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# HOMOGENEOUS [MONO-$^{125}$I-Tyr$^{10}$]- AND [MONO-$^{125}$I-Tyr$^{13}$] GLUCAGON

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SUMMARY

The two monoiodinated forms of glucagon were prepared by lactoperoxidase-catalysed iodination followed by separation by reversed-phase high-performance liquid chromatography. The intramolecular distribution of $^{125}$I was analysed by tryptic and chymotryptic cleavage of the isolated isomers. The results show that [mono-$^{125}$I-Tyr$^{10}$]- and [mono-$^{125}$I-Tyr$^{13}$]glucagon can be separated from each other and from the respective unlabelled polypeptide and thus can be obtained in a pure state with the highest possible specific activity. We have studied the receptor binding ability of both tracer isomers to isolated intact rat hepatocytes. The resulting $K_d$ values were 2.0 ± 0.2 n$M$ for the tyrosine-13-labelled glucagon and 4.2 ± 0.3 n$M$ for the tyrosine-10-labelled glucagon.

INTRODUCTION

The use of radioiodinated peptide hormones as tracers in studies of hormone–receptor interactions requires a precise knowledge of the chemical state of the radiolabelled compounds. Most studies have emphasized the importance of using "monoiodinated" hormones, as more heavily iodinated materials appear to have altered activity[1]. With insulin, which contains four tyrosine residues, even monoiodinated insulin preparations can be heterogeneous mixtures, and it is now clear that the location of the iodine affects the properties of the molecule[2–5]. Glucagon is a 29-amino acid polypeptide hormone and contains two tyrosine residues. Radioactively labelled [$^{125}$I]glucagon is a widely used tracer in receptor assays and many results may depend on the degree of radiochemical homogeneity of the tracer and the distribution of $^{125}$I within the glucagon molecule[13]. A heterogeneous mixture of glucagon species may create the impression of the existence of heterogeneous sets of binding sites. Even monoiodinated materials prepared in different laboratories could possess different properties, because the conditions under which the iodination is performed (oxidizing agent, pH value) have been shown to affect the relative distribution of iodine[6–8]. Most of the commercially available tracer preparations of $^{125}$I labelled glucagon are heterogeneous with respect to the position and/or degree of iodination and/or state of oxidation.

In this paper, we present a convenient technique for the separation of the two monoiodinated glucagon derivatives. This technique employs reversed-phase high-performance liquid chromatography (HPLC) on a $C_{18}$ column with on-line $y$-detection using an isocratic $n$-propanol system and leads to the complete separation of [mono-$^{125}$I-Tyr$^{10}$]- and [mono-$^{125}$I-Tyr$^{13}$]glucagon. The isolated products were characterized by tryptic and chymotryptic cleavage and digestion with pronase. The two monoiodinated glucagon isomers showed high radiochemical purity and specific radioactivity.

## EXPERIMENTAL

### Apparatus

The chromatographic separations and analyses were performed on a DuPont HPLC system, which consisted of a gradient controller (Series 8800), a pump module (Model 870), a thermostatable column compartment, a variable-wavelength UV spectrophotometer and a Rheodyne sample injector (Model 7125) with a 50-$\mu$l sample loop. The system was coupled to an HPLC radioactivity monitor (LB 504, Laboratorium Prof. Berthold), which was connected to a compact computer (Apple II) and a video display (Sanyo). A two-channel recorder (LKB 2210) coupled to the spectrophotometer and to the radioactivity monitor was used for the simultaneous recording of absorbance and radioactivity. Separations were performed on a Knauer 250 × 4.6 mm I.D. LiChrosorb $C_{18}$ cartridge with pre-cartridge.

### Chemicals

Crystalline porcine glucagon was obtained from NOVO (Copenhagen, Denmark). Carrier-free Na$^{125}$I and [mono-$^{125}$I-Tyr$^{10}$]glucagon were purchased from Amersham-Buchler (Braunschweig, F.R.G.). Hydrogen peroxide, sodium hydrogen sulphite, $n$-propanol, potassium dihydrogen orthophosphate, orthophosphoric acid and ammonium hydrogen carbonate were obtained from Merck (Darmstadt, F.R.G.) and Eagle's minimal essential medium and lactoperoxidase from Boehringer (Mannheim, F.R.G.). Trypsin (diphenyl carbamyl chloride-treated), chymotrypsin, pronase and collagenase (type I) were obtained from Sigma (St. Louis, MO, U.S.A.), bacitracin from Serva (Heidelberg, F.R.G.), Bio-Gel P-2 from Bio-Rad Labs. (Munich, F.R.G.), silicone oil (AR20 and AR200) from Wacker-Chemie (Munich, F.R.G.) and Percoll from Pharmacia (Uppsala, Sweden).

### Iodination

A 1-nmol amount of porcine glucagon was iodinated by the lactoperoxidase method with 1 mCi of Na$^{125}$I (IMS 30) in 50 $\mu$l of 0.5 $M$ phosphate buffer (pH 8.0), as previously described[8]. The radioactive iodination mixture was purified as described by Pohl[9]. The specific radioactivity of the tracer was 250 $\mu$Ci/$\mu$g.

### HPLC of [mono-$^{125}$I]glucagon

The monoiodinated glucagon isomers were separated by reversed-phase HPLC on a LiChrosorb $C_{18}$ column with a pre-column using an isocratic elution system consisting of 19.2% of $n$-propanol in 10 m$M$ potassium dihydrogen orthophosphate buffer (pH 2.5) at a flow-rate of 1 ml/min at 40°C. The column eluate was monitored at 220 nm and the radioactivity was measured on-line.

*Enzymatic digestion of purified [mono-$^{125}$I-Tyr$^{10}$]- and [mono-$^{125}$I-Tyr$^{13}$]glucagon*

The HPLC-purified monoiodinated isomers were cleaved in 0.1 $M$ ammonium hydrogen carbonate buffer (pH 8.0) with trypsin (50 $\mu$g/ml) or chymotrypsin (50 $\mu$g/ml) at 37°C.

*HPLC of the tryptic and chymotryptic fragments*

The glucagon fragments were analysed on a LiChrosorb $C_{18}$ cartridge using a linear gradient from 0 to 30% of *n*-propanol in 10 m$M$ potassium dihydrogen orthophosphate buffer (pH 2.5) completed in 30 min at a flow-rate of 1 ml/min at 40°C. The radioactivity was measured on-line.

*Binding of radioiodinated glucagon isomers to isolated intact rat hepacytes*

Isolated intact rat hepatocytes were prepared by the perfusion technique according to Seglen[10] and further purified by isopycnic centrifugation through a continuous Percoll gradient[11]. A 400-$\mu$l volume of the homogeneous cell suspension (1.5 Mio cells/ml) was added to 50 $\mu$l of [$^{125}$I]glucagon (70 000 cpm) and 50 $\mu$l of Eagle's minimal essential medium or unlabelled glucagon (50 f$M$–10 $\mu M$). The incubation was carried out at 37°C for 30 min (pH 7.4) and was terminated by transferring 200-$\mu$l aliquots of the incubation mixture in duplicate into microtubes containing 100 $\mu$l of a 1:1 mixture of silicone oils AR20 and AR 200. The cell-bound hormone was isolated by centrifugation through the silicone oil in an Eppendorf laboratory centrifuge at 10 000 $g$ for 30 sec. Separation of the cells from the incubation medium was complete within a few seconds after centrifugation had begun. The microtubes were frozen in liquid nitrogen and cut through the oil layer. Cell-bound and free hormones were counted in a multi-crystal gamma counter (Berthold, LB 2100). Evaluation of the measured data was performed by a computerized non-linear least-squares curve-fitting procedure according to Peters and Pingoud[12].

RESULTS

Based on our experience with the use of reversed-phase HPLC for separating iodinated glucagon derivatives from one another and from non-iodinated glucagon, we set out to develop a technique that might be useful in separating the two mono-iodinated glucagon isomers. Because glucagon has two tyrosine residues, however, even monoiodinated glucagon is likely to be a heterogeneous mixture of monoiodo species. We recently reported the isolation of mono-, di- and polyiodinated derivatives of glucagon and insulin by a procedure involving lactoperoxidase-catalysed iodination followed by HPLC[8]. [Mono-$^{125}$I]glucagon preparations, obtained by iodinating with lactoperoxidase–$H_2O_2$ as the oxidizing agent, at a molar ratio of iodine to polypeptide hormone of 1:2 performed at 8.0 were purified by adsorption to and desorption from cellulose[10].

The separation of the two possible tyrosine-monoiodinated glucogon isomers was performed by isocratic elution with 19.2% of *n*-propanol in 10 m$M$ phosphate buffer (pH 2.5) on a LiChrosorb $C_{18}$ column at 40°C (Fig. 1a). The unreacted glucagon appears at the indicated arrow. Peak II correlates with commercially available [mono-$^{125}$I-Tyr$^{10}$]glucagon (Fig. 1b) and we therefore assume that peak I represents [mono-$^{125}$I-Tyr$^{13}$]glucagon. The separated isomers were lyophilized and rechromatographed; they showed identical elution positions to those in the mixture.
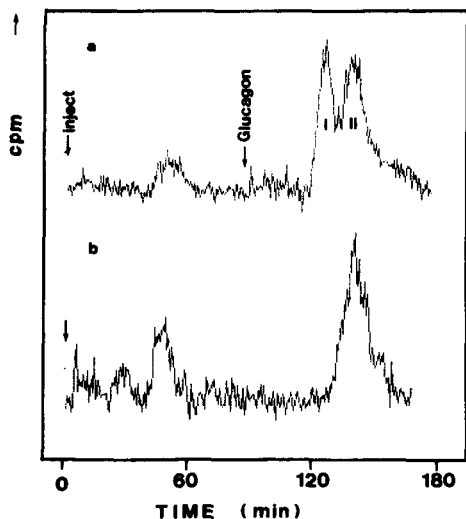
Fig. 1. Reversed-phase HPLC of [mono-$^{125}$I]glucagon. Column: LiChrosorb $C_{18}$ (250 × 4.6 mm I.D.) with pre-column. Eluent: 10 m$M$ $KH_2PO_4$ in 19.2% $n$-propanol (pH 2.5). The isocratic elution was performed for 180 min. The eluate was monitored for $^{125}$I radioactivity in an on-line $y$-detection system. (a) Our mixture of monoiodinated glucagon; (b) elution profile of commercially available [mono-$^{125}$I-Tyr$^{10}$]glucagon.

In order to determine the location of iodine in the radioactively labelled glucagon, enzymatic cleavage of the two separated components with trypsin and chymotrypsin was carried out. The amino acid sequence of glucagon is His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–Asp–Ser–Arg–Arg–Ala–Gln–Asp–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr with the two tyrosine residues at positions 10 and 13, which are the targets of the iodination reaction. Trypsin cleaves glucagon behind Lys and Arg, whereas chymotrypsin cleaves behind the aromatic residues (Phe, Tyr and Trp). The tryptic digestion of glucagon yields three fragments, 1–12 (TS-1), 13–17 (TS-2) and 18–19 (TP)[14]. The tryptic digestion of radioactive tyrosine-10-monoiodinated glucagon (peak II, Fig. 1a) yields one intermediate radioactive fragment, presumably 1–18 or 1–17 (Fig. 2b). The final product must be fragment 1–12 (TS-1, Fig. 2c). The tryptic cleavage of commercially available [mono-$^{125}$I-Tyr$^{10}$]glucagon yields peptides with identical retention times. From chymotryptic digestion of our presumed [mono-$^{125}$I-Tyr$^{10}$]glucagon we obtained one major peptide which must be fragment 7–10 (C-2) according to Bromer et al.[14] (Fig. 2d). The same final product is obtained with commercially available [mono-$^{125}$I-Tyr$^{10}$]glucagon.

Fig. 3a illustrates the chromatographic separation of the peptide resulting from tryptic hydrolysis of peak I (Fig. 1a). The resulting peptide corresponds to fragment 13–17 (TS-2, Fig. 3b). Fig. 3c shows the radioactive chymotryptic peptide of [mono-$^{125}$I-Tyr$^{13}$]glucagon, which corresponds to fragment 11–13 (C-8). The tryptic cleavage of the mixed monoiodinated glucagon isomers as obtained from the radioactive iodination reaction yields two radioactive peaks, each containing nearly 50% of the applied radioactivity. The elution positions of the fragments are identical
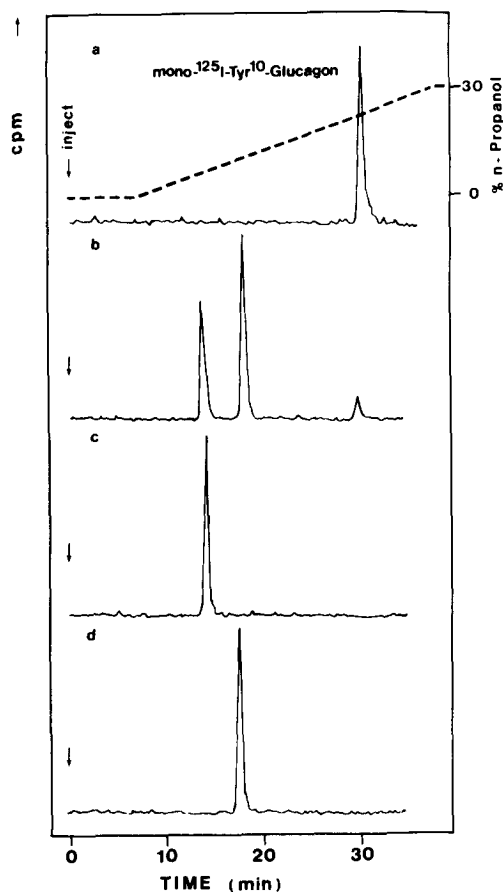
Fig. 2. Reversed-phase HPLC of [mono-$^{125}$I-Tyr$^{10}$]glucagon (a) and the tryptic (b and c) and chymotryptic digests (d) of the tyrosine-10-iodinated glucagon isomer. The elution was performed on a LiChrosorb $C_{18}$ column using a linear gradient from 0 to 30% of $n$-propanol in 10 m$M$ KH$_2$PO$_4$ buffer (pH 2.5) completed in 30 min at a flow-rate of 1 ml/min at 40°C.

with the retention times of the tryptic digestion products of [mono-$^{125}$I-Tyr$^{10}$]glucagon (Fig. 2c) and [mono-$^{125}$I-Tyr$^{13}$]glucagon (Fig. 3b). The radioactive fragments must be 1–12 (TS-1) and 13–17 (TS-2), as the dodecapeptide glucagon 1–12 (containing tyr$^{10}$) elutes from a gel filtration column (Bio-Gel P-2) before the labelled pentapeptide glucagon 13–17 (containing Tyr$^{13}$) (not shown). This result is consistent with peptide I representing [mono-$^{125}$I-Tyr$^{13}$]glucagon and peptide II representing [mono-$^{125}$I-Tyr$^{10}$]glucagon. Chymotryptic hydrolysis also yields two radioactive fragments that show the same retention times as the radioactive cleavage products of isolated tyrosine-10- and tyrosine-13-iodinated glucagon (Figs. 2d and 3c), thus suggesting that they are fragments 11–13 (C-8) and 7–10 (C-2).

The further digestion of the tryptic and chymotryptic fragments with pronase yields [$^{125}$I]tyrosine, which was identified by reference to monoiodotyrosine, and is an indication of substitution of glucagon in the Tyr-10 and Tyr-13 position.
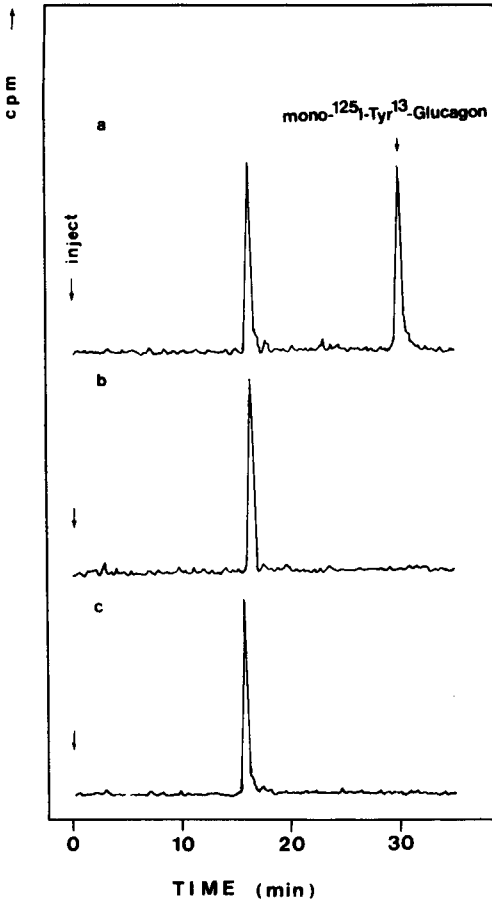
Fig. 3. Reversed-phase HPLC of the tryptic (a and b) and chymotryptic (c) digests of [mono-$^{125}$I-Tyr$^{13}$]glucagon. Chromatographic conditions as in Fig. 2.

With the HPLC technique described here, monoiodinated glucagon can be prepared and separated into the two isomers by a single-step isolation procedure. Both isomers can be obtained without contamination of unlabelled glucagon or other iodinated species, e.g., oxidized forms. The identity of the peaks as the monoiodinated glucagon derivatives (either on Tyr$^{10}$ or on Tyr$^{13}$) was established by correlation with the commercially available product, [mono-$^{125}$I-Tyr$^{10}$]glucagon) and by enzymatic cleavage. The digestion of the mixed monoiodinated isomers with trypsin yields two radioactive fragments corresponding to glucagon 1–12 and glucagon 13–17 (Fig. 4b), whereas the chymotryptic cleavage yields fragments 11–13 and 7–10 (Fig. 4c). The same fragments are obtained after enzymatic cleavage of the two separated monoiodinated isomers.
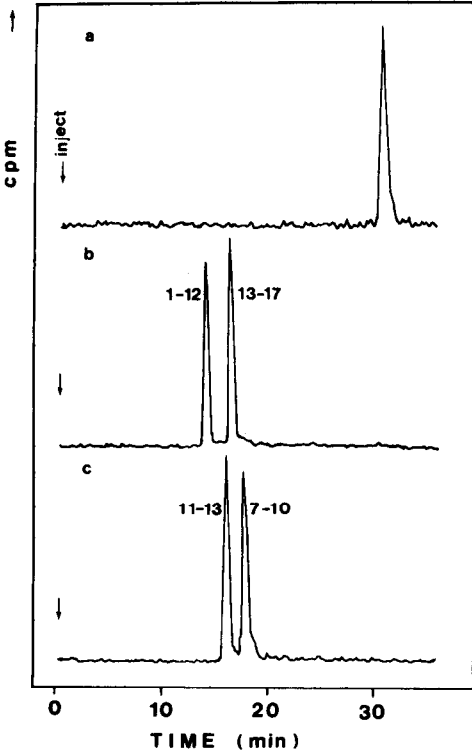
Fig. 4. Tryptic (b) and chymotryptic (c) cleavage of the mixed monoiodinated glucagon isomers (a). HPLC analysis was performed as in Fig. 2.
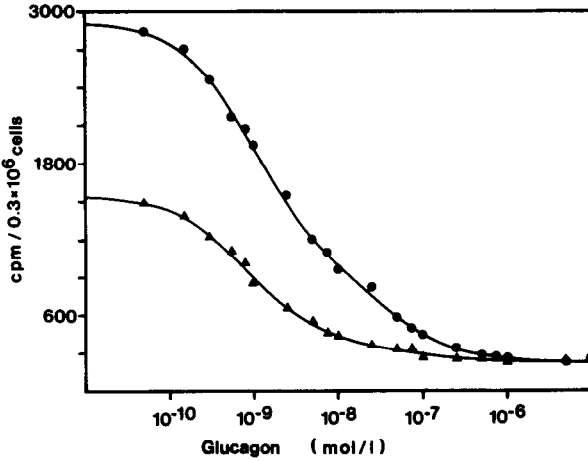


Fig. 5. Competitive binding of the mono-^{125}I-labelled glucagon isomers to isolated intact rat hepatocytes in the presence of increasing concentrations of native glucagon. The incubation was performed as described under Experimental. The binding data were evaluated by a computerized curve-fitting procedure. Displacement of [mono-^{125}I-Tyr[10]]- (●) and [mono-^{125}I-Tyr[13]]glucagon (▲).

*Binding of [mono-$^{125}$I-Tyr$^{13}$]- and [mono-$^{125}$I-Tyr$^{10}$]glucagon to isolated intact rat hepatocytes*

Binding of the purified monoiodinated glucagon isomers to isolated intact rat hepatocytes reached equilibrium after 20 min at 37°C and remained constant for a further 15 min (data not shown). [Mono-$^{125}$I-Tyr$^{13}$]glucagon was displaced by native glucagon with a $K_d$ value of 2.0 ± 0.2 n$M$. Non-specific binding, evaluated in the presence of 10 $\mu M$ glucagon, corresponded to 15% of total binding (Fig. 5). [Mono-$^{125}$I-Tyr$^{10}$]glucagon shows a higher steady-state receptor binding and a decreased affinity according to a $K_d$ value of 4.1 ± 0.3 n$M$. Non-specific binding was evaluated to be 8% of the total binding.

DISCUSSION

This study has demonstrated that the HPLC isolation method will yield, in a rapid, reproducible and selective manner, single-site carrier-free glucagon tracers. The use of isocratic conditions is very convenient (*e.g.*, it can be achieved with a single pump) and the resolution obtained leads to the separation of the iodotyrosine forms of glucagon from the oxidized forms and from the unlabelled hormone. Binding experiments showed that the [mono-$^{125}$I-Tyr$^{13}$]glucagon isomer binds to the hepatic glucagon receptors with an apparent affinity that is twice as great as that for the monoiodinated Tyr$^{10}$ isomer.

ACKNOWLEDGEMENTS

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