*Journal of Chromatography*, 266 (1983) 239–248
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 036

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PREPARATION OF SINGLE-SITE CARRIER-FREE PANCREATIC POLYPEPTIDE HORMONE RADIOTRACERS

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## SUMMARY

The preferred radiotracer for use in radioimmuno- and receptor assays is one which is as similar as possible to the native hormone in both physicochemical and biological properties. Until recently, rapid detailed evaluation of iodination methodology as well as separation of site-specific tracers and their evaluation relative to the native protein has not been possible. Using reversed-phase high-performance liquid chromatographic methodology ($C_8$ or $C_{18}$ columns) we have evaluated a variety of iodination techniques and conditions. Procedures were found that allow the isolation of site-specific radiolabelled protein hormones in a rapid, reproducible and quantitative manner. The radiotracers are of the highest possible specific activity, have very low levels of damage and are more stable than tracers prepared by conventional techniques. This methodology has been applied to preparing tracers of beef, pork and human insulins, proinsulins and C-peptides as well as analogues of these proteins. In addition, the methodology has been applied to somatostatin, glucagon, pancreatic polypeptide, vasoactive intestinal peptide and luteinizing hormone-releasing hormone. The use of these tracers yielded increased sensitivity and reproducibility in a number of radioimmunoassays (*e.g.* C-peptide and glucagon), more reproducible results in radioreceptor assays (*e.g.* insulin receptors) and more defined studies on drug absorption and degradation. Finally, scale-up of our isolation procedures has yielded the $^{127}$I-analogue for homologous binding and displacement studies.

## INTRODUCTION

The use of radioiodinated peptides and proteins in biochemistry is widespread. These modified forms of hormones have utility in radioreceptor assays[1], in radioimmunoassays[2], in drug absorption and degradation studies and in isolation of hormone receptors, as well as many other specific applications. For these applications, the desirability of chemically defined radiotracers of the highest specific activity is obvious. Thus the development of a technique that allows the rapid isolation of specific radiolabels, free from contamination with unlabelled hormone, is very desirable. A number of different techniques, including for example polyacrylamide gel electrophoresis[3] and ion exchange chromatography[4], have been used previously to

prepare specific radiotracers. These methods, however, are not generally applicable and are usually quite laborious. We have undertaken the investigation of the application of high-performance liquid chromatography (HPLC) to this problem, particularly with respect to the pancreatic hormones. This communication describes a general HPLC method that can be used to define the chemistry of the iodination reaction and to rapidly isolate reaction products that are site-specific radiotracers which are free of unlabelled hormone. In addition, the tracers are of high specific activity, are more stable than conventially prepared radiotracers and exhibit very low levels of radiodamage in radioimmunoassays. We have applied this approach to a spectrum of pancreatic polypeptides including insulins, proinsulins, C-peptides, glucagon, somatostatins and pancreatic polypeptide.

## EXPERIMENTAL

All of the proteins used in this study were prepared in the Lilly Research Laboratories either by isolation from pancreatic sources[5], by solid-phase peptide synthesis[6], or by utilizing recombinant DNA technology[7]. Chloramine T (N-chloro-*p*-methylbenzenesulfonamide) was purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.), lactoperoxidase was obtained from Calbiochem (Los Angeles, CA, U.S.A.) and crystalline human serum albumin was purchased from Sigma (St. Louis, MO, U.S.A.). The carrier-free Na $^{125}$I (IMS 300) was purchased from Amersham International (Amersham, Great Britain). All other reagents including HPLC solvents were either analytical or HPLC grade and were used without further purification.

### *Iodination procedures*

All solutions of reagents were freshly prepared. Insulin solutions (1 mg/ml) were prepared by dissolving pork insulin in 0.06 *M* potassium phosphate buffer (pH 7.0). The *tert.*-butoxycarbonyl-L-tyrosine (t-BOC-tyr) human C-peptide was dissolved in the same solvent at a peptide concentration of *ca.* 0.20 mg/ml. For the iodination reactions employing lactoperoxidase, a reaction mixture was prepared by adding the reagents in the following order: 100 $\mu$l of one of the protein solutions, 4 mCi (*ca.* 5 $\mu$l) of Na $^{125}$I (IMS 300), 20 $\mu$l of lactoperoxidase (25 unit/ml) and 10 $\mu$l of a fresh 1:1000 dilution of 3% hydrogen peroxide in phosphate buffer (pH 7.0). The reaction was allowed to proceed for 15 min at room temperature (*ca.* 22°C). At the end of the reaction period, the entire reaction mixture was applied to the HPLC column. When Chloramine-T was employed, the reaction was carried out by adding the reagents in the following order: 100 $\mu$l of the protein solution, 4 mCi (*ca.* 5 $\mu$l) of Na $^{125}$I, 10 $\mu$l of Chloramine-T [2 mg/ml in phosphate buffer (pH 7.0)]. The reaction was allowed to proceed for 45–60 sec at *ca.* 0°C before the entire reaction mixture was placed on the HPLC column. The container for each iodination reaction was a 1.5-ml snap-cap polyethylene vial. Smaller volumes of protein solutions or amounts of Na $^{125}$I were employed when only limited amounts of protein were available. In order to change the iodination profile for proteins containing multiple iodination sites, *e.g.* insulin, solvents of 3.5 *M* urea in 0.06 *M* phosphate (pH 7.0) and/or 50% propylene glycol–0.06 *M* phosphate (pH 7.0) were also employed.

*HPLC systems*

A standard commercial system of the Beckman-Altex Dual Pump Gradient System with a Model 420 programmer and a 50-$\mu$l injector loop was employed. A 0.46 $\times$ 25 cm Beckman-Altex ODS ($C_{18}$, 5 $\mu$m) column was employed.

For both analytical and preparative purposes a system developed in the Lilly Research Laboratories is routinely used[8]. This system consists of a LP-1/$C_{18}$ silica gel (10–20 $\mu$m, 20% C) packed in 50 $\times$ 1.2 cm glass columns (Ace Glass, Vineland, NJ, U.S.A.) fitted with PTFE end-fittings. All tubing is made of PTFE, and solvents are pumped by a FMI (Fluid Metering) Explosion Proof Lab Pump (Oyster Bay, NY, U.S.A.). This system is equipped with a three-way slider–injector valve with a 0.2–1.0-ml sample loop. These columns are usually eluted isocratically.

*Detection systems*

To either of the two HPLC systems described above, the following detection systems were attached: a UV monitor [either a Spectromonitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) or an ISCO UA-5] in tandem with a Baird-Atomic Ratemeter equipped with a JP-200 Johnson Probe. The radioactivity and UV monitors are connected to a three-channel strip chart recorder. The third channel is connected to the event marker of an ISCO Fraction Collector (Model 328). The column eluate fractions are collected in 100 $\times$ 16 mm polystyrene tubes.

*Sequencing of radiolabelled peptides*

In order to establish the position at which $^{125}I$ is attached in the peptide of interest, a small aliquot (usually 50 $\mu$l containing *ca.* 500,000 cpm) of a given HPLC peak was diluted with 1 ml of a solution containing the unlabelled peptide at 1 mg/ml. This was then lyophilized and subsequently redissolved for application to the sequenator cup of the Beckman Sequenator, Model 890C (Beckman Instruments, Palo Alto, CA, U.S.A.). The individual cycles were collected and counted directly in a gamma counter. This method is based on that described by Chan *et al.*[9].

## RESULTS

Our initial investigation of the different iodination procedures available confirmed the observations of earlier investigators[10] that Chloramine-T rapidly oxidizes methionine residues to methionine sulfoxide and iodinates protein. We did not observe such phenomena occuring to any significant degree with the lactoperoxidase–hydrogen peroxide-catalyzed iodinations. Since we desired to introduce only a single chemical change in the radiotracers, and in addition wished to use the mildest iodination conditions available, we usually employed the lactoperoxidase method. Since this reaction is slower than the Chloramine-T-catalyzed iodination, we found that we could also more readily control the degree of iodination.

*Insulin*

Insulin contains four tyrosine residues: at positions 14 and 19 of the A-chain, and at positions 16 and 26 of the B-chain. Thus there are four possible monoiodotyrosine forms of the radiolabelled hormone. In aqueous medium[3], iodination of pork insulin results in about 85% of the iodine being introduced into the A-chain, and the

remaining 15% into the B-chain. If increased iodination of the B-chain tyrosines is desired, the iodination reaction can be carried out in 3.5 *M* urea–0.06 *M* phosphate (pH 7.0). Under these conditions 40–45% of the iodine is introduced onto the B-chain tyrosines and 55–60% on the A-chain tyrosines[4]. Fig. 1 shows the isocratic elution profile of a reaction mixture of pork insulin, after iodination in this urea system. In general, >90% of the radioactivity applied to the column is recovered. The identification of each of the $^{125}$I-insulin peaks was achieved by automated sequencing of the particular peak fractions. An example of the sequencing data for the $^{125}$I (A-14)-insulin* is shown in Fig. 2. The radioactivity is obviously released at cycle 14. Trailing of the peak is due to the incomplete coupling that occurs with each cycle of the sequenator. No peak of radioactivity is observed at positions 16, 19 or 26 which correspond to the other possible tyrosine iodination positions.

Rechromatography of each of the peaks shown in Fig. 1 yielded single symmetrical peaks. Moreover, rechromatography of either the B-26, B-16 or A-14 peaks did not change the receptor binding properties of these tracers (see below). In addition, subsequent chromatography on the commercial system discussed below yielded only single symmetrical peaks of radioactivity for each of these three tracers. Significant overlap of the $^{125}$I (A-19)-insulin elution position with the unlabelled insulin would be noticeable. The B-26, B-16, and A-14 tracers are eluted far enough away from the unreacted insulin to be essentially carrier-free. We have verified this by isolating each of the $^{127}$I-forms of these isomers and then subjecting them to polyacrylamide gel electrophoresis under conditions where the native insulin and iodinated insulins can be separated[3]. This technique would detect 0.5–1% contamination by native insulin in the iodinated insulins. In no instance was native insulin detected

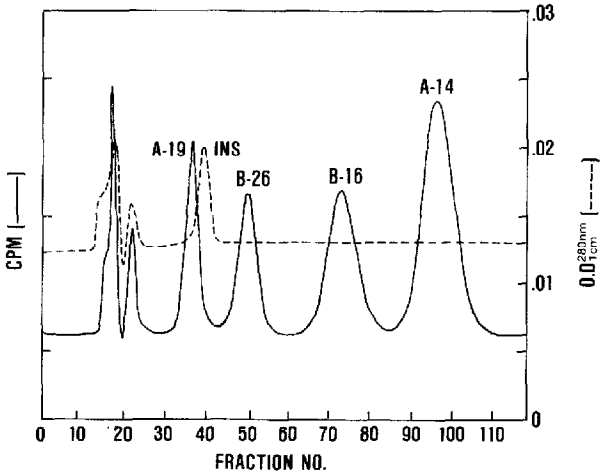


Fig. 1. HPLC isocratic elution profile for pork insulin iodination mixture. See text for experimental details. Iodination reaction performed in 3.5 *M* urea–0.06 *M* phosphate (pH 7.0). Flow-rate, *ca.* 2.0 ml/min; temperature, 25°C; cpm scale is arbitrary and used only for a qualitative radioactivity profile. Eluent of 29% acetonitrile in 0.2 *M* ammonium acetate (pH 5.5).

* Numbers refers to amino acid residue in sequence to which the iodine is attached.

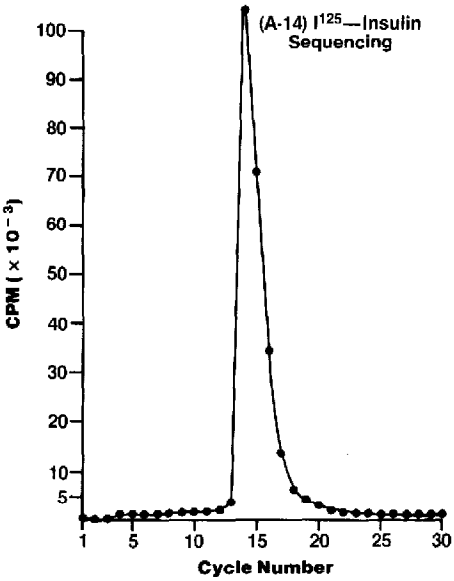


Fig. 2. Amino acid sequencing of $^{125}$I (A-14)-pork insulin.

above this level. In addition, a 100-$\mu$g sample of native insulin was chromatographed under the same conditions and the column fractions were assayed by an insulin radioimmunoassay. No significant amount of native insulin was found in the region where the B-chain and the A-14 tracers are eluted. Thus the tracers obtained are essentially carrier-free and the specific activity of these tracers can be calculated from the molecular weight of the protein and the specific activity of the $^{125}$I used in the iodination reaction. The specific activities of these insulin tracers normally are in the range of 300–350 $\mu$Ci/$\mu$g.

The ability to rapidly and reproducibly obtain the carrier-free insulin tracers described above has allowed us, for example, to undertake detailed receptor binding studies on each of these tracers. In agreement with and in extension of previous results[4], we found that these tracers each exhibited different binding characteristics in different receptor systems (see Fig. 3). Thus we were able to clearly demonstrate non-homologous behavior of the tracers used in most insulin receptor studies. Moreover, our results again demonstrated that $^{125}$I (A-14)-insulin is very similar in properties to native insulin[3]. We have also found that the reproducibility of insulin radioimmunoassays is significantly greater when tracers prepared in this manner are used than with the conventionally prepared tracers. The amount of "damaged" tracer, *i.e.* tracer that is not bound in the presence of a large excess of antibody, is reduced from 5–9% for conventionally prepared materials to 1–2% for the HPLC-isolated materials. The stability of the tracer preparations is also significantly greater, the useful lifetime of the tracer being in fact limited by the decay rate of $^{125}$I rather than by increased levels of "damaged" tracer. Finally, on the basis of the conventional measure of tracer integrity, trichloroacetic acid (TCA) precipitability, the HPLC-isolated tracers are usually >98% TCA precipitable.

Having established the usefulness of our HPLC system for separation of in-

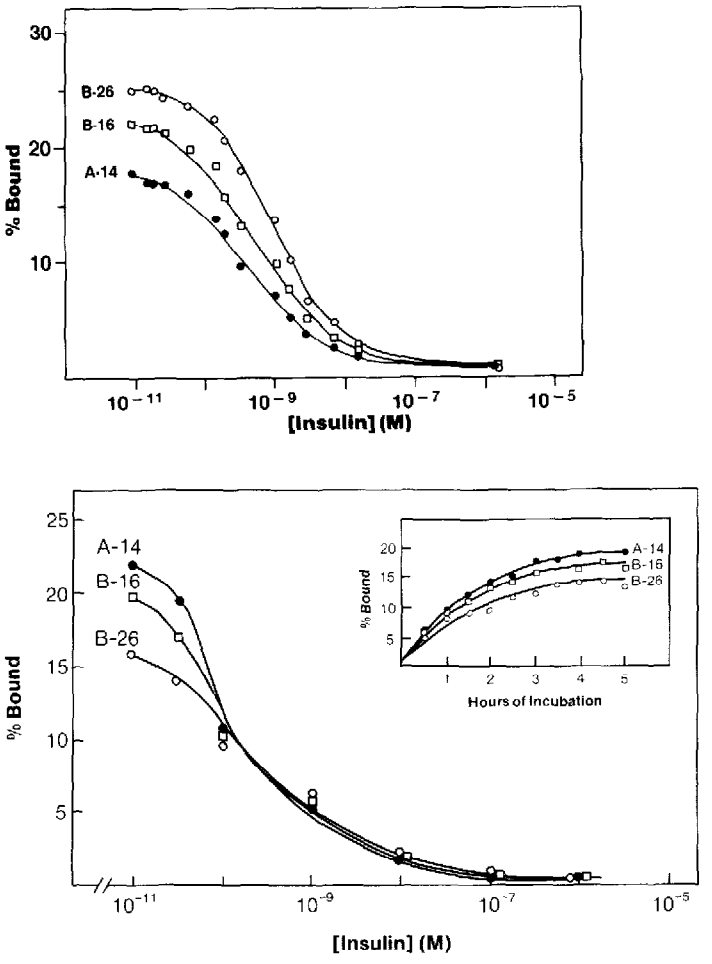


Fig. 3. Competitive binding of $^{125}$I-insulin isomers to IM-9 lymphocytes (above) and to isolated rat hepatocytes (below). Binding studies carried out at 15°C. Insert demonstrates time course of specific binding to rat hepatocytes. Displacement of individual tracers was with pork insulin.

sulin tracers, we also undertook an examination of commercial HPLC systems for this purpose. Fig. 4 shows the elution profile of a reaction mixture after aqueous iodination of insulins with $^{127}$I, applied directly to the HPLC column. The elution position of the $^{125}$I (A-14)-insulin [or $^{127}$I (A-14)insulin] is indicated on the graph along with the elution position of unreacted insulin. The peak at approximately 4 min is due to solvent breakthrough and also contains any unreacted iodine (detected by its radioactivity). The small peaks between native insulin and the $^{125}$I (A-14)-insulin are the monoiodo-insulin forms with iodine on either of the B-chain tyrosines. The peaks eluted at about 33 min represent a mixture of diiodo-insulin forms. The separation of the $^{125}$I (A-14)-insulin from the other protein species, though not as good as in the glass column systems described above, is still sufficient to yield this tracer in good purity. Thus this commercial system can be used readily to prepare this particular

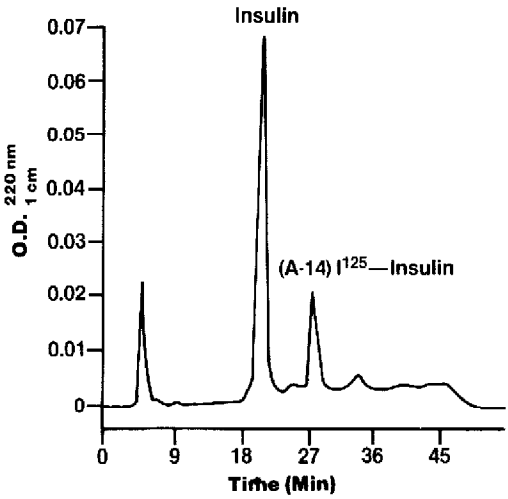


Fig. 4. HPLC gradient elution profile for pork insulin iodination ($^{127}$I) mixture. Beckman-ODS ($C_{18}$, 5 $\mu$m) column. Solvent A is 10% acetonitrile and solvent B is 35% acetonitrile, both in 0.3 *M* ammonium sulphate (pH 5.5). Gradient from 51% to 53% B for 10 min followed by a gradient to 70% B over the next 30 min. Temperature, 25°C. 50-$\mu$l sample injector loop. Reaction mixture consisted of a 4:1 insulin–iodine mole ratio. Flow-rate, 1 ml/min.

tracer of insulin. We have detected no differences in the receptor binding behavior of $^{125}$I (A-14)-insulin isolated by either of these HPLC techniques. We would note that isolation of the B-26 and B-16 tracers on the commercial columns in pure form is much more difficult than with our glass column systems. Thus far we have been unable to achieve the resolution of all four tracers in homogeneous form, directly from reaction mixtures with this commercial system.

*t-BOC-Tyr ($C_{32}$) human C-peptide*

The determination of C-peptide levels in human sera by radioimmunoassay is clinically very important since these levels are assumed to be an indicator of the functional state of the $\beta$-cell of the pancreas[11]. Variability in the results of the C-peptide radioimmunoassay have been shown to be due to a number of factors including the quality of the radiotracer used in the assay[12]. Thus the development of an isolation procedure which yields pure tracers of t-BOC-tyr human C-peptide in a reproducible manner would have great utility.

We investigated both our glass-column systems and commercial HPLC systems for the isolation of radioiodinated human C-peptide. Fig. 5 shows the isocratic elution profile of a C-peptide iodination mixture which contained both monoiodo- and diiodo-t-BOC-tyrosyl human C-peptide on our glass-column system. Since the introduction of iodine into the peptide changes the hydrophobic nature of the t-BOC-tyr human C-peptide, any unreacted peptide is eluted at the solvent front. The identity of these two iodinated species was confirmed by synthesizing the $^{127}$I-analogue and then demonstrating that they are eluted with the individual peaks shown in Fig. 5. The specific activities of the monoiodo- and diiodo-t-BOC-tyr human C-peptides were *ca.* 550 and 1100 $\mu$Ci/$\mu$gm, respectively. The behavior of these two tracers

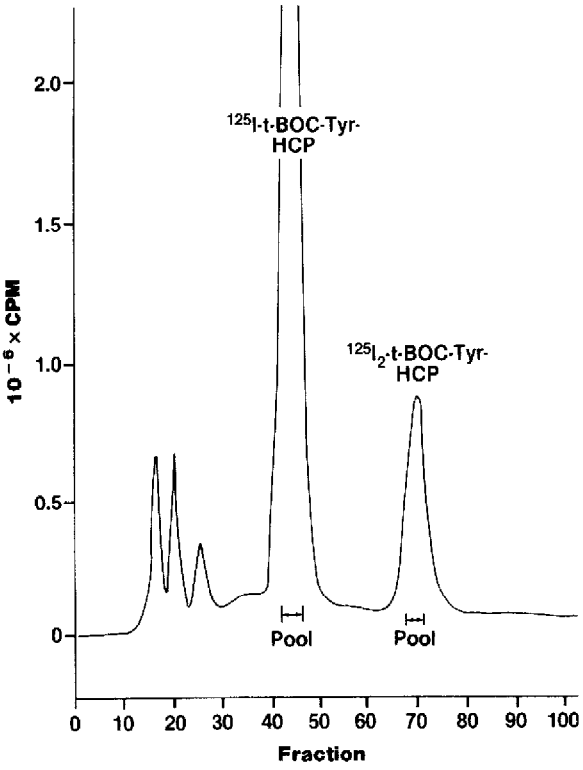


Fig. 5. Isocratic elution profile of a t-BOC-tyr human C-peptide iodination mixture. See text for iodination conditions and column details. Flow-rate, 2.0 ml/min; 1-min fractions. Temperature, 25°C. Eluent of 33% acetonitrile in 0.2 *M* ammonium acetate (pH 5.5). Identity of individual carrier-free tracers shown. Unreacted iodine and peptide are eluted in fractions 15–25.

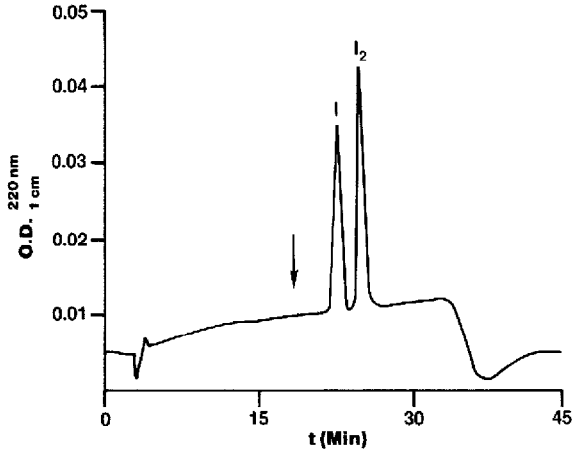


Fig. 6. HPLC gradient elution profile for $^{127}$I-forms of t-BOC-tyr human C-peptide. Solvent A is 15% acetonitrile and solvent B is 35% acetonitrile, both in 0.07 *M* ammonium acetate (pH 7.1). Flow-rate, 1 ml/min. Temperature, 25°C. Gradient from 15% B at $t = 0$ to 40% at $t = 30$ min. Arrow indicates elution position of unreacted t-BOC-tyr human C-peptide. I is the position of monoiodinated tracer and $I_2$ is the position of the diiodo tracer.

in the radioimmunoassay for C-peptide was excellent and, in fact, identical. Because the diiodo tracer has such a high specific activity, we have been able to substantially reduce (five-fold) the amount of serum required for the assay, and to use the C-peptide antibody at 2–4 times greater dilution in order to achieve reliable and reproducible assay results[13].

Because the use of the C-peptide assay is so widespread, we have also determined conditions under which a commercial HPLC system could be used to prepare C-peptide tracers. Fig. 6 shows the elution profile of an iodination reaction mixture of the mono- and diiodo-t-BOC-tyr human C-peptides. The elution position of unreacted t-BOC-tyr human C-peptide also is indicated. Obviously under the iodination conditions where molar excesses of iodine are used, no unreacted starting material is observed. The peaks shown are for a mixture of the $^{127}$I-forms. When the $^{125}$I-forms are applied to this column, they are eluted with the respective $^{127}$I-forms. Labeled C-peptide, isolated by reversed-phase HPLC, is stable for up to 5 months.

The two proteins discussed above are just two examples of our general approach to preparing site-specific, carrier-free radiotracers of pancreatic hormones. Using this methodology, we prepare on a routine basis a number of tracers of pancreatic hormones and their analogues. Included are the four monoiodo forms of bovine, human and sheep insulin, monoiodo (A-14) human, bovine and porcine proinsulins, monoiodo (either 10 or 13) glucagon, mono and diiodo tyr(1)-somatostatin, monoiodo (27 or 36) bovine, porcine or human pancreatic polypeptide, monoiodo (A-14) des Asn (A21)-des Ala (B30) bovine insulin and monoiodo A-14-desoctapeptide insulins.

## CONCLUSIONS

The data presented for both insulin and t-BOC-tyr human C-peptide tracer preparations illustrates the general approach we have developed in order to prepare single-site carrier-free radiotracers of these pancreatic peptides. In addition we have illustrated the enormous utility of reversed-phase HPLC for the purpose of isolating these radiotracers directly from the radioiodination reaction mixtures. Two different HPLC systems have been used: one developed in our laboratories and the second a widely available commercial HPLC system. Thus we have an approach whereby the detailed chemistry of the iodination of the particular pancreatic peptide hormone can be rapidly investigated. Then the individual site tracers are isolated and sequenced to establish the iodination site. Finally the suitability of each tracer for any given biological application (for example receptor studies, radioimmunoassays and metabolism studies) can be established. We have accomplished this for each of the major pancreatic hormones, namely insulin, proinsulin, glucagon, pancreatic polypeptide, as well as somatostatin. In the "Lilly" HPLC system, the only variation in the HPLC methodology is to increase or decrease the amount of acetonitrile in the isocratic eluent. For example, whereas insulins require *ca.* 28–29% acetonitrile in 0.2 *M* ammonium acetate (pH 5.5), glucagon requires *ca.* 32% acetonitrile in 0.2 *M* ammonium acetate (pH 5.5) in order to achieve separation of monoiodinated glucagon from unreacted glucagon. We would note that, as one would expect, the separation achieved in our isocratic systems are better than in gradient systems. One advantage of

the "Lilly" HPLC system is that it can be readily and directly scaled up. This has allowed us to prepare mg quantities of the $^{127}$I-forms of our radiotracers. Using these we have been able to undertake homologous displacement studies in insulin receptor systems[1]. These studies have clearly demonstrated both the importance of using homogeneous radiotracers and the non-equivalence of various monoiodo-insulin forms in receptor binding. Further, we have been able to undertake a physicochemical characterization of the $^{127}$I-insulins, which suggests the structural non-equivalence of individual monoiodo-insulins (unpublished data).

In contrast to conventionally prepared tracers, the radiotracers isolated by reversed-phase HPLC are more stable, have a much lower level of "damaged" material and yield greater sensitivity in biological assays. The rapidity and ease with which tracers can be prepared by HPLC should allow much broader use of pure tracers in the future.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. M. A. Root, R. E. Chance, D. E. Peavy and W. C. Duckworth and Ms. C. S. Hooker for their helpful discussions and assay support. In addition, we wish to acknowledge the very able technical assistance of M. G. Johnson and A. H. Pekar.

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