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ONE-STEP ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATO-GRAPHIC PURIFICATION OF RADIOIODINATED AND RADIOIODINA-TED-PHOTOACTIVABLE DERIVATIVES OF CHOLECYSTOKININ

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SUMMARY

N-Hydroxysuccinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionate (the Bolton–Hunter reagent) was conjugated with (Thr 34, NLeu 37) cholecystokinin $(CCK)_{31-39}$ in anhydrous dimethylformamide–pyridine in 20% yield. The radiolabelled peptide was purified from the reaction mixture in one step, by isocratic elution from a C_{18} high-performance liquid chromatographic (HPLC) column with 35% aqueous acetonitrile–0.13% heptafluorobutyric acid as eluent. The concentration of the radiolabelled peptide was estimated by UV monitoring. The acylating photoactivable radioiodinated reagent N-hydroxysuccinimidyl-N-(4-azido-2-nitrophenyl)-3-[^{125}I]iodotyrosine was synthesized, purified on a C_{18} HPLC column by isocratic elution with 65% acetonitrile–1 mM hydrochloric acid, then conjugated with (Thr 34, NLeu 37) CCK_{31-39}. The resulting photoactivable radioiodinated CCK analogue was purified by isocratic elution on a C_{18} HPLC column with 39% aqueous acetonitrile–0.1 M triethylamine phosphate (pH 3.5). The binding ability of both tracers and their non-radioactive analogues to CCK receptors was tested on rat pancreatic plasma membranes. As compared to a K_D of 4.5 nM for unmodified (Thr 34, NLeu 37) CCK_{31-39}, the K_D of the radioiodinated Bolton–Hunter derivative was 3 nM, and that of the photoactivable radioiodinated derivative was 19 nM.

INTRODUCTION

Cholecystokinin (CCK), a hormone and neurotransmitter, has been isolated in several molecular forms from gastrointestinal and neural tissues. The number of amino acid residues in the CCK family of peptides varies from 4 to 39, counting from the C-terminal end[1]. All biological activities are located on the seven C-terminal amino acids. The C-terminal octapeptide CCK_{32-39} (CCK-8) is the most potent and

most likely physiological form[2,3]. Appropriate peptide modifications may preserve the biological properties of the C-terminal heptapeptide moiety, and be successfully applied to introduce a radioactive label into natural CCK peptides, all of which, except CCK_{1-39}, lack a tyrosine residue[4]. This was achieved by coupling a iodinated N-hydroxysuccinimidyl ester to the free amino group of CCK-33 (CCK_{7-39})[5] and CCK-8[6] or iodinated methylimidate to the free amino group of CCK-8[7]. A photosensitive group was similarly coupled to CCK-8 through a N-hydroxysuccinimidyl ester[8].

In general, the study of receptors requires a pure tracer of known concentration, an aim that is not easily achieved. In the present case, the above CCK derivatives were purified either by simple gel filtration (that separates free from coupled reagents but poorly separates labelled from unlabelled peptides[5] or by laborious multi-step techniques, *e.g.*, ion-exchange chromatography followed by gel filtration, high-performance liquid chromatography (HPLC) combined with paper chromatography[5] and electrophoresis combined with paper chromatography[7].

Fourmy *et al.*[9] conjugated N-hydroxysuccinimidyl 3-(4-hydroxy-3-[^{125}I]iodophenyl) propionate (the Bolton–Hunter reagent, ^{125}I-BH-NHS) with (Thr 34, NLeu 37) CCK_{31-39} (or CCK-9N) in borate buffer with a 4–6% yield. The synthetic CCK analogue was well designed as a receptor probe: it possesses full biological activity; its N-terminal arginine residue facilitates acylation, and it does not contain the two methionine residues of CCK-9 that are easily oxidized. The conjugated product was separated by HPLC, on a C_{18} μBondapak column by a two-step gradient of acetonitrile, buffered with 0.25 *M* triethylamine phosphate (TEAP) (pH 3.5). Unfortunately, when switching the acetonitrile concentration from 26 to 50%, various products that were accumulated on the column were readily eluted so that the concentration and homogeneity of the ^{125}I-BH-CCK-9N collected could not be estimated. In the present study, we improved this method after observing that (a) the yield of acylation of CCK-9N with ^{125}I-BH-NHS in anhydrous dimethylformamide (DMF)–pyridine was four-fold higher than in borate buffer, and (b) the products of conjugation of CCK-9N could be separated by a one-step isocratic elution on a C_{18} μBondapak HPLC column when a 35% aqueous acetonitrile–0.13% heptafluorobutyric acid solution was used.

Our second aim was to design a tool to study the molecular structure of CCK receptors. The identification of the protein component(s) of hormone receptors is greatly facilitated after specific cross-linking with a radioactive ligand[10]. This approach for CCK receptors has already been investigated with ^{125}I-BH-CCK-33 which is readily photoactivable *per se*[11] or by chemical binding of CCK-33 with N-hydroxysuccinimidyl suberate[12,13]. Pure CCK-33 is, however, relatively unstable, not routinely available, and the yield of spontaneous cross-linking is low[11]. Synthetic CCK-9N is more readily available but has only one major reactive site (the N-terminal amino group of Arg) and no tyrosine residue, so that a reagent must be prepared that introduces the radioactive label together with the (photo)activable group. N-Hydroxysuccinimidyl 4-azido-3-[^{125}I]iodosalicylate, synthesized and conjugated by Ji and Ji[14] with concanavalin A, was useless, in our hands, when tested for conjugation with CCK-9N. We prepared another reagent bearing a radioactive label and photoactivable group: N-hydroxysuccinimidyl-N-(4-azido-2-nitrophenyl)-3-[^{125}I]iodotyrosine (^{125}I-NAP-Tyr-NHS). After conjugation with CCK-9N, the product was purified by isocratic HPLC on a C_{18} μBondapak column.

The binding properties of the two new radioiodinated and radioiodinated photoactivable CCK derivatives were tested *in vitro* on CCK receptors from rat pancreatic plasma membranes.

MATERIALS AND METHODS

Apparatus

The isocratic HPLC system consisted of a M 6000A solvent-delivery unit, a U6K injector and a 440 absorbance detector (280 nm) with flow cell of 10-mm path length from Waters Associates (Milford, MA, U.S.A.). In addition, absorbance at 206 nm was monitored with a Uvicord S with flow cell of 2.5-mm path length from LKB (Uppsala, Sweden). A Tricarb 320 monitor, equipped with a KI-containing flow detector, was from Packard (Downers Grove, IL, U.S.A.).

The stainless-steel C_{18} μBondapak column (3.9 × 250 mm) and radial compression module with the Radial-Pak A (C_{18}) cartridge were purchased from Waters Associates.

Chemicals

Synthetic nonapeptide (Thr 34, NLeu 37) CCK_{31-39} (CCK-9N) was synthesized by one of us (L.M.). The synthetic C-terminal octapeptide of cholecystokinin-pancreozymin CCK_{32-39} (CCK-8) was a generous gift from Dr. S. J. Lucania (Squibb Institute for Medical Research, Princeton, NJ, U.S.A.). N-Hydroxysuccinimidyl 3-(4-hydroxy-3-[^{125}I]iodophenyl)propionate (^{125}I-BH-NHS; iodinated Bolton–Hunter reagent) and $Na^{125}I$ were from Amersham Radiochemical Centre (Bucks, U.K.). High-performance thin-layer chromatographic (HPTLC) silica plates were from Merck (Darmstadt, F.R.G.). Acetonitrile (HPLC grade) was from Carlo Erba (Milan, Italy); heptafluorobutyric acid (HFBA) for sequential analysis was from Fluka (Buchs, Switzerland). N,N-Dimethylformamide (DMF), pyridine (silylation grade) and N-hydroxysuccinimidyl 3-(4-hydroxyphenyl)propionate (BH) were from Pierce (Rockford, IL, U.S.A.). Human and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Preparation of 3-(4-hydroxy-3-iodophenyl)propionyl (Bolton–Hunter) derivatives of CCK-9N

Unlabelled ^{127}I-BH derivative of CCK-9N. To 0.2 μmol of N-hydroxysuccinimidyl 3-(4-hydroxyphenyl)propionate and 0.1 μmol of $K^{127}I$ in 50 μl of acetonitrile–water (80:20) were added 20 μl of 20 mM chloramine T in 0.2 M phosphate buffer (pH 7.5); after 1 min, 10 μl of 50 mM sodium sulphite were added, the mixture was acidified with 10 μl of 1 M hydrochloric acid and immediately chromatographed on a C_{18} μBondapak column using water–acetonitrile (50:50) + 1 mM hydrochloric acid as eluent. The elution was performed at 1 ml/min and the peak at retention time $t_R = 6.5$ min was collected. Acetonitrile was quickly evaporated under a nitrogen stream and ^{127}I-BH extracted with 1% DMF in benzene. After evaporation of benzene under nitrogen, 50 μl of 0.2 mM CCK-9N, in 2% pyridine in DMF, were added. After standing overnight at 4°C, in a phosphorus pentoxide-dried atmosphere, the mixture was diluted in 50 μl water, acidified with 10 μl of 10% HFBA, then chromatographed on C_{18} μBondapak using 35% aqueous acetonitrile–0.13% HFBA as

eluent (1 ml/min). The peak at t_R = 22 min, identified as ^{127}I-BH-CKK-9N, was collected.

The yield of coupling in regard to ^{127}I-BH was 40%, while the yield of coupling, performed in parallel, in 0.1 *M* borate buffer (pH 8.5), was only 13%. The concentration of ^{127}I-BH-CCK-9N was assayed by chromatography on C_{18} μBondapak, using UV-transparent acetonitrile–water (35:65) + 0.1 *M* TEAP (pH 3.5) as eluent. Absorbancies at 280 nm and 206 nm were monitored and the areas of the ^{127}I-BH-CCK-9N peak (t_R = 11.5 min) were compared to peak areas obtained with CCK-9N solutions of known concentration. In this computation the absorption coefficient of the BH moiety was taken into account.

Radiolabelled ^{125}I-BH derivative of CCK-9N. 1 mCi (0.5 nmol) of commercial ^{125}I-BH was evaporated to dryness under a gentle stream of nitrogen. Then, 40 μl of 0.5 m*M* CCK-9N (20 nmol), in 2% pyridine in DMF, were added. The mixture was allowed to stand overnight at 4°C in a dry atmosphere. The coupling efficiency was followed by high-performance thin-layer chromatography (HPTLC) as described in Fig. 1. The mixture was then diluted in 100 μl water and acidified with 10 μl of 10% HFBA, before purification, as described in Fig. 2.

Under these conditions as well as in all the other chromatographic experiments (see below), the recovery of the applied amount of radioactivity was at least 90%. After each isocratic chromatography, the system was washed out with 75% of aqueous acetonitrile and the radioactivity remaining on the precolumn was negligible, as determined by counting the packing of the precolumn after ten successive elutions.

Preparation of unlabelled and labelled N-(4-azido-2-nitrophenyl)-3-iodotyrosyl derivatives of CCK-9N (I-NAP-Tyr-CCK-9N)

All operations were conducted in subdued light.

Synthesis of the N-hydroxysuccinimidyl ester of NAP-Tyr (NAP-Tyr-NHS). N-(4-Azido-2-nitrophenyl)tyrosine (NAP-Tyr) was first prepared by coupling 4-fluoro-3-nitrophenyl azide with tyrosine in dimethyl sulphoxide (DMSO), in the presence of triethylamine, as described by Levy[15]. The product was purified on a silica gel column (3.5 × 60 cm, silica gel 40 for column chromatography, Merck) eluted with 2 l of chloroform. The purity of each fraction was checked by TLC on silica gel plates, eluted with chloroform–methanol–25% ammonium hydroxide (48:48:2). The R_F value of NAP-Tyr was 0.4.

NAP-Tyr-NHS, the N-hydroxysuccinimidyl ester of NAP-Tyr, was then prepared in dichloromethane by using dicyclohexylcarbodiimide as coupling reagent. The ester was purified by chromatography on Whatman No. 3MM paper with dichloromethane–*n*-heptane (1:1) as eluent. The R_F value of the ester was 0.3, while the unreacted acid remained at the origin. The ester was eluted from the paper with dichloromethane and stored in this solvent. It was used within 1 week, older solutions having to be repurified by paper chromatography.

Preparation of ^{127}I-NAP-Tyr. Unlabelled iodinated ^{127}I-NAP-Tyr was prepared by dissolving 42 μmol of NAP-Tyr and 12 μmol of ^{127}I in 3 ml of 0.1 *M* phosphate buffer (pH 7.5), followed by addition of 2 × 0.5 ml of 20 m*M* chloramine T (20 μmol) at 1-min intervals. After addition of 50 μmol of sodium sulphite, the reaction mixture was acidified with HCl to pH 1. All NAP-Tyr derivatives were extracted with diethyl ether; then ^{127}I-NAPTyr was purified with a C_{18} Radial-Pak

A cartridge, using 50% aqueous acetonitrile–3 mM HCl as eluent (0.7 ml/min): retention volumes were 11 ml, 18 ml and 30 ml respectively for non-iodinated, mono-iodinated and diiodinated NAP-Tyr (up to 5 μmol of the NAP-Tyr mixture could be injected at a time with good resolution). Monoiodinated [127]I-NAP-Tyr-NHS was purified by paper chromatography (R_F = 0.5), as described for the non-iodinated ester (see above).

Preparation of unlabelled [127]*I-NAP-Tyr-CCK-9N*. To 48 nmol of dry [127]I-NAP-Tyr-NHS were added 60 μl of 1 mM CCK-9N (60 nmol) in 2% pyridine in DMF. The reaction mixture was allowed to stand for 2 days in a P_2O_5-dried atmosphere at 4°C. The mixture was then diluted in 200 μl of 40% aqueous acetonitrile, acidified with 20 μl of 1% phosphoric acid and chromatographed on a C_{18} μBondapak column, using acetonitrile–water (41:59) + 0.1 M TEAP (pH 3.5) as eluent (Fig. 3).

Preparation of radiolabelled ^{125}I-NAP-Tyr-CCK-9N

Radioiodination of NAP-Tyr-NHS for direct coupling. To 2.5 mCi of carrier-free Na[125]I (1.25 nmol) in 10 μl of 0.1 M phosphate buffer (pH 7.5) were added 10 μl of 0.1 mM NAP-Tyr-NHS (1.0 nmol) in acetonitrile, followed by rapid addition of 10 μl of 1 mM chloramine T (10 nmol) and 10 μl of 2.5 mM sodium sulphite, both in 0.1 M phosphate buffer (pH 7.5). The iodination, conducted in less than 30 sec, was immediately followed by addition of 20 μl of 1 mM CCK-9N. After 5 min, the mixture was acidified with 20 μl of 1% phosphoric acid and promptly chromatographed, as described in Fig. 4.

Radioiodination of NAP-Tyr-NHS followed by purification of the iodinated ester before coupling. To 5 mCi in 50 μl of carrier-free Na[125]I (2.5 nmol) were added 10 μl of 0.2 M phosphate buffer (pH 7.5), 50 μl of 0.3 mM NAP-Tyr-NHS (15 nmol) in acetonitrile, 10 μl of 2 mM chloramine T in 0.2 M phosphate buffer and 15 μl of 5 mM sodium sulphite in 0.3 M hydrochloric acid. The mixture was applied to a C_{18} Radial-Pak A cartridge and eluted with 65% aqueous acetonitrile–0.5 mM HCl (1 ml/min). About 80% of the radioactivity was eluted as ^{125}I-NAP-Tyr-NHS with t_R = 8 min. Immediately after peak collection, 0.2 ml of 0.1 mM CCK-9N in 0.1 M phosphate buffer (pH 7.5) were added. The acetonitrile was evaporated under a nitrogen stream and the mixture was chromatographed on C_{18} μBondapak using acetonitrile–water (39:61) + 0.1 M TEAP (pH 3.5) as eluent (Fig. 5).

Binding of radioiodinated derivatives of CCK-9N to rat pancreatic plasma membranes

Purified rat pancreatic membrane proteins (10–20 μg)[16] were incubated in duplicate for 15 min at 37°C in 120 μl (total volume) of a medium consisting of 20 mM Tris–HCl (pH 7.4), 0.5 mg/ml bacitracin, 1% human serum albumin, 0.5 mM o-phenanthroline, 5 mM MgCl$_2$ and 20,000–50,000 cpm ^{125}I-BH-CCK-9N or ^{125}I-NAP-Tyr-CCK-9N. In the latter case, the incubation was conducted in subdued light. All incubations were terminated by rapid filtration on Whatman GFC filter (diameter 2.5 cm) and three washings with 2 ml of 20 mM Tris–HCl (pH 7.4) containing 0.2% bovine serum albumin. Non-specific binding was measured in the presence of 1 μM CCK-8 and subtracted from total counts to obtain specific binding.

RESULTS

Preparation of ^{125}I-BH-CCK-9N

Bolton and Hunter[17] conjugated their reagent with proteins in aqueous borate buffer (pH 8.5). Alternatively, the conjugation of this reagent can be performed in an anhydrous medium of DMF, in the presence of an organic base such as pyridine or triethylamine[6,7]. We compared, therefore, the yield of conjugation of ^{125}I-BH-NHS with CCK-9N in borate buffer and DMF–2% pyridine. As shown in Fig. 1, the yield of conjugation was much higher under anhydrous conditions. Various conjugated contaminants were separated, in addition to ^{125}I-BH-CCK-9N and the acid derived from the activated ester. Their number and importance increased when the chemicals were not of the highest purity.

The purification of ^{125}I-BH-CCK-9N from the reaction mixture was achieved, in one step, by isocratic elution on a C_{18} µBondapak column (Fig. 2). The eluent was 35% aqueous acetonitrile–0.13% HFBA, HFBA being required to separate the radiolabelled peptide from CCK-9N and irrelevant radiolabelled products. The UV monitoring at 280 nm of ^{125}I-BH-CCK-9 allowed us to calculate the specific radioactivity and the concentration, a value of importance in experiments on binding to CCK receptors (see below). In five preparations, the yield of ^{125}I-BH-CCK-9N varied from 10 to 32% (mean 20%) with respect to the ^{125}I-BH-NHS added.

I·BH-CCK-9N ———

CCK-9N ———

Fig. 1. Comparison of the yield of ^{125}I-BH coupling in aqueous and anhydrous media. 0.25 mCi of commercial ^{125}I-BH were coupled with 10 nmol of CCK-9N in either 20 µl of DMF–2% pyridine (lane 1) or 20 µ of 0.1 *M* borate buffer (pH 8.5) (lane 2). After standing overnight at 4°C (in a dry atmosphere) each mixture was diluted in 80 µl of 50% aqueous acetonitrile and analyzed by silica gel HPTLC. The plate was first developed to half-height with butanol–pyridine–acetic acid–water (30:20:3:12), then development was completed (total plate height) with chloroform–methanol (2:1). The HPTLC plate was subjected to autoradiography on X-Omat S Kodak film. The arrows show the position of ^{125}I-BH-CCK-9N, as compared to a non-labelled CCK-9N standard (revealed by the TRP-specific *p*-dimethylaminobenzaldehyde reagent).
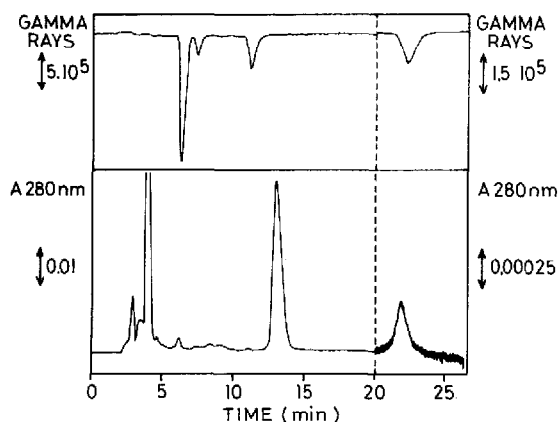
Fig. 2. Isocratic HPLC purification of ^{125}I-BH-CCK-9N. The coupling of ^{125}I-BH with CCK-9N was performed in DMF–2% pyridine, as described under Materials and methods. After acidification, the reaction mixture was applied to a C_{18} μBondapak column, eluted at 1 ml/min with 35% aqueous acetonitrile–0.13% HFBA. The absorbance at 280 nm and the radioactivity (arbitrary units) were monitored. After 20 min the sensitivity of A_{280} monitoring was increased 40 times and the sensitivity of radioactivity monitoring was increased 3 times. Free CCK-9N was eluted at 13 min, and conjugated ^{125}I-BH-CCK-9N was eluted at 22 min. The yield was 17% with respect to the ^{125}I-BH ester used in the reaction.

Preparation of [127]I-NAP-Tyr-CCK-9N and ^{125}I-NAP-Tyr-CCK-9N

The conjugation of CCK-9N with a photoactivable iodinated probe was performed at first using the non-radioactive [127]I isotope. The reagent [127]I-NAP-Tyr-NHS was prepared in dry form (see Materials and methods) so as to conjugate CCK-9N in anhydrous DMF–2% pyridine. The separation of the products was accomplished by isocratic elution from a C_{18} μBondapak column (Fig. 3). Peak 1, when collected, migrated as a single spot on silica gel HPTLC, under the conditions described in Fig. 1. This spot, containing the [127]I-NAP-Tyr chromophore, was yellow and reacted with the tryptophan-specific reagent p-dimethylaminobenzaldehyde. Peak 1 was, thus, identified as the [127]I-NAP-Tyr-CCK-9N that contains one Trp residue, and its biological activity was tested (see below). As shown in Fig. 3 the yield of peptide acylation was very high, 76% of the iodinated photoactivable chromophore being incorporated into CCK-9N.

In our first attempt to prepare a radioiodinated carrier-free ^{125}I-NAP-Tyr-NHS reagent, we proceeded as for the non-radioactive material: radioiodinating NAP-Tyr, then purifying and drying monoiodinated ^{125}I-NAP-Tyr and esterifying this compound with NHS, in an anhydrous medium. Unfortunately, concentrating the NAP-Tyr chromophore to dryness, after iodination with carrier-free ^{125}I, led to radiolysis. We were forced, therefore, to iodinate NAP-Tyr-NHS directly. The iodination of phenols is known to proceed rapidly in an aqueous medium at pH > 7, but in our case, where a phosphate buffer (pH 7.5) was used, these conditions led to the hydrolysis of as much as 50% of NAP-Tyr-NHS in 1 min. To slow down this hydrolysis, we turned to acetonitrile–phosphate buffer (pH 7.5) (1:1): there was a rapid iodination of NAP-Tyr-NHS (all operations took less than 30 sec) and the hydrolysis (less than 10%) was, this time, much lower than in 100% phosphate buffer.

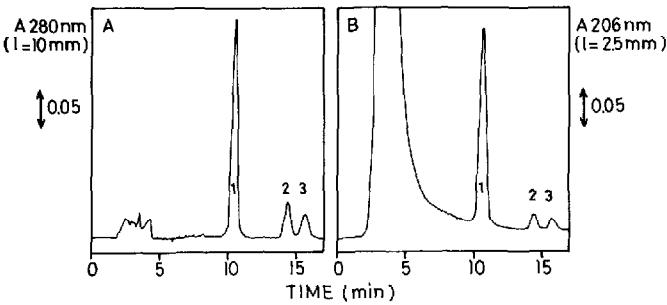Two procedures were then tested for coupling [127]I-NAP-Tyr with CCK-9N:

Fig. 3. Isocratic HPLC purification of unlabelled [127]I-NAP-Tyr-CCK-9N. The coupling of CCK-9N with the photosensitive reagent was performed in DMF–2% pyridine, as described under Materials and methods. The reaction mixture was chromatographed on a C_{18} μBondapak column, eluted at 1 ml/min with acetonitrile–water (41:59) + 0.1 M TEAP (pH 3.5). Absorbances at 280 nm (flow-cell path length = 10 mm) and 206 nm (flow-cell path length = 2.5 mm) were monitored. CCK-9N was eluted ahead of various contaminants. Peak 1 was identified as [127]I-NAP-Tyr-CCK-9N, peak 2 as [127]I-NAP-Tyr acid and peak 3 was not identified. The yield was 76% with respect to the amount of [127]I-NAP-Tyr-NHS ester used.

(a) adding the peptide immediately to the total iodination mixture, and (b) purifying the monoiodinated ester before coupling.

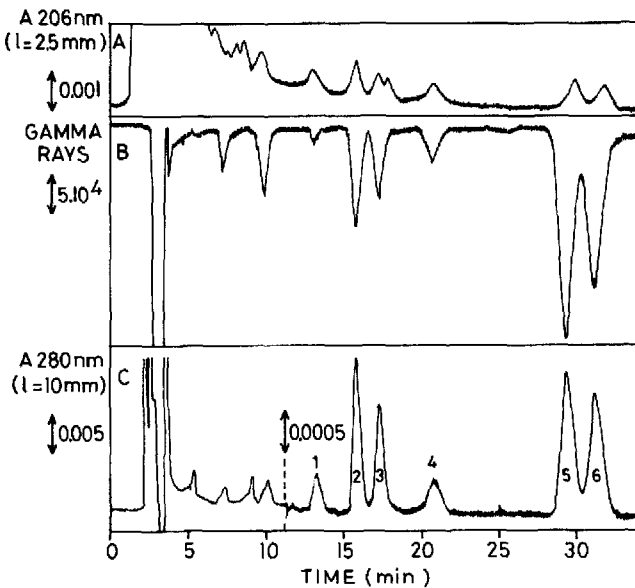(a) CCK-9N, when added to the iodination mixture, coupled with all active

Fig. 4. Isocratic HPLC purification of monoiodinated ^{125}I-NAP-Tyr-CCK-9N after direct coupling of CCK-9N with the reaction mixture of NAP-Tyr-NHS. Radioiodination was performed as described under Materials and methods. The final mixture was injected on a C_{18} μBondapak column, eluted at 1 ml/min with acetonitrile–water (39:61) + 0.1 M TEAP (pH 3.5). The absorbances at 280 nm and 206 nm and radioactivity (in arbitrary units) were monitored. Fractions of 0.5 ml were collected at 0°C in tubes containing 0.5 ml of a protective solution, consisting of 10 mg/ml bovine serum albumin, 0.5 mg/ml soy bean trypsin inhibitor, 1 mM K[127]I and 20 μM NAP-Tyr. Peak 1 was monoiodinated ^{125}I-NAP-Tyr-CCK-9N and peak 4 was diiodinated [125]I_2-NAP-Tyr-CCK-9N. Peak 3 was monoiodinated ^{125}I-NAP-Tyr, and peak 6 diiodinated [125]I_2-NAP-Tyr. Peaks 2 and 5 were not identified.

esters present in the medium. The chromatographic profile of this reaction mixture is shown in Fig. 4. Several radioactive peaks were present whose comparison with non-radioactive standards was difficult, due to salt effects on t_R. This difficulty was circumvented when taking into account the fact that the radioactivity: absorbance ratio of diiodinated products was twice that of monoiodinated products. In addition, the absorbance of CCK-9N was ten-fold higher at 206 nm than at 280 nm, whereas the absorbances of the NAP-Tyr chromophore at 206 nm and 280 nm were similar, so that the A (206 nm): A (280 nm) ratio was relatively high in all peptidic materials. Based on these observations, the small peak 1 was identified as mono-iodo ^{125}I-NAP-Tyr-CCK-9N and peak 4 as di-iodo ^{125}I$_2$-NAP-Tyr-CCK-9N. Peaks 3 and 6 were, respectively, mono-iodo and di-iodo NAP-Tyr. Peaks 2 and 5 were unknown products. The identity of the CCK-9N derivatives was confirmed by HPTLC on silica gel (by comparison with non-radioactive standards) and in binding experiments on rat pancreatic CCK receptors (see below). In conclusion, this direct coupling method with CCK-9N allowed a only small portion of iodine-125 to be incorporated as mono-iodo activated ester.

(b) To obtain mono-iodinated ^{125}I-NAP-Tyr-NHS, before coupling, a six-fold excess of NAP-Tyr-NHS over iodine was used. This allowed the production of ^{125}I-NAP-Tyr-NHS with a 80% yield with respect to the ^{125}I used. The pure mono-radioiodinated ester was collected in 1–2 ml of acetonitrile–water (65:35) + 1 mM hydrochloric acid, as described under Materials and methods. Using this eluent, the hydrolysis of the ester was relatively slow (no more than 50% in 30 min), but all attempts to concentrate the ester led to its hydrolysis. The ester was, therefore, coupled immediately after collection, by adding CCK-9N in phosphate buffer (this buffer giving, in our hands, better results than the usual borate buffer). The separation
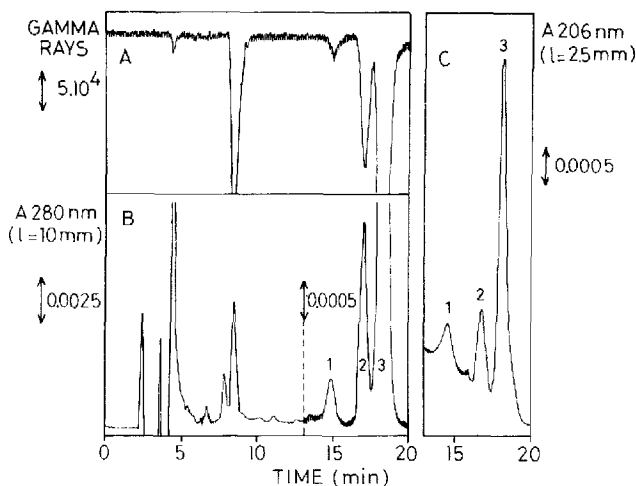

Fig. 5. Isocratic HPLC purification of ^{125}I-NAP-Tyr-CCK-9N, prepared by coupling of CCK-9N with purified monoradioiodinated ^{125}I-NAP-Tyr-NHS, as described under Materials and methods. The reaction mixture was chromatographed on C$_{18}$ μBondapak, eluted at 1 ml/min with acetonitrile–water (39:61) + 0.1 M TEAP (pH 3.5). The absorbances at 280 nm and 206 nm and radioactivity (in arbitrary units) were monitored. Fractions of 0.5 ml were collected in tubes containing 0.5 ml of a protective solution (composition as in Fig. 4). Peak 1 was identified as ^{125}I-NAP-Tyr-CCK-9N, and peak 3 as ^{125}I-NAP-Tyr. Peak 2 was not identified.

of the reaction mixture is illustrated in Fig. 5. There were fewer peaks than with the preceding method (Fig. 4) and their identification was easier, as all of them were mono-iodinated. Peak 1 proved to be ^{125}I-NAP-Tyr-CCK-9N, as confirmed by HPTLC and biological activity. Peak 2 was an unknown product (its importance increased at pH > 8). Peak 3 was ^{125}I-NAP-Tyr.

Binding of CCK-9N derivatives to rat pancreatic CCK receptors

The binding of ^{125}I-BH-CCK-9N (0.2–1 nM) to rat pancreatic plasma membranes reached equilibrium after 10 min at 37°C and was constant for a further 10 min period (data not shown). Non-specific binding, evaluated in the presence of 1 μM CCK-8, corresponded to 5% of total binding. ^{125}I-BH-CCK-9N was displaced by [127]I-BH-CCK-9N with a K_D value of 3.2 ± 0.6 nM and by CCK-9N with a K_D value of 4.5 ± 0.3 nM. Increasing concentrations of [127]I-NAP-Tyr-CCK-9N (in subdued light) inhibited the binding of ^{125}I-BH-CCK-9N with a K_D value of 19 ± 3 nM (Fig. 6A).

^{125}I-NAP-Tyr-CCK-9N was also able to bind to rat pancreatic membranes: in subdued light a plateau was attained after 8 min at 37°C, and binding remained stable for a further 5–8 min. However, non-specific binding, measured with 1 μM CCK-8 or 1 μM [127]I-NAP-Tyr-CCK-9N, corresponded to 45–60% of total binding. This high non-specific binding was not significantly decreased when 0.1 mM NAP-Tyr was added to the incubation medium. The K_D value of [127]I-NAP-Tyr-CCK-9N, derived from competition curves of ^{125}I-NAP-Tyr-CCK-9N with increasing concentrations of [127]I-NAP-Tyr-CCK-9N (Fig. 6B), was 6–14 nM. The small discrepancy between this value and the value of 19 nM, derived from competition experiments between ^{125}I-BH-CCK-9N and [127]I-NAP-Tyr-CCK-9N, perhaps reflects the relatively low accuracy of the determination of the [127]I-NAP-Tyr-CCK-9N concentration (due to some adsorption to glassware at low concentrations, despite the storage
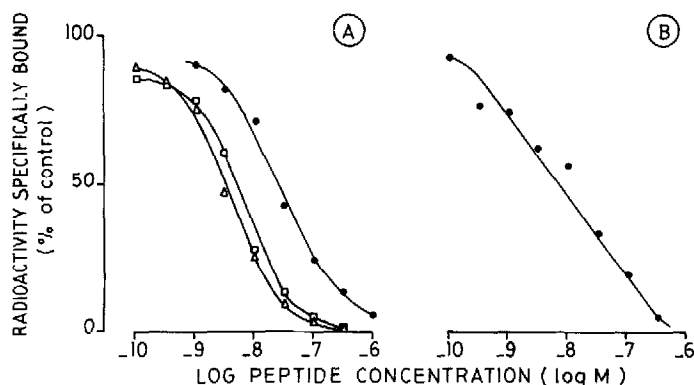

Fig. 6. Competition of ^{125}I-labelled and unlabelled analogues of CCK-9N for binding to rat pancreatic CCK receptors. A, Pancreatic membranes were incubated for 15 min at 37°C, as described in Materials and methods, with 35,000 cpm of ^{125}I-BH-CCK-9N in the presence of increasing concentrations of unlabelled CCK-9N (□), [127]I-BH-CCK-9N (△) or [127]I-NAP-Tyr-CCK-9N (●). B, Pancreatic membranes were incubated for 15 min at 37°C with 27,000 cpm of ^{125}I-NAP-Tyr-CCK-9N in the presence of increasing concentrations of [127]I-NAP-Tyr-CCK-9N in a total volume of 60 μl of the binding medium as described in Materials and methods. The results (one representative of three similar experiments) are expressed as per cent of radioactivity specifically bound in the presence of tracer only.

conditions indicated in Fig. 4) and the use of different batches of [127]I-NAP-Tyr-CCK-9N in the two series of experiments.

Stability of the CCK-9N derivatives

The stability of radiolabelled CCK-9N derivatives was checked by HPTLC, performed as in Fig. 1, and by binding experiments, as in Fig. 6. ^{125}I-BH-CCK-9N, stored at −18°C, was stable for at least 2 months. By contrast, ^{125}I-NAP-Tyr-CCK-9N was rapidly radiolysed when stored in unprotected concentrated solution, but when 10 μM NAP-Tyr was present in this storage solution, the photosensitive peptide remained unchanged for at least 2 weeks.

DISCUSSION

The techniques employed to purify biologically active radioiodinated tracers, free from unlabelled hormone, have greatly benefited from the introduction of HPLC[18]. The separation, by HPLC on a C_{18} μBondapak column, of a peptide where iodine has been introduced on a phenol ring, is facilitated by the increased interaction of this modified peptide with the C_{18} stationary phase. We tried to use this type of column without resorting to an expensive gradient system that would be devoted only to the purification of highly radioactive material. The development of appropriate isocratic elution must, however, take account of the tendency of peptides to develop multisite binding interactions with the C_{18} stationary phase under these conditions[19].

Adding an acidic modifier to the eluent is as a rule useful in this respect[20], but the TEAP-containing eluent (pH 3.5), used by Fourmy *et al.*[9], allowed quantitative elution of ^{125}I-BH-CCK-9N from a C_{18} column only after an abrupt increase in acetonitrile concentration so that several contaminants were also eluted, preventing the determination of the ligand concentration. Perfluorinated acids of various carbon chain lengths are also utilized for the reversed-phase HPLC of peptides because of their good solubizing properties (for review see ref. 18) and their capacity to alter the relative t_R of peptides[21]. We found, indeed, that HFBA allowed isocratic purification of ^{125}I-BH-CCK-9N (Fig. 2) of known concentration and well defined biological activity (Fig. 6).

Our HPLC detectors were able to monitor UV absorbances at 280, 254 and 206 nm, so that the ratio of absorbances at 206 and 280 nm could be used to identify some of the reaction products (see below). Thus, the use of a perfluorinated acid, such as HFBA, should be avoided, because it absorbs at 206 nm. Moreover, HFBA was described to have corrosive action on a C_{18} silica stationary phase[22]. We were fortunate, therefore, to observe that, with TEAP as acidic modifier in the mobile phase, the hetero-bifunctional photoactivable agent ^{125}I-NAP-Tyr-CCK-9N could be purified by isocratic elution (Figs. 4 and 5). This 206 nm-transparent eluent allowed the determination of a 280 nm: 206 nm ratio that led to the identification of the separated ^{125}I-NAP-Tyr derivatives.

Only a few photoactivable radiolabelled agents have been prepared so far and some have only a low specific radioactivity[23] or cannot be coupled with peptides[24,25]. Ji and Ji[14] prepared N-hydroxysuccinimidyl 4-azido-3-[^{125}I]iodosalicylate, which was iodinated in acetone solution and purified by TLC on silica gel. Our attempt to obtain

a iodinated 4-azidosalicylate derivative of CCK-9N by this approach was unsuccessful. Another reagent, the N-hydroxysuccinimide ester of 4-azidobenzoylglycyltyrosine, similar to NAP-Tyr-NHS, was also considered by Ji and Ji[14], but its iodination and/or purification leads to breakdown into several derivatives. Similarly, we found that dry silica gel (and also alumina) quickly hydrolyzed NAP-Tyr-NHS (iodinated or not) to the corresponding acid (this phenomenon was easily monitored, the ester being yellow and the liberated acid red). We then resorted to paper chromatography or cellulose TLC: both procedures led to poor but sufficient resolution to allow the isolation of active unlabelled ^{127}I-NAP-Tyr-NHS under anhydrous conditions. Even so, these separation techniques could not be applied to carrier-free dry ^{125}I-NAP-Tyr-NHS because of rapid radiolysis. The problem was finally solved by purifying ^{125}I-NAP-Tyr-NHS on a C_{18} μBondapak column under slightly acidic conditions that secured reasonable stability of the active ester (see Results).

The advantage of using purified ^{125}I-NAP-Tyr-NHS was the limited number of coupling products and the easy identification of mono-^{125}I-NAP-Tyr-CCK-9N in the HPLC eluent (Fig. 5). The only inconvenience of this approach was that, with the 35% aqueous eluent utilized, the coupling yield to CCK-9N was lower than in an anhydrous medium.

The coupling efficiency of the two N-hydroxysuccinimidyl esters utilized in the present study with CCK-9N depended on: (a) the nature of the active ester, I-NAP-Tyr-NHS being more reactive and less stable than I-BH-NHS, and (b) the relative concentrations of ester, peptide and water. The yield was satisfactory under anhydrous conditions: 20% with 12.5 μM ^{125}I-BH-NHS (Fig. 2) and as high as 70% with 0.8 mM ^{127}I-NAP-Tyr-NHS (Fig. 3), when the same concentration (0.5 mM) of CCK-9N was used. To limit the formation of secondary products, strictly anhydrous solvents of high purity were essential, especially when coupling was performed at tracer (micromolar) concentration (Fig. 1). Indeed, the yield of coupling was only 1% when ^{125}I-NAP-Tyr-NHS was used at a concentration < 1 μM and then purified in aqueous medium before coupling (Fig. 5). Fortunately, the purification of the mono-radioiodinated ^{125}I-NAP-Tyr-NHS reagent, before coupling, was so efficient that a high 80% iodination yield of CCK-9N as monoiodinated ester resulted, the final yield of labelling comparing well with that obtained when unpurified ^{125}I-NAP-Tyr-NHS was used (Fig. 4).

The binding characteristics of the present CCK-9N derivatives to rat pancreatic plasma membranes indicated that: (a) the introduction of I-BH on the N-terminal moiety of CCK-9N modified neither the affinity (Fig. 6A) nor the binding capacity (not shown) of this peptide; (b) by contrast, the introduction of I-NAP-Tyr —a larger and more hydrophobic group— into CCK-9N increased the K_D from 4.5 to 6-19 nM when examined in subdued light. We are at present investigating ^{125}I-NAP-Tyr-CCK-9 as a photoactivable radioactive probe of CCK receptors.

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