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PURIFICATION OF RADIOIODINATED PHOTOACTIVABLE GLUCAGON BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

2-Nitro-4-azidophenylsulphenyl-glucagon, a specific photoaffinity label for glucagon receptors, was synthesized and radioactively labelled with ^{125}I. The radio-labelled peptide was purified from the reaction mixture by high-performance liquid chromatography in one step by isocratic elution from a C_{18} column with 20.4% *n*-propanol in 10 mM phosphate buffer (pH 2.5) as eluent. This glucagon derivative can be used to attach a label specifically to the glucagon receptor. The binding ability of the photoaffinity derivative was tested on isolated intact rat hepatocytes. Compared with a K_d of 3 nM for unmodified monoiodinated glucagon, the K_d value of the photoaffinity labelled monoiodinated glucagon tracer was 7 nM.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) has already been reported as a rapid reproducible and selective method for the purification of radioactively labelled peptide hormones, and as insulin[1–4], glucagon[4–6], somatostatin[7], MSH[8] and angiotensins[9]. Only a few photoactivable radiolabelled peptide hormones have been purified so far by HPLC[10].

Our aim was to study the molecular structure of glucagon receptors. The identification of protein components of hormone receptors is greatly facilitated after specific cross-linking with a radioactive ligand. The synthesis of a homogenous radio-labelled photosensitive glucagon derivative with high specific radioactivity would provide the possibility of isolation of labelled receptor proteins and of identification of peptide fragments from the covalently labelled binding sites during the cellular processing of membrane receptors. In addition, labelled components could be identified, even if the isolation procedure caused loss of biological activity.

In general, the study of receptors requires a pure tracer of known concentration and chemical state, an aim that is not easily achieved.

In the present case 2-nitro-4-azidophenylsulphenyl-glucagon (NAPS-glucagon) was synthesized according to Demoliou and Epand[11]. The synthetic photosensitive glucagon derivative was modified at the tryptophan residue in position 25. The radioactive iodination reaction with the lactoperoxidase method[4] leads to substitu-

tion of the two tyrosine residues in positions 10 and 13, as estimated by enzymatic digestion. We report here a simple one-step purification procedure of [^{125}I]NAPS-glucagon by reversed-phase HPLC on a LiChrosorb C_{18} column. The binding properties of the radioiodinated photoactivable glucagon derivative were tested on isolated intact rat hepatocytes.

MATERIALS AND METHODS

Chemicals

Crystalline porcine glucagon and bacitracin were obtained from Serva (Heidelberg, F.R.G.), carrier-free sodium [^{125}I]iodide from Amersham-Buchler (Braunschweig, F.R.G.), and lactoperoxidase from Boehringer (Mannheim, F.R.G.). Trypsin (diphenylcarbamylchloride-treated), chymotrypsin and collagenase (type I) were obtained from Sigma (St. Louis, U.S.A.). 4-Chloro-3-nitroaniline was purchased from Janssen (Beerse, Belgium). All other reagents were obtained from Merck (Darmstadt, F.R.G.) and were of highest available commercial grade.

Methods

2-Nitro-4-azidophenylsulphenyl chloride (NAPS-Cl) was synthesized according to Demoliou and Epand[11].

Coupling of NAPS-Cl to glucagon. NAPS-glucagon was prepared in the dark by the method of Demoliou and Epand[11] and Veronese *et al.*[12], with the exception that the coupling reaction was carried out but with a 2–3:1 molar ratio of reagent to glucagon. Unchanged reagent was removed by gel filtration on a Sephadex-G-10 (fine) column. The fractions were pooled, lyophilized and stored at −20°C in the dark. The purity of NAPS-glucagon was studied by HPLC.

Radioactive labeling of NAPS-glucagon with sodium [^{125}I]iodide. NAPS-glucagon was iodinated by the lactoperoxidase method at pH 8.5. A 20-μl volume of sodium [^{125}I]iodide (2 mCi, 1 nmol) in 20-μl of 0.5 M K_2HPO_4 buffer (pH 8.5) was mixed with 20 μl of NAPS-glucagon (20 nmol in 0.01 M hydrochloric acid) and 5 μg of lactoperoxidase (in 10 μl of 0.5 M K_2HPO_4 buffer, pH 8.5). The reaction was started by the addition of 20 μl of hydrogen peroxide (20 nmol). The reaction was allowed to proceed for 3 min at room temperature under vigorous stirring. The whole mixture was applied to HPLC.

High-performance liquid chromatography. The separation was performed with an HPLC system that consisted of a Milton-Roy pump, a Rheodyne sample injector (Model 7125) with a 180-μl sample loop, a LiChrosorb C_{18} cartridge with precolumn (Knauer) and a fraction collector (ISCO, Model 1850). The iodination reaction was analysed using an isocratic elution buffer, which consisted of 20.4% of *n*-propanol in 10 mM KH_2PO_4 buffer (pH 2.5). The flow-rate was 0.5 ml/min, and fractions of 1 ml were collected. The radioactivity of the column eluate was counted off-line in a curimeter (Kapentec), and the absorbance in a variable-wavelength spectrophotometer (Zeiss) at 220 nm.

Enzymatic digestion of purified mono[^{125}I]NAPS-glucagon. The HPLC-purified monoiodinated NAPS-glucagon was cleaved in 0.1 M ammonium hydrogen carbonate buffer (pH 8.0) with trypsin (50 μg/ml) or chymotrypsin (50 μg/ml) at 37°C.

HPLC of tryptic and chymotryptic fragments. The [^{125}I]NAPS-glucagon frag-

ments were analysed 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-μl sample loop. The system was coupled to a 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 LiChrosorb C_{18} cartridge with pre-cartridge using a linear gradient from 0 to 30% of n-propanol in 10 mM potassium phosphate buffer (pH 2.5) completed in 30 min with a flow-rate of 1 ml/min at 40°C. The radioactivity was measured on-line.

Binding of [^{125}I]NAPS-glucagon to isolated intact rat hepatocytes. The binding experiments were performed as previously described[14], and the evaluation of the measured data was performed by a computerized non-linear least-squares curve-fitting procedure according to Peters and Pingoud[13].

RESULTS

Purification of mono[^{125}I]NAPS-glucagon
    NAPS-glucagon was iodinated with ^{125}I by the lactoperoxidase method at pH 8.5. The radioactive reaction mixture was directly applied to reversed-phase HPLC. Fig. 1 illustrates the UV absorbance and the radioactive elution profile obtained on
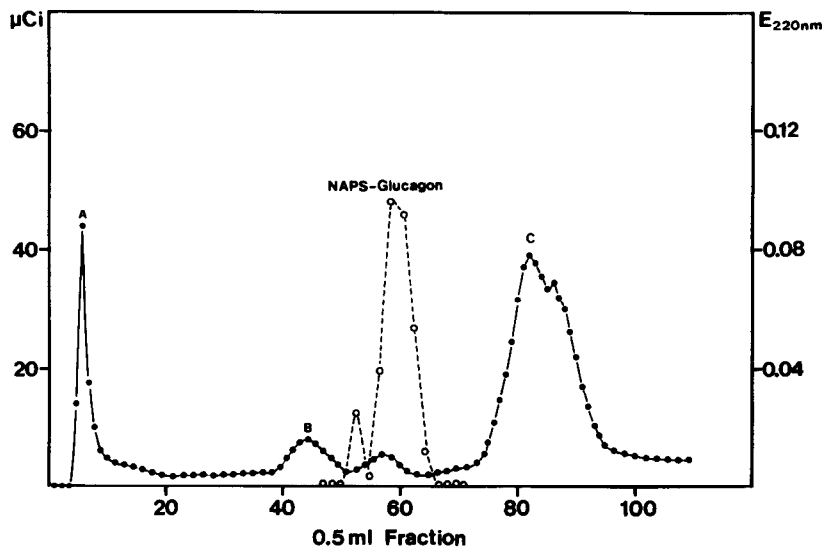

Fig. 1. Isocratic HPLC purification of monoiodinated [^{125}I]NAPS-glucagon directly after the iodination reaction. Radioiodination was performed as described under Materials and Methods. The final mixture was injected on a LiChrosorb RP-18 column, eluted at 0.5 ml/min with n-propanol–10 mM phosphate buffer (20.4:79.6, pH 2.5). Fractions of 1 ml were collected. Peak A represents unchanged ^{125}I, peak B oxidized [^{125}I]NAPS-glucagon and peak C monoiodinated [^{125}I]NAPS-glucagon. Unlabelled NAPS-glucagon is indicated.

a C_{18} column under isocratic conditions (20.4% n-propanol in 10 mM KH_2PO_4 buffer, pH 2.5). A complete separation of unchanged iodine (peak A), oxidized mono [^{125}I]NAPS-glucagon (peak B), unlabelled NAPS-glucagon and mono[^{125}I]NAPS-glucagon (peak C) can be achieved. From Fig. 1 it is evident that the main radioactive peak consists of the two possible Tyr[10] and Tyr[13] monoiodinated isomers because a slight separation tendency is observable. The incorporation of radioactive iodine into the glucagon molecule exceeds 80%, and the specific radioactivity of the obtained tracer is 2000 μCi/nmol. Electrophilic substitution with radioactive iodine leads to an increase in hydrophobicity of NAPS-glucagon. In Fig. 2 it is shown that the coupling of NAPS-Cl to the Tryp-25 of the glucagon molecule leads to an increase in the retention time on a C_{18} column. Peak A represents native glucagon and peak B NAPS-glucagon. The iodination of both hormones (native glucagon and NAPS-glucagon) results in the production of derivatives with higher hydrophobicity towards the octadecylsilyl stationary phase (peak C: [^{125}I]glucagon, peak D: [^{125}I]NAPS-glucagon).
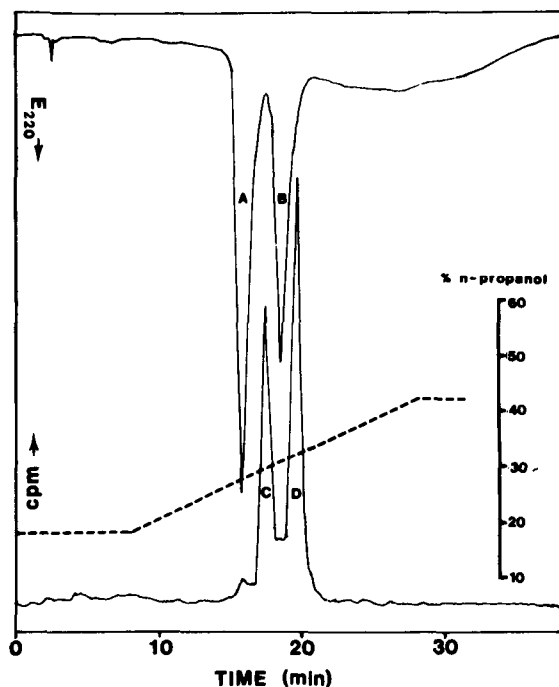

Fig. 2. Elution profile of unlabelled glucagon (peak A), NAPS-glucagon (peak B) and the respective radioactively monoiodinated derivatives (peaks C and D). The syntheses were performed as described under Materials and Methods. The elution was performed on a LiChrosorb C_{18} column using a linear gradient from 30% to 70% of B-buffer (A, 10 mM KH_2PO_4, pH 2.5; B, 10 mM KH_2PO_4 in 60% n-propanol, pH 2.5), completed in 20 min with a flow-rate of 1 ml/min at 40°C. The absorbance at 220 nm and the radioactivity were monitored on-line.

Enzymatic digestion of the monoiodinated NAPS-glucagon

In order to characterize the obtained radioactive NAPS-glucagon derivative concerning the position and degree of iodination, tryptic and chymotryptic cleavage
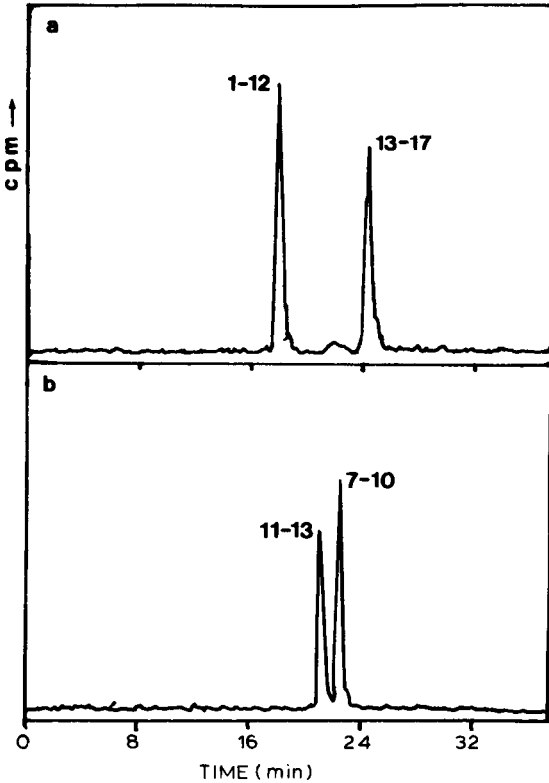
Fig. 3. Reversed-phase HPLC of the tryptic (a) and chymotryptic (b) digests of mono[^{125}I]NAPS-gluca-gon. The elution was performed on a LiChrosorb C_{18} column using a linear gradient of 0 to 30% of buffer B completed in 30 min.

was performed. The result is shown in Fig. 3. Tryptic digestion (Fig. 3a) of [^{125}I]NAPS-glucagon yields two radioactive peaks, each containing nearly 50% of the applied radioactivity.

The radioactive fragments show retention times identical with those of frag-ments obtained from tryptic cleavage of mono[^{125}I]glucagon under the given chro-matographic conditions. The digestion products therefore must be fragment 1–12 and fragment 13–17 originating from Tyr^{10} and Tyr$_{13}$ monoiodinated NAPS-glu-cagon. Digestion of the lyophilized fragments with pronase yields a product that can be correlated with monoiodinated tyrosine by HPLC (not shown) and is an indication for monoiodination. Chymotryptic digestion of mono[^{125}I]NAPS-glucagon (Fig. 3b) also produces two radioactive fragments with retention times identical with those from chymotryptic fragments from mono[^{125}I]glucagon. The cleavage products therefore must be fragments 7–10 and 11–13. These results suggest that the two tyrosine residues of the intact NAPS-glucagon molecule are equivalently reactive towards iodine labelling.

Binding of mono[^{125}I]NAPS-glucagon to isolated intact rat hepatocytes

The binding of mono[^{125}I]NAPS-glucagon to isolated intact rat hepatocytes

was studied in relation to mono[^{125}I]glucagon. For the determination of the apparent dissociation constants, hepatocytes were incubated in the presence of mono[^{125}I]NAPS- and mono[^{125}I]glucagon (*ca.* 70 000 cpm) and increasing concentrations of glucagon (50 f*M*–10 μ*M*) until a steady state is reached (30 min). The competitive binding assay data were analysed by a non-linear least-squares curve-fitting procedure (Fig. 4) as previously described[13]. The apparent dissociation constant for the binding of mono[^{125}I]NAPS-glucagon is larger by a factor of 2–3 than the K_d value for the binding of mono[^{125}I]glucagon.
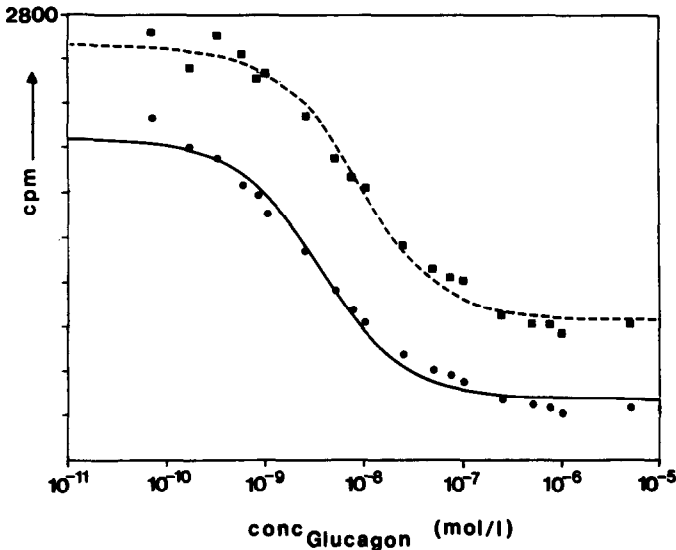

Fig. 4. Competitive binding of the mono[^{125}I]labelled glucagon and NAPS-glucagon to isolated intact rat hepatocytes in the presence of increasing concentrations of native glucagon. The incubation was performed as described previously[14]. Binding data were evaluated by a computerized curve-fitting procedure. Displacement of [^{125}I]glucagon (●—●) and [^{125}I]NAPS-glucagon (■---■).

Stability of the HPLC-purified mono[^{125}I]NAPS-glucagon

The stability of radiolabelled NAPS-glucagon was tested by reversed-phase HPLC. The photosensitive peptide was stable for at least 2 months, when neutralized and stored in Tris–HCl buffer containing 1% bovine serum albumin. It was rapidly radiolysed when stored neutralized but in an albumin-free medium.

DISCUSSION

The technique reported for the purification of biologically active radioiodinated photosensitive glucagon tracer employed reversed-phase HPLC on a C_{18} column using an isocratic elution system. This technique allows the rapid recovery of the tracers and the direct calculation of their specific activity. As the method permits complete separation of the unchanged peptide, we choose to use experimental conditions under which only a small amount of the peptide is labelled in order to minimize the amount of di- and polyiodinated peptide. The iodinated NAPS-glucagon

was more strongly bound to the column phase than the unlabelled peptide, which coincides with iodinated glucagon and native glucagon. As the main radioactive product we obtained a mixture of Tyr[10] and Tyr[13] monoiodinated NAPS-glucagon with the highest possible specific radioactivity.

The binding characteristics of the mono[^{125}I]NAPS-glucagon to isolated intact rat hepatocytes indicated that the introduction of an NAPS residue (a hydrophobic group) increased the K_d value from 3 nM to 7 nM under subdued light. We are at present investigating [^{125}I]NAPS-glucagon as a photoactivable radioactive probe for glucagon receptors.

REFERENCES

1 B. S. Welinder, S. Linde and B. Hansen, *J. Chromatogr.*, 265 (1983) 301.
2 D. E. Peavy, J. D. Abram, B. H. Frank and W. C. Duckworth, *Endocrinology*, 114,5 (1984) 1818.
3 B. S. Welinder, S. Linde, B. Hansen and D. Sonne, *J. Chromatogr.*, 298 (1984) 41.
4 V. Pingoud and I. Trautschold, *Anal. Biochem.*, 140 (1984) 305.
5 F. J. Rojas, T. L. Swartz, R. Iyengar, A. J. Garber and L. Birnbaumer, *Endocrinology*, 113,2 (1983) 711.
6 W. A. Hagopian and H. S. Tager, *J. Biol. Chem.*, 259,14 (1984) 8986.
7 H. Antoniotti, P. Fagot-Revurat, J. P. Esteve, D. Fourmy, L. Pradayrol and A. Ribet, *J. Chromatogr.*, 296 (1984) 181.
8 N. G. Seidah, M. Dennis, P. Corvol, J. Rochemont and M. Chretien, *Anal. Biochem.*, 109 (1980) 185.
9 M. N. Guy, G. L. Roberson and L. D. Barnes, *Anal. Biochem.*, 112 (1981) 272.
10 M. Svoboda, M. Lambert, L. Moroder and J. Christophe, *J. Chromatogr.*, 296 (1984) 199.
11 C. D. Demoliou and R. M. Epand, *Biochemistry*, 19, 20 (1980) 4539.
12 M. Veronese, E. Boccu and A. Fontana, *Int. J. Prot. Res.*, II (1970) 67.
13 F. Peters and V. A. Pingoud, *Biochim. Biophys. Acta*, 714 (1982) 442.
14 V. A. Pingoud, F. Peters, T. D. U. Haas and I. Trautschold, *Biochim. Biopohys. Acta*, 714 (1982) 448.