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Short communication

Selective stability-indicating high-performance liquid chromatographic assay for recombinant human regular insulin

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Abstract

This report presents a selective HPLC assay capable of separating recombinant human regular insulin from insulin decomposition and transformation products. The assay utilizes an isocratic delivery of mobile phase, a C_{18} peptide column, UV detection and is performed at ambient temperature. The standard curve ranges from 0.2 to 2.5 U/ml. The inter-day and intra-day variabilities are less than 7 and 5%, respectively, at the concentrations studied. The accuracy and precision are within 5% over the range of the standard curve.

1. Introduction

Insulin, a pancreatic peptide hormone, is utilized for the treatment of insulin dependent diabetes mellitus. The currently accepted method of determining the potency (i.e., concentration) of insulin in a given preparation is based on biological assays [1]. Modern analytical assay methodologies such as HPLC have not been commonly employed for the analysis of insulin. Some investigators [2,3] have demonstrated that HPLC methods correspond well with the mouse blood glucose method and the rabbit bio-assay for determining the concentration of insulin. HPLC has also been used to study the effect of sodium hydrogensulfate on insulin in solution [4]. In addition, the purity of various insulin preparations has also been studied with the aid of HPLC [5]. Recently HPLC has been applied to determine the concentration of insulin in various preparations [6]. Although HPLC has been utilized in these studies, there have been several problems associated with the published assays. Retention times of 25 min [4] and 40 min [7] for the compound of interest may be considered excessive when it is necessary to analyze multiple samples. In addition, one published method supplied no documentation of the assay utilized in their studies [4]. Furthermore, no researcher has established the stability-indicating nature of the assays utilized. Documented stability-indicating HPLC methods utilized for the assay of insulin have been lacking in the literature. The movement toward non-animal testing whenever possible may encourage in vitro methods of stability testing such as HPLC.

The purpose of this paper is to present an HPLC assay that is capable of separating recombinant human regular insulin from insulin breakdown and transformation products. Insulin in

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aqueous solutions is treated with heat, freezing, fluorescent light and acidic or basic solutions to cause the formation of breakdown products. The method presented is based on isocratic delivery of solvent, UV detection, and has short retention times.

2. Materials and methods

2.1. Materials

All organic solvents employed in this assay were B + J ChromPure HPLC solvents (Burdick & Jackson solvents; Baxter, Muskegon, MI, USA). The water utilized for the mobile phase was glass distilled, treated with ion-exchange resins, charcoal filtered, and stored under UV light. Benzoic acid and KH_2PO_4 were analytical grade compounds (Mallinckrodt, Paris, KY, USA). Recombinant human regular insulin (Humulin R) in both injectable form and analytical dry powder were generous gifts from Eli Lilly & Co. (Indianapolis, IN, USA).

2.2. Instrumentation and chromatographic conditions

The HPLC system utilized for this assay consisted of a 25 cm × 0.46 cm column packed with a octadecylsilane-coated 5-μm silica particle matrix with a 300 Å pore size for the separation of proteins and peptides (Vydac); a Spectra-Physics Isochrom LC pump, a Spectra Focus detector, a SP8880 autosampler, and a ChromJet integrator (all from Spectra-Physics, San Jose, CA, USA).

The chromatographic separation performed in this assay was based on a mobile phase consisting of 0.05 *M* KH_2PO_4 (pH 2.4)–CH_3CN (75:25, v/v) and was delivered at 1 ml/min. A detection wavelength of 230 nm was chosen. Although this wavelength does not maximize sensitivity, it does eliminate some interferences associated with the injectable form of Humulin R. These interferences were not seen with solutions made from insulin powder.

Insulin standards were made from injectable Humulin R solution. The concentrations of the standards for the standard curve performed on this series of experiments ranged from 0.2 to 2.5 Units/ml. A comparison of analytical recombinant human regular insulin powder with the Humulin R injection solution shows that 1 Unit of insulin is equal to 0.035 mg of analytical powder. A standard curve was performed during each analytical run. In addition to the standards, a zero control (blank) and accuracy controls (spiked standards) were analyzed during each analysis. The spiked standards were prepared separately by a third person to concentrations unknown to the HPLC operator. All standards, controls, and unknowns were analyzed in duplicate and the values averaged. Benzoic acid (50 μg/ml) in water was used as the internal standard. Internal standard (50 μl) and 250 μl water were injected into duplicate screw capped test tubes containing 100 μl of standard, control, or unknown. The tubes were vortexed briefly to achieve complete mixing and a portion (50 μl) was injected onto the HPLC column for analysis. The concentrations of unknown solutions were determined by back calculating from the best fit line as established by linear regression based on the standard concentrations and the insulin/internal standard peak area ratios.

2.3. Stability-indicating determination

In order to determine that an assay is stability-indicating, it is necessary to subject the compound of interest to extreme conditions that will cause the decomposition of the compound. The assay must then demonstrate the ability to separate the parent compound from its decomposition products. Separate 0.5 U/ml solutions of Humulin R were subjected to one of the following conditions for 72 h: (1) freezing temperatures (−15°C); (2) heat (75°C); (3) three cycles, 24 h per cycle, of freezing and heating to 75°C; (4) acid pH < 1; (5) alkaline pH > 13 and (6) room temperature in darkness or in exposure to fluorescent lighting 10 h per day. In addition, a 0.5 U/ml solution of Humulin R was refrigerated at 4°C for seven days. Humulin R concentrations were determined in triplicate samples of each solution after 48 and 72 h. A decrease in

Humulin R/internal standard peak height ratio of 10% [8] is considered a significant decrease in parent compound. Although, ideally, a stability-indicating assay would be capable of detecting the entire realm of decomposition products, in the case of a large polypeptide or protein such as insulin, many products may not be detectable within the same chromatographic conditions designed to monitor the parent compound. The peak of the parent compound should not have an appreciable distortion of peak shape or alteration in retention time as a result of exposure to conditions designed to alter it.

2.4. Assay validation

Intra-day variation was calculated from the average concentration and standard deviations of seven spiked standards assayed on the same day. Inter-day variation was calculated from the average concentration and standard deviation of each spiked standard determined in five consecutive chromatographic analyses. Accuracy and precision were determined from five consecutive chromatographic assays by comparing calculated versus actual concentrations of standards spanning the range of the standard curve. The means, standard deviation, percent relative standard deviation and percent difference between means were calculated to validate the assay.

3. Results

The ambient, heating block, and freezer temperatures were monitored hourly for 10 h per day for three consecutive days. The average room temperature was 22°C ± 1.0°C, the average temperature of the heating block was 75°C ± 0.5°C, and the average freezer temperature was −15°C ± 0.5°C. Table 1 shows the effects that the various treatments had on insulin peak area. Insulin proved to be stable [9] for 72 h under these separate conditions: room temperature in the presence or absence of fluorescent light, freezing (−20°C), and in the presence of 2 *M* HCl. There was no significant (>10%) decline in insulin exposed to 75°C for 72 h. However, there was the formation of small amounts of compounds not seen in the control samples. It was found that three cycles of freezing and heating (75°C) a sample within 72 h resulted in a decrease of insulin by 11.8%. The treatment that resulted in the greatest decrease of insulin was 72-h exposure to 2 *M* NaOH. Chromatograms of insulin exposed to several of these conditions are shown in Fig. 1. The chromatograms demonstrate the ability of this assay to separate degradation and transformation products of insulin from the parent compound.

The intra-day and inter-day variability of the assay at the concentrations 0.47 and 2.27 U/ml are presented in Table 2. The intra-day vari-

Table 1
Stability-indicating nature of insulin assay

Treatment	Mean peak area	Change in peak area (%)
Original solution ($n = 5$)	11 237 737 (S.D. 493 818, R.S.D. 4.39%)	
Refrigerated (4°C, 7 days)	11 305 840	+0.6
Three cycles −15°C to 75°C	9 911 174	−11.8
2 *M* NaOH (72 h)	347 688	−96.9
2 *M* HCl (72 h)	11 055 598	−1.6
Freezing 15°C (72 h)	11 301 120	+0.6
Heating 75°C (72 h)	11 576 628	+3.0
25°C with light (72 h)	11 018 273	−2.0
25°C without light (72 h)	11 292 986	+0.5

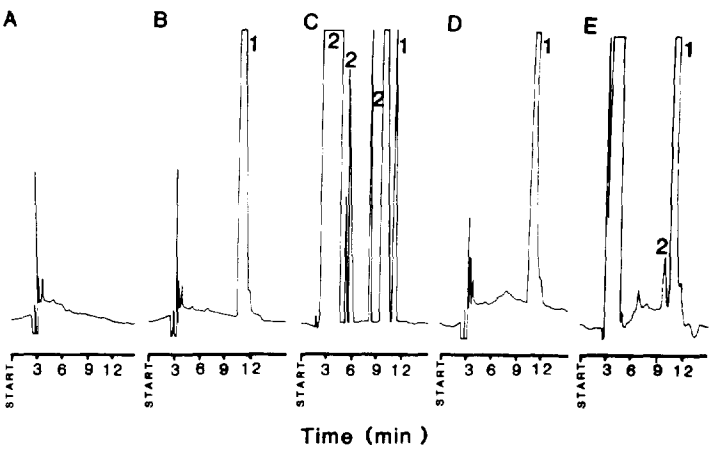

Fig. 1. Chromatograms of (A) a 50-μl injection of water; (B) a 50-μl injection of insulin (0.5 U/ml) control solution (note that the early peaks are similar to A); the peak at 11.16 min represents insulin; the shoulder on the downslope of the insulin peak and the small peak at 7.21 min may represent contaminants of the original solution; (C) a 50-μl injection (0.5 U/ml) of insulin incubated in 2 *M* NaOH for 48 h [note the reduced size of the peak at 11.10 min (insulin) and the appearance of peaks not seen in the previous panels]; (D) a 50-μl injection of insulin (0.5 U/ml) control solution; (E) a 50-μl injection of insulin (0.5 U/ml) heated at 75°C for 48 h (note some breakdown products, the quantitated amount of insulin remaining was >98% of controls). Peaks: 1 = insulin; 2 = insulin breakdown products.

ability for the two spiked sample concentrations was calculated using the concentration of seven samples determined during the same analytical operation. The intra-day relative standard deviations for the 0.47 and 2.27 U/ml spiked samples were 4.2 and 3.2%, respectively. The inter-day variability of each concentration was calculated from the concentration of insulin in the spiked samples as determined by five analyses performed on consecutive days. The inter-day relative standard deviations were 6.4 and 4.2% for the 0.47 and 2.27 U/ml spiked samples, respectively.

Table 2
Intra-day and inter-day variability of insulin assay

Spiked concentration (U/ml)	Mean calculated concentration (U/ml)	S.D.	R.S.D. (%)
Intraday variation (n = 7)			
0.47	0.43	0.02	4.2
2.27	2.09	0.07	3.2
Interday variation (n = 5)			
0.47	0.50	0.03	6.4
2.27	2.23	0.05	4.2

The precision and accuracy of the assay as calculated utilizing the actual and calculated concentrations are shown in Table 3. The accuracy of the assay is good throughout the range tested. The percent difference in mean concentrations (actual concentration versus the calculated concentration) does not exceeded 5% for any concentration. The precision of the assay as estimated from the relative standard deviation is less than 5% for all concentrations. The low variation seen in the precision and accuracy of the assay may be related to the fact that these data are determined from the standard curves where as the intra-day and inter-day assay variability were determined with standards prepared by an independent laboratory.

4. Discussion

Previously published HPLC assays have not been documented as stability-indicating as determined by the rigorous methods suggested by Trissel [8]. This assay is stability-indicating as

Table 3
Precision and accuracy ($n = 5$) of insulin assay

Actual concentration (U/ml)	Mean calculated concentration (U/ml)	S.D.	Difference between means of actual and calculated concentrations (%)
0.2	0.21	0.01	5.0
0.5	0.50	0.01	0.1
1.0	1.00	0.01	0.0
1.5	1.50	0.01	0.2
2.0	2.00	0.01	0.2

demonstrated by its ability to separate recombinant human regular insulin from products formed under various stressful conditions such as 2 *M* NaOH, and repeated freezing and heating. The assay exhibits low variability in that both inter-day and intra-day variation are lower than 6.5% at the concentrations utilized. In addition, the accuracy and precision established for this assay are good.

Fig. 1 shows the separation of insulin and products formed from recombinant human regular insulin under stressful conditions. The products formed have not been identified. All the peaks on the chromatograms display symmetrical peak shape and demonstrate minimal tailing. Several of the peaks seen in chromatogram B have longer retention times than insulin and may represent the insulin transformation products monodesamido insulin and insulin dimer [10]. The late-eluting peaks seen after repeated freezing and heating do not coincide with those obtained after alkaline treatment. It cannot be excluded that these products are also formed during other treatments but at concentrations below the detection limit. In order to assure the ability to detect degradation products of insulin, the chromatographic run was extended to 30 min. However, in the presentation of Fig. 1 the length of the chromatograms has been restricted as degredation products were not detected past a retention time of 15 min.

We have presented an HPLC assay that has been demonstrated to be stability-indicating for recombinant human regular insulin as defined by Trissel. The assay has been validated and documented. The assay has some benefits when compared to previously published HPLC assays [2–6]. Of primary importance, this assay is stability-indicating. Additionally, the assay has a relatively short insulin retention time and is based on an isocratic mobile phase. Although this assay was not utilized for quantitation of unknown concentrations of insulin it should be suitable for this application. Previously published studies [5,6] have demonstrated the accuracy of HPLC methods in determining insulin concentration and thereby activity.

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