since 1978 several RP-HPLC systems for analysis of insulin have been described [6–27]. RP-HPLC is the most commonly used mode of analysis for peptides and proteins. In this method, the hydrophobic interactions between the column packing and the hydrophobic regions of the protein are exploited [4]. Most of these RP-HPLC determinations of insulin controlled the degree of ionization on insulin by using a mobile phase with low pH and high salinity. An alternative approach to get good peak shape and reproducible retention time with better resolution is to use an ion-pairing reagent. However, some of these methods suffer to resolve adequately insulin

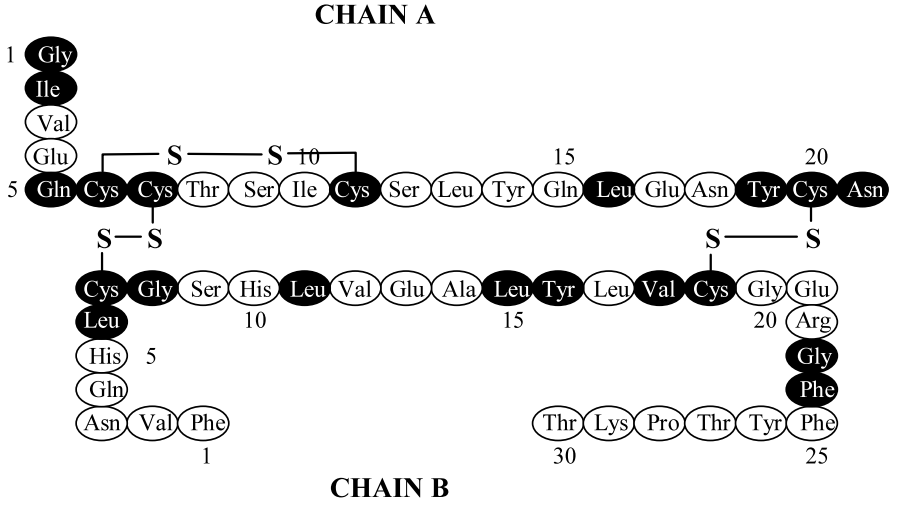

Fig. 1. The primary structure of human insulin. The black residues designate the amino acids which are invariant among species of insulin.

from its desamidated form, because of inadequate selectivity or efficiency [6–9]. Some are time consuming and retention time of insulin related peak is too long [10–13]. Using of a special column or column temperature controller which are not popular in most of the analytical laboratories, is another limitation [6,10,14–18].

In this study a simple and specific method for simultaneous analysis of human insulin and its main decomposition product, A21-desamido insulin, has been developed by using of an ion-pair RP-HPLC technique which is easily available in most of laboratories.

2. Materials and methods

2.1. Materials

Tetrametylammonium hydroxide 10% solution (Merck, Germany), HPLC-grade acetonitrile (Merck), phosphoric acid (Merck), and hydrochloric acid (Merck) were of analytical reagent grade. Samples of crystalline Human insulin (Lot. No. 038EX9, Lilly, France) and Human insulin injections (Batch No.7907006) were kindly denoted by Lorestan Pharmaceautical Co. (Lorestan, Iran).

2.2. HPLC technique

The HPLC apparatus consisted of a model 510 HPLC pump, 486 tunable absorbance detector and 746 data module (Waters; Milford, MA). The RP-HPLC analysis was performed using a Waters Assoc. μBondapak C18 (average particle size 10 μm) column (300 mm × 3.9 mm).

The mobile phase consisted of 3.9% tetrametylammonium hydroxide solution in water and acetonitrile (70:30, v/v), pH 2.5 adjusted by phosphoric acid, filtered through a 0.22 μm membrane filter and degassed prior to use. The column effluent was monitored at 214 nm (AUFS = 1). The flow rate of 1 ml/min and the sample size of 20 μl were carried out at room temperature all over the study.

2.3. Standard curve

Crystalline insulin was dissolved in 0.01 M HCl to obtain concentrations of 10, 20, 40, 60, 80 and 100 g/ml. Prepared samples were injected directly to HPLC column ($n = 3$). Standard curve constructed using peak areas versus known concentrations of insulin. The resulting regression line data were used to determine the concentration of the samples.

2.4. Sample preparation

The injection formulation, containing 3.8 mg/ml human insulin, was diluted with 0.01 M HCl to achieve an appropriate concentration.

2.5. Analysis validation tests

2.5.1. Intra- and inter-day variations

All of the concentrations used for construction of standard curve were prepared as six replicates and analyzed in one day or 6 different days in order to evaluate intra-day or inter-day variations respectively. Then, the coefficients of variations (CV%) of responses were calculated in each case.

2.5.2. Absolute recovery

Three samples from each of the concentrations used for construction of standard curve were prepared and the concentration of insulin in each sample was determined using standard curve. Then, the percent ratios of measured concentration to known added concentration were calculated in each case.

2.5.3. Limits of detection and quantitation

20 successive injections of 0.01 M HCl (blank) were carried out and the average areas of noise peaks for each of the samples were determined. Then, the S.D. of the peak areas was calculated and multiplied by 3 and 10 to obtain limits of detection and quantitation, respectively. To validate the estimated values, six samples of each of the calculated concentrations were prepared and analyzed.

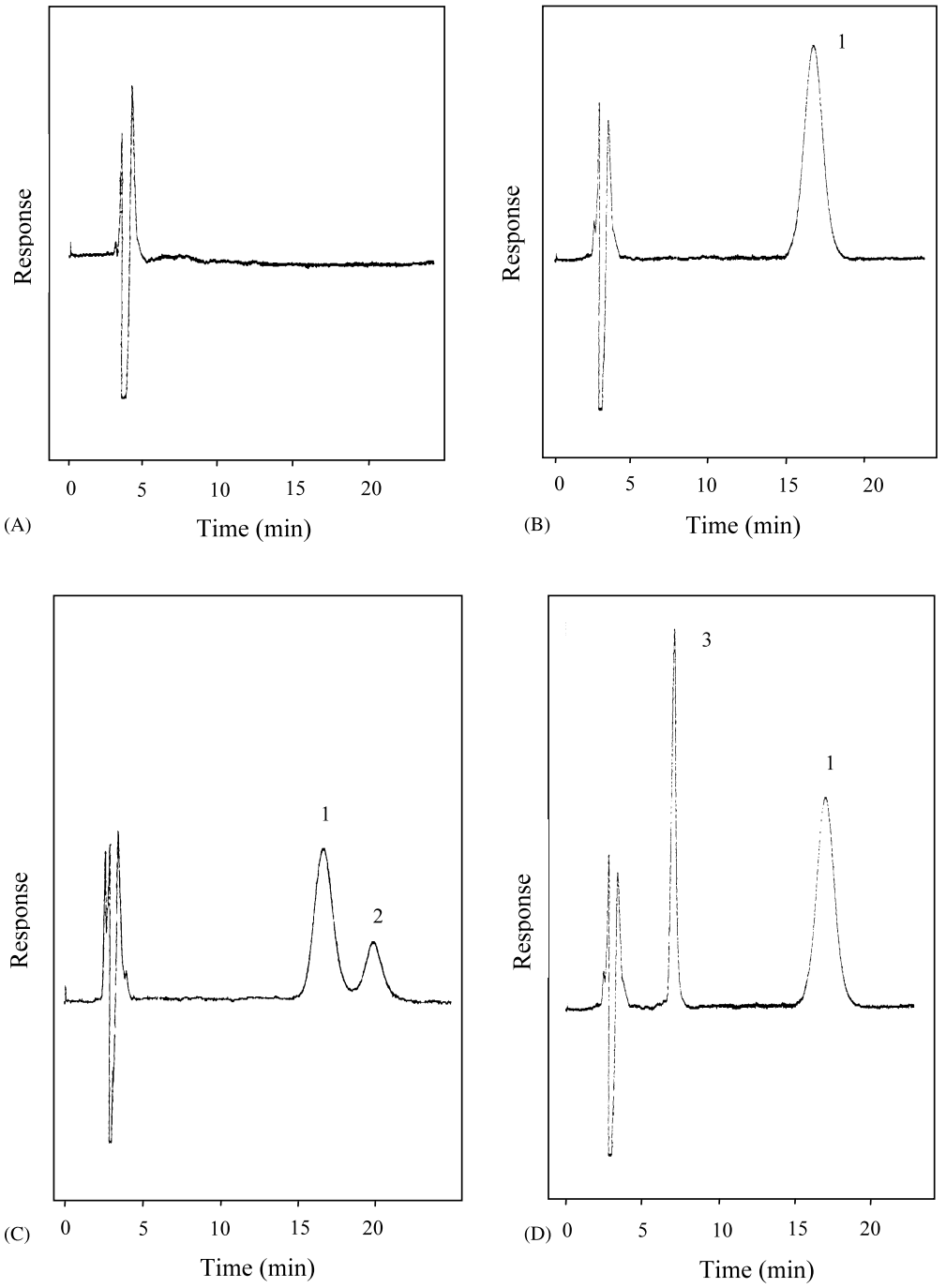

Fig. 2. Chromatograms of: HCl 0.01 M as blank [A], human insulin [B], human insulin and A21-desamido insulin [C]; *m*-cresol and human insulin [D]. Peaks: 1, human insulin (16.76 min); 2, A21-desamido insulin (19.99 min); 3, *m*-cresol (7.01 min).

2.5.4. Stability

To evaluate the stability of samples during analysis time and also upon storage for a limited time, samples with concentrations of 10, 40 and 100 μg/ml of insulin were analyzed immediately after preparation as well as after 12, 24 and 72 h in room temperature. Then the percent ratios of concentrations determined in each case to known added concentrations were calculated.

3. Results and discussion

3.1. Method development

The method is based on separation of insulin and A-21 desamido insulin on a hydrophobic packing (alkyl silica) by a hydroorganic eluent containing a cationic ion pair reagent. It was claimed that the small tetraalkylammonium ion could block the residual OH groups of the reversed phase packing and also possibly act as an ion-pairing agent interacting with the carboxylate groups of insulin [28]. Several parameters such as composition of mobile phase, flow rate and detection wavelength were evaluated for their effect on location and shape of the insulin and its related desamido form. A-21 desamido insulin was prepared by storing human insulin in 0.01 M HCl at 40 °C for 48 h.

Considering the final optimized method, a mobile phase consisting of 3.9% tetrametylammonium hydroxide solution in water and acetonitrile (70:30, v/v), pH 2.5 adjusted by phosphoric acid, and flow rate of 1 ml/min, the retention times for human insulin and A21-desamido insulin were 16.76 and 19.99 min, respectively. Fig. 2 shows a typical chromatogram. It should be noted that the retention times varied considerably unless the relative content of acetonitrile in the mobile phase was carefully kept constant, but slightly changes in pH or the concentration of tetrametylammonium hydroxide were not critical. These observations are similar to those reported by Terabe [9].

Insulin injections like other multiple dose injection formulations contain preservative(s). Many preservatives have characteristics that interfere with analytical methods that work extremely well for the protein in their absence. For example, many preservatives exhibit high ultraviolet adsorption, making it necessary to have analytical techniques capable of separating the protein active from the preservative. Ideally, it would be desirable to have quantitative separation methods capable of resolving the preservative(s) from the protein with no interfering interactions from any of the components. Under the conditions described, formulations containing *m*-cresol (as a preservative) may be analyzed without special preparation. When an acetonitrile concentration of 30% was used, *m*-cresol was eluted well before insulin and did not interfere with the analysis (Fig. 2), however, in the absence of preservatives the acetonitrile concentration could be raised to 33% to allow a more rapid separation.

Table 1
Between- and within-run variations of the HPLC method for determination of insulin[a]

Concentration (μg/ml)	Within-run CV[b] (%)	Between-run CV (%)
10	1.39	6.20
20	2.59	4.66
40	1.83	5.59
60	0.88	4.79
80	0.40	4.61
100	0.62	5.58
Mean	1.29	5.24

[a] $n = 6$.
[b] Coefficient of variation.

Table 2
Absolute recovery of the HPLC method for determination of insulin[a]

Added concentration (μg/ml)	Measured concentration (μg/ml)	Recovery (%)
10	9.55	95.50
20	19.42	97.10
40	38.79	96.98
60	58.30	97.17
80	78.26	97.83
100	96.85	96.85
Mean ± S.D.		97.35 ± 1.36

[a] $n = 3$.

Table 3
Sample stability in the room temperature and analysis condition[a]

Concentration (μg/ml)	Recovered (%)			
	Fresh sample	12 h sample	24 h sample	72 h sample
10	95.06±1.32[b]	93.40±1.81	87.97±1.13	79.30±1.47
40	96.73±1.77	94.77±1.88	89.53±1.83	78.59±1.15
100	99.33±0.62	96.93±2.67	89.98±0.80	81.33±1.19

[a] $n = 6$.
[b] Mean±S.D.

3.2. Method validation tests

3.2.1. Linearity

The peak area for human insulin varied linearly with amount injected over the range 10–100 μg/ml (slope 42 673, intercept −46 213, standard error of slope 374, standard error of intercept 29 142, correlation factor 0.9997). Since higher concentrations cause overlapping of insulin and A21-desamido insulin related peaks, it is recommended to dilute the injection test samples to desired concentration.

3.2.2. Intra- and inter-day variations

The resulted variations of the method through the concentration range of 10–100 g/ml are summarized in Table 1. These data indicate a considerable degree of precision and reproducibility for the method both between and within analytical runs.

3.2.3. Absolute recovery

The mean absolute recovery values of the method are shown in Table 2.

3.2.4. Limits of detection and quantitation

The limits of detection and quantitation of the method were 0.25 and 0.75 μg/ml, respectively. The CV% values of the peak heights of six successive injections in each case were 7.58 and 5.69, respectively.

3.2.5. Stability

Results of the stability of different insulin concentrations at room temperature are shown in Table 3. About 10% decrease in concentration of samples after 24 h storage at room temperature was observed, but it was negligible after 12 h. Therefore, it is recommended to use freshly prepared samples.

As the results show the method is remarkably accurate and this ensures obtaining reliable results.

4. Conclusion

The most remarkable advantages of this method are: (1) simultaneous analysis of insulin and A-21 desamido insulin; (2) the wide range of linearity with considerable accuracy and precision; (3) appropriate resolution and reproducibility and (4) the lack of need for particular requirements such as column temperature controller. Therefore, the developed RP-HPLC system can be used successfully in evaluation of formulations as well as bulk insulin.

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