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PREPARATIVE AND ANALYTICAL SEPARATION OF INSULIN-DEXTRAN CONJUGATES FROM NATIVE INSULIN: APPLICATION TO PREPARATION AND CHARACTERIZATION OF INSULIN-DEXTRAN CONJUGATES

Reza Mehvar
College of Pharmacy and Health Sciences, Drake University, Des Moines, IA, USA

ABSTRACT

A high-performance size exclusion chromatographic (HPSEC) method was developed for analysis and separation of insulin-dextran conjugates and native insulin. The separation is achieved on a size-exclusion analytical column with a mobile phase of 0.05 M phosphate buffer (pH 7.0): acetonitrile (80:20, v/v) delivered at a flow rate of 0.5 ml/min. The conjugates are detected using a UV detector at a wavelength of 280 nm, based on the UV absorbance of the attached insulin. Under these conditions, the conjugates of insulin with dextrans with MWs of 500 kD, 70 kD, and 40 kD eluted at 4.3, 4.6, and 4.9 min, respectively. The conjugates were resolved from the native insulin which eluted at 6.3 min. Additionally, a method for preparative separation of the conjugates from the native insulin was developed. The method is based on a glass column packed with silica gel powder and elution of the conjugates with distilled water in less than 80 min.

The application of these methods to preparation and characterization of conjugates of insulin with various MWs of dextrans

was also demonstrated. The conjugates were prepared using the periodate method and the reaction was monitored using the HPSEC method. After 72 h of reaction, the insulin content of the conjugates (w/w) were independent of the MW of dextrans and ranged from 20.2 to 22%. Reaction samples containing 44-50% of native insulin were purified using the preparative columns, resulting in less than 5% free insulin impurity in the final samples.

INTRODUCTION

Dextrans are glucose polymers which have been suggested as vehicles for delivery of drugs and hormones (1). Two major applications for dextrans have been to increase the blood longevity of therapeutic agents with short half-life (2-3) and to target drugs to certain organs or tissues (4-5).

Covalent conjugates of dextrans and insulin have been previously prepared and used for studies of insulin action and determination of the cellular location of insulin receptors (6-8). Further, a study by Torchilin et al. (9) indicated that the hypoglycemic effects of Sephadex-bound insulin was much longer than that of native insulin after iv administration of the products to dogs. Insulin-dextran conjugates may also have other utilities in delivery of insulin because they can potentially alter the biopharmaceutic characteristics of insulin, and, therefore, change the tissue distribution of the hormone.

Previous studies using insulin-dextran conjugates (7-9) have used gel chromatography for preparation and characterization of the conjugates. These methods are lengthy and not suitable for analytical separation of larger sample numbers. In this communication, a high-performance size exclusion chromatographic (HPSEC) method is reported for separation and measurement of insulin-dextran conjugates in samples containing free insulin. Additionally, a new preparative method for separation of the conjugates from free insulin is described. The methods have been applied to preparation and characterization of the conjugates of insulin with various molecular weights of dextrans.

MATERIALS AND METHODS

Materials

Insulin powder from bovine pancreas, dextrans with average MWs of 39,100 (40 kD), 72,600 (70 kD), and 503,000 (500 kD), sodium m-periodate, and sodium borohydride were obtained from Sigma Chemical Co. (St. Louis, MO). Silica gel powder (60-200 mesh) was purchased from J. T. Baker (Phillipsburg, NJ). For chromatography, HPLC grade acetonitrile (Mallinckrodt, Inc., Paris, KY) was used. All other reagents and solvents were analytical reagent grade.

High-performance size exclusion chromatography

The HPLC instrument (Waters; Milford, MA) consisted of a 501 pump, a 712 Wisp autosampler, a 484 variable wavelength UV detector, and a 745 Data Module. The detector was set at a wavelength of 280 nm. Insulin-dextran conjugates were separated from native insulin at ambient temperature utilizing a 4.6 mm x 25 cm analytical column containing 5 μm size exclusion packing material (Hydropore-5-SEC, Rainin Instrument, Woburn, MA) attached to a Mini-Cartridge (4.6 mm x 1.5 cm; Rainin) of the same packing material as a guard column. The mobile phase consisted of 0.05 M phosphate buffer (pH 7.0): acetonitrile (80:20) delivered at a flow rate of 0.5 mL/min.

Peak areas were used for determination of the concentration or amount of free or dextran-conjugated insulin in the sample.

Preparative chromatography

Sephadex of various sizes (G-25, G-75, and G-150) and silica gel were used as packing materials for construction of columns to separate unreacted insulin from insulin-dextran conjugates after reacting dextrans with insulin described below. The optimum separation was achieved using silica gel material. The column (48 x 1.5 cm) was packed with 60-200 mesh silica gel, and the sample components were

eluted using distilled water at a flow rate of 1 mL/min. Before applying the sample to the column, 4 ml of a 0.1 M solution of monobasic potassium phosphate was added to 5 ml of the sample to adjust its pH to ~7. The fractions were collected every 2 min using a FRAC-100 Fraction Collector (Pharmacia Fine Chemicals, Sweden), and an aliquot of 3-5 μl was injected into the HPSEC for determination of the contents of free insulin and insulin-dextran conjugates in the fractions.

Preparation of insulin-dextran conjugates

The conjugates of insulin with dextrans were prepared after oxidation of dextran according to the periodate method (1). Briefly, 2 g of each dextran was dissolved in 10 ml of distilled water, and 200 mg of sodium periodate was added to convert the dextrans to dextran aldehyde. The solution was left at room temperature in the dark for 24 h. Afterward, the resultant dextran aldehydes were dialyzed for 24 h against 1 L of distilled water using a dialysis membrane with molecular weight cut-off of 12,000-14,000. The dialysate was replaced with fresh distilled water at 12 h. The samples (approximately 20 mL) were then freeze-dried and kept at -20 ^{o}C until further use.

For preparation of conjugates, 200 mg of each dextran aldehyde was dissolved in 10 ml of 0.1 M dibasic sodium phosphate (pH 9.2), and 100 mg of insulin was added to the solution. The samples were kept at 4 ^{o}C with constant stirring. Although insulin was not completely dissolved immediately after its addition to the solution, complete solubility was achieved within 12 h. The reaction of insulin with dextran aldehyde was monitored by measurement of the conjugate at 24, 48 and 72 h after the start of the reaction using the HPSEC method described above. After 72 h of reaction, the produced Schiff base was converted to a covalent conjugation by addition of 16 mg of sodium borohydride and leaving the samples at room temperature for 2 h. The formed conjugates were separated from the unreacted insulin using the preparative method described above with silica gel as packing material, and fractions 22-32 were pooled. The conjugates were then freeze-dried and stored at -20 ^{o}C until used for further studies.

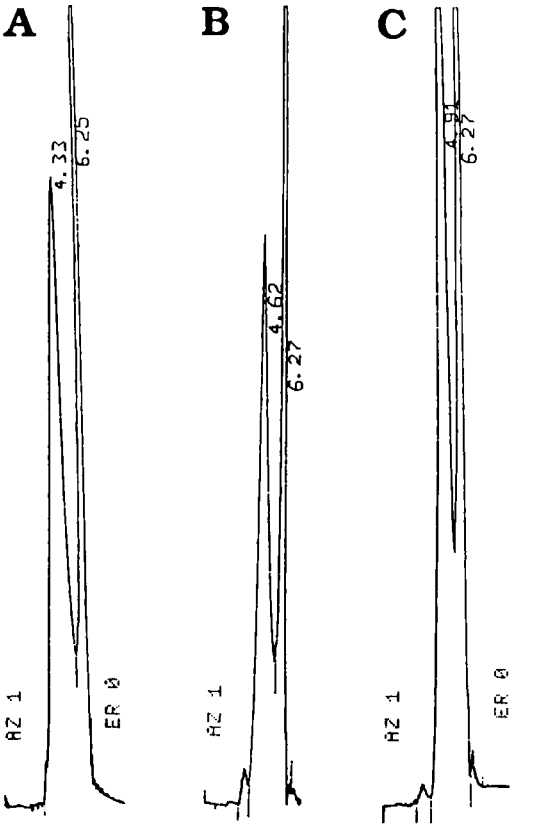

FIGURE 1

High-performance size exclusion chromatograms of insulin-dextran conjugates and free insulin after 72 h of reaction of 100 mg insulin with 200 mg dextran-aldehyde with MWs of 500 kD (A), 70 kD (B), and 40 kD (C). In all chromatograms, the first and second eluting peaks represent the conjugates and the free insulin, respectively. The numbers associated with peaks are retention times in minutes.

Characterization of the conjugates

The insulin content of insulin-dextran conjugates was determined from the area of the conjugate peak compared to that of the unreacted insulin. The MW of the conjugates were estimated from molar ratios of insulin:dextran conjugates and MWs of the parent compounds (dextrans and insulin).

RESULTS AND DISCUSSION

Figure 1 depicts HPSEC chromatograms of samples after 72 h of reaction of insulin with the three MWs of dextrans and further reduction of the formed Schiff bases using sodium borohydride. Under the conditions stated above, the retention times of the conjugates were 4.3, 4.6, and 4.9 min for conjugates of insulin with dextrans with MWs of 500 kD, 70 kD, and 40 kD, respectively. The conjugates were resolved from the native insulin which eluted as a very sharp peak at 6.3 min (Fig. 1).

In the presence of low percentages (0-15%) of acetonitrile in the mobile phase, the native insulin peak eluted at a later time and was broad. Additionally, in the absence of acetonitrile, the retention time and area of the insulin peak would vary substantially from column to column. However, with the use of 20% acetonitrile in the mobile phase, reproducible results were obtained and the insulin peak was very sharp (Fig. 1).

Dextrans are devoid of any chromophore group in their structure. Consistent with this, injection of samples of native dextrans, even at very high concentrations, into the HPSEC did not result in any detectable peak. Therefore, the peak area of the insulin-dextran conjugates could be attributed to the UV absorbance of the attached insulin. Furthermore, preliminary studies indicated that the insulin attached to dextrans would have the same absorbance coefficient as the native insulin; the peak area of the insulin-dextran conjugate plus unreacted insulin was the same as the area for the total native insulin added to the system at time zero. Hence, the insulin content of the conjugates was calculated based on the peak area of the conjugates relative to that of the unreacted insulin. The estimated values at various times after the reaction of dextran aldehydes with insulin are depicted in Figure 2.

As shown in Fig. 2, at 72 h after the reaction of insulin with dextran aldehydes, 100 mg of the conjugates of insulin with 40 kD, 70 kD, and 500 kD dextrans contained, respectively, 20.9, 20.2, and 22.0 mg of insulin. The similar values for the three different MWs of

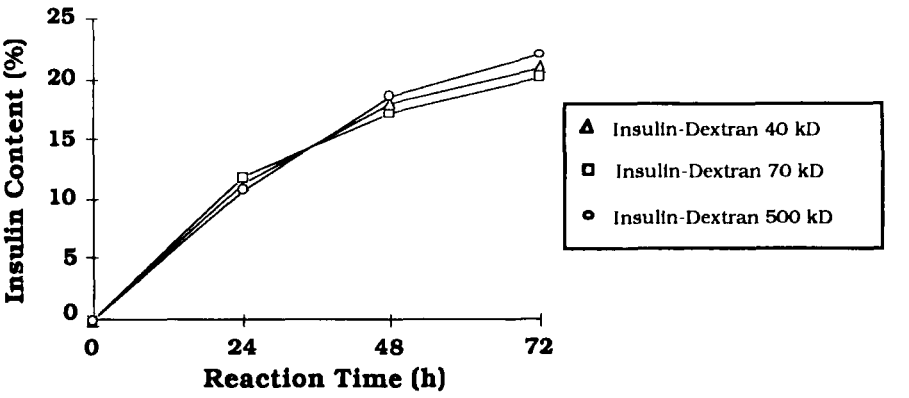

FIGURE 2

Insulin content (w/w) of the conjugates of insulin with different MWs of dextrans as a function of reaction time. Each point is an average of 3 measurements.

dextrans indicate that the conjugation of insulin with dextrans is independent of the MW of dextrans. If expressed in terms of molar ratio, however, the insulin:dextran ratios are 1.77:1, 3.06:1, and 23.9:1 for dextrans with MWs of 40 kD, 70 kD, and 500 kD, respectively. The larger molar ratio for higher MW dextrans is due to the availability of more aldehyde groups for reaction in these dextrans. Based on the above molar ratios, the estimated MWs of the conjugates are 49,100, 90,100, and 639,400 when dextrans with MWs of 40 kD, 70 kD, and 500 kD are used.

The insulin contents of the conjugates in our experiments (~20%) are much higher than those reported (9) for attachment of insulin to modified Sephadex with MWs of 10,000-150,000 (3-6.2%). This difference may be attributed to solubility differences between the carrier used in our study (soluble dextran) and that used in the above investigation (insoluble Sephadex). Furthermore, the reaction of insulin with the modified Sephadex was stopped after 20 h (9), whereas, we allowed the reaction to proceed for 72 h. This is consistent with the data reported in Figure 2, indicating a greater degree of substitution at 72 h, compared with 20 h.

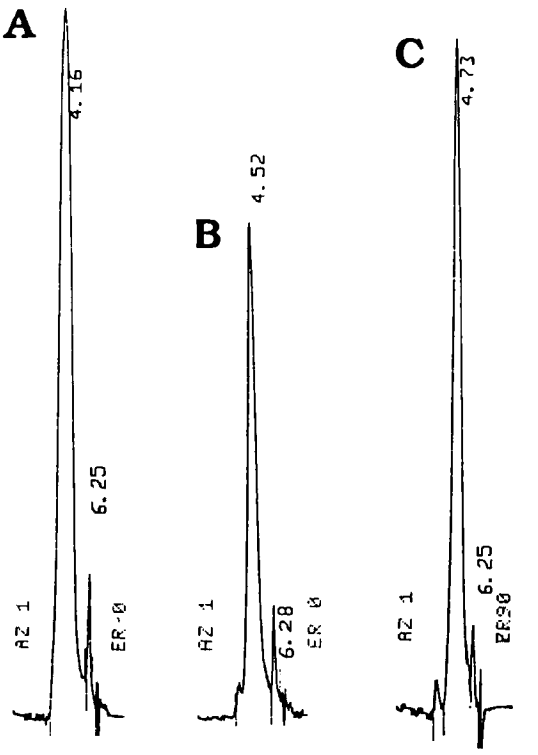

FIGURE 3

High-performance size exclusion chromatograms of pooled fractions after preparative chromatography of the samples depicted in Figure 1 to remove the free insulin. The first eluting peaks in the chromatograms are conjugates of insulin with dextrans with MWs of 500 kD (A), 70 kD (B), and 40 kD (C). The small, second eluting peaks represents the insulin impurities in the samples. The numbers associated with peaks are retention times in minutes.

Gel chromatographic separation of the conjugates of insulin with dextrans (7,8), Sephadex (9), or cellulose (10) has been previously reported using columns packed with Sephadex G-25 to G-150. Using these media, we were not able to satisfactorily separate the native, unreacted insulin from the dextran-insulin conjugates; in some collected fractions, the free insulin accounted for more than 50% of the total insulin. However, when silica gel was used as packing material, the free insulin content of the fractions were consistently less than

10% of the total insulin. High-performance size exclusion chromatograms of the pooled samples (fractions 22-32) after preparative chromatography using silica gel are presented in Figure 3. The free insulin impurities in these samples were only 3.2%, 5.4% , and 2.9% for insulin-dextran conjugates with MWs of 40 kD, 70 kD, and 500 kD, respectively. This is compared to impurities of 47%, 50%, and 44% for the same samples before their application to the preparative column (Fig. 1). Nevertheless, the method offers the advantages of short separation time (<80 min), good separation capability, and low cost.

A comparison of Figures 1 and 3 also indicates that the pooled samples collected from the preparative column (Fig. 3) had shorter retention times (larger MWs) compared with their respective samples before the preparative separation (Fig. 1). This may be due to the fact that only fractions 22-32 eluted from the preparative column were pooled; although, in most cases, the conjugates could be detected in later fractions, because of their low concentrations of the conjugates, fraction numbers ≥33 were not included in the sample pool. Interestingly, when analyzed by HPSEC, the conjugates in these later eluting fractions also showed longer retention times. These data may suggest some degree of size exclusion behavior for silica gel.

CONCLUSION

Analytical and preparative methods for separation and analysis of insulin-dextran conjugates and free insulin were developed. Using the rapid analytical method, the conjugates were separated from free insulin in less than 8 min. Using the preparative method, insulin is retained on the column, while the conjugates elute in a reasonable time (less than 1.5 h). The methods can be applied to *in vitro* studies of insulin-dextran conjugates.

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REFERENCES

1. C. Larsen, Adv. Drug Deliv. Rev., 3, 103 (1989).
2. T. S. Wileman, R. L. Foster, and P. N. C. Elliott, J. Pharm. Pharmacol., 38, 264 (1986).
3. W. H. Ko, C. C. Wong, H. W. Yeung, M. H. Yung, P. C. Shaw, and S. C. Tam, Biochem. Pharmacol., 42, 1721 (1991).
4. H. Sezaki and M. Hashida, Critic. Rev. Ther. Drug Carrier Sys., 1, 1 (1984).
5. C. Larsen, E. Harboe, M. Johansen, and H. P. Olesen, Pharm. Res., 6, 995 (1989).
6. P. Cuatrecasas, Proc. Natl. Acad. Sci., 63, 450 (1969).
7. F. Suzuki, Y. Daikuhara, M. Ono, and Y. Takeda, Endocrinology, 90, 1220 (1972).
8. Y. Sakamoto, Y. Akanuma, K. Kosaka, and B. Jeanrenaud, Biochem. Biophys. Acta, 498, 102 (1977).
9. V. P. Torchilin, E. V. Il'Ina, A. V. Mazaev, B. S. Lebedev, V. N. Smirnov, and E. I. Chazov, J. Solid-Phase Biochem., 2, 187 (1977).
10. M. Singh, P. Vasudevan, T. J. M. Sinha, A. R. Ray, M. M. Misro, and K. Guha, J. Biomed. Material. Res., 15, 655 (1981).