A Rapid Means of Separating A_{14}-^{125}I-Insulin from Heterogeneously Labeled Insulin Molecules for Biologic Studies

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SUMMARY

We have used two methods for the preparation of a highly homogeneous insulin with high specific activity. After iodination with chloramine T, the labeled peptides were retained on a disposable Sep Pak cartridge and subsequently eluted. The eluted labeled insulins were further purified by either DEAE cellulose or high performance liquid chromatography (HPLC) to separate A_{14}-^{125}I- from A_{19}-^{125}I-insulin. Both methods of chromatography were effective, but HPLC offered the advantage of better resolution in less time and higher yields of A_{14}-^{125}I-insulin, which is suitable for biologic studies in various target tissues. DIABETES *31:*1128–1131, December 1982.

In the routine iodination of insulin, random labeling of the four tyrosine molecules occurs, resulting in multiple ^{125}I-insulins with distribution of iodine atoms dependent on the average iodine atoms incorporated per insulin molecule.[1] Studies by Gliemann and co-workers,[2] however, showed that of the four tyrosine molecules in insulin, A_{14} is the most similar to native insulin in regard to biologic activity and properties. Although attempts have been made to purify this material by numerous methods,[3–4] including high performance liquid chromatography (HPLC),[5] the details of these procedures have not been reported and widely disseminated to enable various laboratories to adopt such a procedure for reproducible results.

In this study, we evaluated two methods of column chromatography for separation and purification of the A_{14} species of insulin. The two systems, consisting of disposable Sep Pak cartridge with either HPLC or DEAE cellulose ion-exchange chromatography, resulted in comparable purity and homogeneity of the A_{14} peak. However, on HPLC it was possible to separate and purify the insulin in less than 1 h with resolution of A_{14}-^{125}I- from A_{19}-^{125}I-insulin, while 15–20 h were required for separation of A_{14} insulin by ion-exchange chromatography. A preliminary report on comparative binding data on two species of labeled insulin has also been included in this paper.

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MATERIALS AND METHODS

Highly purified porcine insulin was kindly provided by Dr. Ronald Chance of Eli Lilly Company (Indianapolis, Indiana). Storage of insulin in diluted acid for more than 6 mo led to deterioration of the insulin molecule and subsequent poor yield of tyrosine A_{14}-^{125}I-monoiodoinsulin (A_{14} insulin). Therefore, all insulin was prepared in 0.01 N HCl (1 mg/ml) monthly.

Na ^{125}I (IMS. 30), with a specific activity as indicated by the manufacturer of 14–17 mCi/mg iodine, was obtained from Amersham Company (Arlington Heights, Illinois) immediately as fresh lots became available. Chloramine T from Fisher Scientific (Fairlawn, New Jersey) was stored in the dark and weighed out on the day of iodination. DEAE cellulose (Whatman DE52) was purchased from Bodman Chemicals (Media, Pennsylvania). Trifluoroacetic acid (TFA), sequanal grade, was obtained from Pierce Chemicals (Rockford, Illinois); acetonitrile (ACN), distilled in glass suitable for HPLC, was obtained from Burdick and Jackson Laboratories (Muskegon, Michigan); Sep Pak C_{18} cartridges and μ Bondapak C_{18} (10 μm) liquid chromatography columns for use in conjunction with a Waters HPLC were obtained from Waters Associates (Milford, Massachusetts). Bovine serum albumin (BSA) and all other chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri). A_{14}-^{125}I-insulin (S.A. 225 μCi/μg) was purchased from Novo Research Institute (Copenhagen, Denmark).

Iodination procedure. Insulin was iodinated to 90–110 μCi/μg (i.e., 0.25–0.32 mol iodine/mol insulin) with chloramine T at room temperature by the modified method of De Meyts.[6] Reagents were added in the following order: 40 μl

0.3 M sodium phosphate, pH 7.5; 10 μg monocomponent porcine insulin in 10 μl of 0.01 N HCl; 2 mCi of Na ^{125}I in 16 μl of 0.01 N NaOH, carrier-free; 0.6–1 μg chloramine T (40 μg/ml dissolved immediately before use in 0.3 M sodium phosphate, pH 7.5) added stepwise in 5-μl aliquots over 2 min with agitation using a 50-μl Hamilton syringe through the septum. Sufficient chloramine T was added to incorporate 45–55% ^{125}I into TCA precipitable insulin. Na ^{125}I was a fresh batch used within 3 days of availability.

The final concentrations of reactants were: insulin, 2.0×10^{-5} M; Na ^{125}I, 1.2×10^{-5} M; chloramine T, 4.0×10^{-5} M. At the end of iodination, 0.1 ml of 2.5% BSA (dialyzed overnight against Krebs-Ringer bicarbonate, pH 7.4) was added to the reaction mixture to inhibit adsorption of labeled insulin to the glassware during transfer. Each half of the reaction mixture (90 μl or 1 mCi) was diluted with 2 ml of 0.1% TFA and loaded onto a Sep Pak C_{18} cartridge. The Sep Paks had been prewashed with 5 ml of ACN followed by 2 ml of 0.1% TFA. After loading the sample, each of the Sep Paks was washed with 2 ml 0.1% TFA followed by 2 ml ACN: 0.1% TFA (1:9, vol/vol) to remove BSA, Na ^{125}I, and damaged insulin as assessed by incomplete TCA precipitability. Intact ^{125}I-insulin was eluted from the Sep Pak with three 1-ml aliquots of ACN: 0.1% TFA (1:1, vol/vol). The majority of labeled intact insulin eluted in the first milliliter, which also had the highest TCA precipitability. The labeled insulin was either placed directly onto a reverse phase C_{18} column or lyophilized and then placed on a DEAE cellulose column followed by chromatography of the major peak on HPLC (Table 1).

Ion-exchange chromatography. DEAE cellulose was obtained in a preswollen microgranular condition and suspended in 0.5 M ammonium acetate, pH 9; the column (0.9 × 39 cm) was packed and washed with a suspension buffer. The column was then equilibrated with 0.05 M sodium acetate, pH 9. Iodinated insulin (100–200 μCi) free of Na ^{125}I (accomplished by elution from Sep Pak C_{18} cartridges) was placed on top of the previously prepared DEAE cellulose column. The material was then eluted with a slightly concave gradient of 0.1–1 M ammonium acetate, pH 9, generated by placing 125 ml of 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M buffer in each of the six chambers of a Buchler multichambered gradient maker, at a rate of approximately 0.3 ml/min for 15–20 h at room temperature. The peak fractions (3.8 ml each) of the radioactivity were pooled, BSA was added, and the mixture was then lyophilized and stored at −20°C. Further documentation of A_{14}-^{125}I-insulin was accomplished by rechromatography on HPLC and/or sequencing of the labeled insulin.

TABLE 1
Profile of specific activity of labeled insulin during different stages of purification

Step	Yield (μCi)	Specific activity (mCi/mg)	% TCA precipitable
1. Iodination mixture	1000	100 ± 10	50
2. Sep Pak	800	130 ± 10	96
3. DEAE cellulose	220*	275 ± 25	99
4. HPLC	200	360	99

* A higher yield may be obtained if the third step is omitted and one proceeds directly from Sep Pak to HPLC.

HPLC chromatography. The lyophilized material from the Sep Pak cartridge was resuspended in 100 μl of ACN: 0.1% TFA (34:66, vol/vol) and injected into the two HPLC columns in tandem and eluted isocratically with the above solvent system at a flow rate of 1 ml/min (2000 psi). The chromatography was run for 40 min and 1-ml fractions were collected. The peak of radioactivity was assessed by counting in a gamma spectrometer and concomitant monitoring of eluate absorption at 205 and 254 nm. Authentic monoiodinated A_{14} insulin (Novo) and porcine insulin were used as standards. Identification of the homogeneity of A_{14}-^{125}I-insulin was confirmed by rechromatography in the same solvent system and by sequencing of the radioactive peak material eluting with the same retention time as A_{14}-^{125}I-insulin (Novo) standard on a Beckman Sequencer, Model 890C, using the SLOW PEPTIDE-DMAA (071472) program of Beckman Instruments (Fullerton, California).

Binding and degradation of ^{125}I-insulin. Specific insulin binding of labeled insulin to red blood cells,[7] fibroblasts,[8] and liver cell membranes[9] was assessed by the previously published methods. Degradation studies using 5% TCA solubility to determine the generation of ^{125}I-insulin breakdown products were as described by us earlier.[7]

RESULTS

Controlled iodination of insulin with chloramine T without the use of sodium metabisulfite has proven to be a reliable procedure for preparation of monoiodinated insulin. Prior preparations using sodium metabisulfite resulted in variable yields, variability in the amount of labeled A_{14} insulin, and multiple species of labeled insulin separated chromatographically. Table 1 shows the specific activity and TCA precipitability of the labeled insulin at various steps of purification. Use of the Sep Pak column was a distinct advantage in preparation of samples for column chromatography as this economical, small disposable cartridge removed large- and small-molecular-weight radioactive and nonradioactive materials by selectively absorbing more than 90% of the labeled insulin. Subsequent elution and lyophilization allowed concentration of the ^{125}I-insulin in an appropriate solvent for chromatography. The solvent system selected for elution (ACN: 0.1% TFA 1:1, vol/vol) extracted virtually all the labeled insulin from the Sep Pak cartridge and thus provided an effective and efficient means of preparing the labeled peptide for further purification and/or identification. The same solvent system has also been shown to be effective in isolating native insulin (unpublished results). Other solvents such as ethanol-HCl require larger volumes and are therefore less efficient for isolating small polypeptides in the concentrations needed.[10]

Figure 1 depicts a chromatographic profile of heterogeneously labeled insulin species separated on DEAE cellulose with corresponding peaks for A_{14}- and A_{19}-^{125}I-insulin, Na ^{125}I, and ^{125}I albumin.

On HPLC, A_{14}-^{125}I-insulin had a retention time of 26 min ($K'_1 = 3.3$) and A_{19}-^{125}I-insulin had a retention time of 17 min ($K'_2 = 1.8$) (Figure 2).

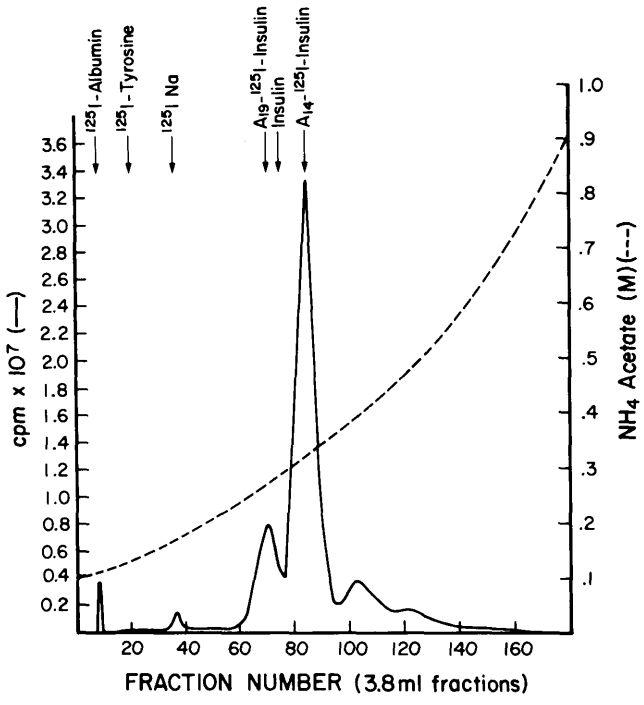

FIGURE 1. DEAE chromatographic profile from a representative insulin iodination. (See METHODS.)

Rechromatography on HPLC of A_{14}-^{125}I-insulin peaks purified by both methods produced a single peak with a retention time of 26 min and no detectable absorbance at 15 min, the retention time of unlabeled insulin, indicating a specific activity of 360 mCi/mg. Specific activity assessed by TCA

FIGURE 2. ^{125}I-insulin chromatograph on HPLC after Sep Pak purification. (See METHODS.)

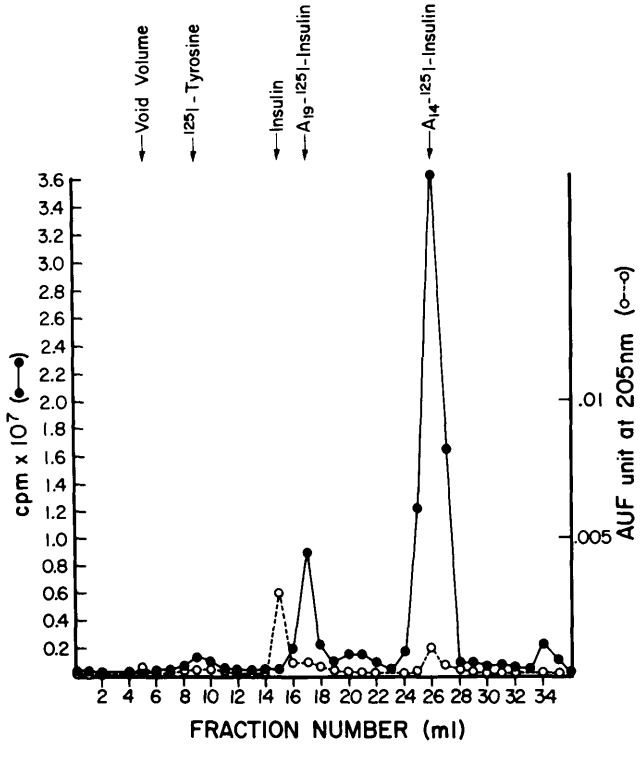

TABLE 2
Binding and degradation properties of A_{14}- and A_{19}-^{125}I-insulin

	% Specific binding of ^{125}I-insulin			
	Red blood cells	Liver membrane	Fibroblasts	Insulin degradation by fibroblasts*
A_{14}-^{125}I-insulin	13	14	4.1	0.20
A_{19}-^{125}I-insulin	6	7	3.1	0.17

* fmole insulin degraded/min/flask at 37°C.

precipitation and radioimmunoassay at each step of purification is given in Table 1 along with the yield of labeled insulin.

^{125}I radioactivity measured after 30 cycles of sequencing of the A_{14}-^{125}I-insulin peak on a Beckman Sequencer was found only in the products of cycle 14, confirming the position of ^{125}I at tyrosine 14.

Table 2 shows results of studies comparing the binding and degradation properties of A_{14}- and A_{19}-^{125}I-insulin isolated by HPLC on three different target tissues. A_{14}-^{125}I-insulin was clearly superior to A_{19}-^{125}I-insulin in these tissues.

DISCUSSION

The use of homogeneously labeled insulin with a high specific activity is desirable since recent work with a heterogeneous mixture of insulins has suggested that the iodinated insulin species exhibit different binding properties depending on the tissues selected.[11] Therefore, the use of a homogeneously labeled insulin that is biologically comparable to native insulin[12] is essential for an accurate estimation of binding kinetics.

Although, theoretically, it is possible to purify other species of insulin labeled at B_{16} or B_{26} positions, the profiles from our HPLC studies suggest that under our experimental condition of chloramine T iodination, very few B_{16} or B_{26} tyrosine molecules are iodinated. However, small amounts of iodination (10–15%) other than A_{14}-^{125}I-insulin could be easily removed with a one-step purification by either DEAE cellulose or HPLC chromatography.

Other methods of iodination such as lactoperoxidase may provide proportionately greater B chain labeling or a different mixture of labeled insulin than the modified method of chloramine T we have used and thus might not be comparable with our purification procedures.

Presentation of a simple procedure for HPLC in combination with the Sep Pak provides a means of labeling and purifying insulin in a few hours. This reproducible method produces a high yield of A_{14}-^{125}I-insulin, which has high specific activity, a long shelf life, and biologic and binding characteristics similar to native insulin.[2,4] In the absence of HPLC, DEAE cellulose can be used with comparable results and parallel biologic activity, but a much longer period (more than 15 h) is needed for resolution of different peaks.

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