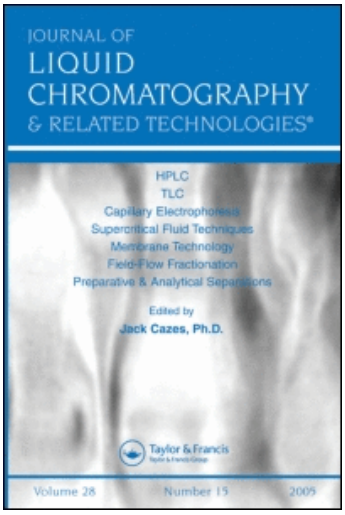

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PURIFICATION OF RADIO-IODINATED CHOLECYSTOKININ PEPTIDES BY REVERSE PHASE HPLC

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ABSTRACT

The purification of the iodinated tracers of CCK peptides using Sphadex G50 chromatography does not allow for a good separation between non-modified peptides and labelled peptides. We present, in this paper a simple and rapid purification method using reverse phase HPLC with a C-18 column for four of these tracers. The biological characteristics of the molecules obtained demonstrate their strong specific radioactivity and their high degree of purity.

INTRODUCTION

The use of radio-iodinated peptides as tracers in radioimmunoassay (RIA) and receptor binding studies has gained wide acceptance due to the ease of preparation and the high specific activities obtainable (1). In the particular case of peptides belonging to the cholecystokinin family (CCK), the Bolon-Hunter method for iodination reaction under conditions avoiding oxidation,

is the only one which permits the use of a tracer in radio-immunoassay that is capable of biological activity.

Up to now, the purification of these tracers has been achieve on sephadex which is not totally satisfactory. In addition, this method does not give good chromatographic differentiation of the iodinated peptide from the non-iodinated peptide, the latter being added in excess.

We propose, in this paper, a simple and rapid method for the purification of four iodinated polypeptides belonging to the CCK-family.

EXPERIMENTAL

Peptides

Highly purified CCK_{1-39} was a gift of Professeur V. Mutt (Karolinska Institutet, Stockholm, Sweden) ; the C-terminal decapeptide (CCK_{30-39}) and a derivative of the C-terminal nonapeptide namely (Thr_{34}, N Leu_{37})-CCK_{31-39} were synthetized by Prof. E. Wünsch from Max Planck Institute für Biochemie, Muncheen, West Germ. The C-terminal tetrapeptide CCK_{36-39} was purchased from Interchim (Montluçon, France). Peptides used in this study were homogenous in reverse phase HPLC. They were rechromatographied before labelling if and when necessary.

Iodination precedure

CCK peptides were radio-iodinated by conjugation of the hormone to ^{125}I - Bolton-Hunter reagent[N-succinimidyl 3-(4-hydroxy,

5 -(^{125}I) iodophenyl) propionate] of specific activity ≃ 2 Ci/ µmol. (Amersham France) according to Rehfeld (2). About five micrograms of peptide in 15 µl of 0.1 M sodium borate, pH ≃ 8.5 were added to 0.5 mCi of dried Bolton-Hunter (BH^{125}I) reagent. After a 45 min stirring in an ice bath, 500 µl of 0.2 M glycine in 0.1 M Sodium borate pH = 8.5 was added to the reaction mini-vial.

HPLC

Purification of CCK-BH-^{125}I tracers was performed on a µ-Bondapak C-18 column (3.9 x 30 cm). The mobil phase was composed of a triethylammonium phosphate (TEAP) buffer 0.25 N, pH = 3.5 combined with acetonitril. Triethylamin and acetonitril were supplied by Fluka-Lab. Water was deionized and then distilled in glass. The waters associates liquid chromatograph model 204 consisted of a U6K injector, 6000 A pump, Schoeffel model 770 multiwave lenght detector and omniscribe chart recorder.

HPLC was run isocratically in a TEAP/acetonitril (74/26) buffer for at least 15 minutes, then elution of the radiolabelled peptides was performed by increasing the acetonitril ratio of the mobil phase to 50 %. Flow rate and back pressure were respectively 2 ml/min and 1500 PSI. Non-labelled peptides in the eluant were monitored by absorption at 210 nm, fractions of 1 ml were collected and ratio-activity was determined in a Packard PGD auto gamma counter.

Biological studies

The purity of the CCK radio-iodinated peptides was tested by the study of their binding characteristics to isolated pancreatic

acini. Acini were prepared, as previously reported, from guinea-pigs (5). About 50 000 cpm of radioactive fractions were incubated alone or in combination with caerulein (Farmitalia, Milan) for 40 min at 37°C in a 24.5 mM Hepes [4-(2-hydroxyethyl)-1-piperazine ethane salfonic acid] buffer pH = 7.4 containing 1.5 % (w/v) bovine ablumin, 0.1 % (W/V) bacitracin, 50 microg/ml streptomycin sulfate, 100 U/ml potassium penicillin G and 0.01 % soybean trypsin inhibitor. After incubation, an aliquot of the suspension was removed, washed with medium plus 12 % albumin at 4°C and centrifuged. Radioactivity associated with cells was then measured. Specific binding was defined as the excess binding over that in blanks containing 10^{-6}M caerulein and I_{50} as the concentration of cerulein that inhibited 50 % of specific binding.

RESULTS

Figure 1a and 1b illustrate the elution profils of the Bolton-Hunter reagent and the iodinated product of glycine. The BH-^{125}I reagent injected in solution in benzene is not eluted with the 26 % acetonitril buffer in less than 15 minutes. However with a change to 50 % acetonitril a total elution of the reagent takes place.

Under isocratic conditions (26 % acetonitril), the radioactivity of the labelled glycine product is eluted as a major peak with t_R = 4 min ; this corresponds to gly-BH-^{125}I, and to a secondary peak with a t_R = 7.5 min, which is most likely a hydrolysis product of BH-^{125}I. It was observed that after a 26 %

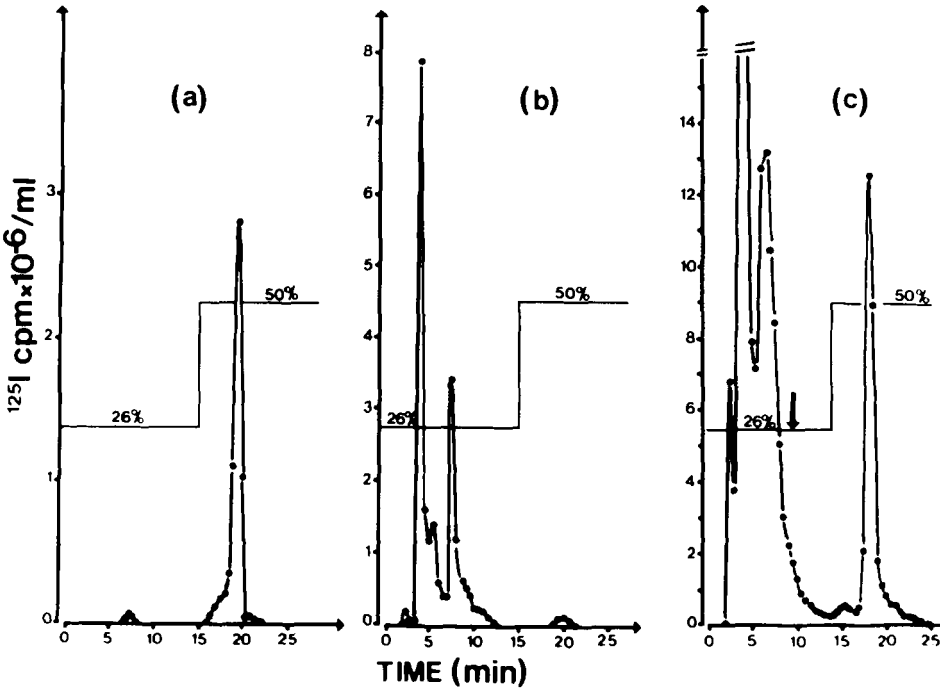

FIGURE 1. Radioactive profiles of the eluted fractions from HPLC chromatography using C-18 column.
Solutions injected :
a) 5.5 10^6 cpm of Bolton-Hunter-^{125}I reagent (BH-^{125}I)
b) 18.10^6 cpm of the iodinated product of glycine
c) 260.10^6 cpm of the iodinated solution of CCK_{30-39}
Step gradient with triethylamin phosphate buffer 0.25 N pH = 3.5 /acetonitril (74/26) during 15 min and then triethylamin phosphate buffer/acetonitril (50/50). Flow rate : 2 ml/min ; Back pressure: 1500 PSI ; volume of each eluted fraction : 1 ml
↓ : t_R of CCK_{30-39}

chromatographic run, the quasi-total radioactivity injected is eluted, and no observable residual peak is eluted when going to a 50 % acetonitril buffer (fig. 1b).

The radioactivity profiles of collected fractions from the HPLC of an aliquot of the CCK_{30-39} labelled solution used is

shown in figure 1c. Four peaks are resolved ; one peak close to Vo whose identity is not known ; another very dominant peak at 4 min, which corresponds to gly - BH - ^{125}I ; a peak at 7.5 min probably containing a hydrolysis product of BH-^{125}I and a last peak eluted with the 50 % acetonitril buffer. This last peak is the only one that contains molecules exclusively with characteristics of the CCK_{30-39} -BH-^{125}I tracers, since these molecules bind specifically to the high affinity receptors of CCK in the pancreatic acini.

All the results dealing with the tracer HPLC runs are summarized in table 1. Isocratic runs using 26 % acetonitril buffer permit the differentiation and elution of the unlabelled peptides. Using the same buffer system, the retention of the labelled peptides produces a total separation of the tracers from non-labelled peptide moiety. The choice of the 50 % acetonitril buffer within the 15 min period under isocratic conditions results in a compromise between two requisites : 1) the certainty of the total elution of the non-iodinated species and 2) the assurance of a good tracer recovery. The limiting values of the percent radioactivity incorporated in the species demonstrating a specific affinity with pancreatic acini, are reported in table 1 as well. In addition, during the course of the experiments, an average chromatographic recovery calculated on the basis of the percent radioactivity eluted within 30 minutes between 85.0 $\pm$ 3.4 % was obtained.

When CCK_{1-39}-BH^{125}I is placed in the presence of isolated pancreatic acini, a total binding of 5.7 $\pm$ 0.9 % is found. The

TABLE I

Chromatographic results of four labelled peptides belonging to CCK-family. Column - Bondapak C-18. Step gradient with triethylamin phosphate buffer 0.25N pH : 3.5 (acetonitril : 74/26, during 15 min and then, triethylamin phosphate buffer/acetonitril : 50/50 ; flow rate : 2 ml/min ; back pressure 1500 PSI ; volume of each eluted fraction : 1 ml.

Peptides	CCK_{1-39}	CCK_{30-39}	$(Thr_{34}, NLeu_{37})$ - CCK_{31-39}	CCK_{36-39}
t_R (min) un-modified peptides	7.10	9.60	8.25	3.20
t_R (min) radio-labelled peptides	19.0	19.0	19.5	20.0
% of total radioactivity recovered in biologically active fractions (limiting values)	12-30	10	4-6	16-30
% of total radioactivity eluted at 30 min	85.0 ± 3.4 ($\pm$ SEM)			

non-specific binding and the I_{50} are respectively 0.6 $\pm$ 0.2 % and 0.1 nM (6). With CCK_{30-39} BH-^{125}I and (Thr_{34}, $NLeu_{37}$) CCK_{31-39} - BH-^{125}I tracers a slightly weaker specific binding took place with I_{50} values comparable to that of CCK_{1-39} - BH-^{125}I. Regardless of which tracer used, non-specific binding values of about 10 % of the total binding were obtained. Only CCK_{36-39} BH-^{125}I, alone does not bind to pancreatic acini.

DISCUSSION

HPLC results of BH-^{125}I and gly-BH-^{125}I reveal a strong hydrophobic ability on the part of the Bolton-Hunter reagent. Its combination with peptides of CCK lead to the formation of molecules which are highly retained on the C-18 phase of the column. It is this characteristic property which led us to use a step gradient of 26 % to 50 % acetonitril.

Application of the least step gradient is sufficient enough for elution of the labelled peptides with, however a drop in the chromatographic yield as well as for the elution of a larger volume.

Our objective was not optimisation of the percent incorporation of iodine ^{125}I in peptides ; nevertheless the values were obtained are compatible with those found by other authors (2, 3).

Our method has the distinct advantage of producing a good separation of tracers from the unsubstituted peptide, the latter being perfectly eluted in the 26 % acetonitril buffer ; tracers of high specific activity could thus be anticipated.

The results of the first studies on the binding of these tracers with pancreatic acini proove their strong specific binding affinity as well as to their high degree of purity. In fact, a greater percentage of the specific binding of CCK_{1-39} BH-^{125}I than that obtained from CCK_{4-39} - BH-^{125}I prepared from chromatography using Sephadex (4,7) and a weaker non-specific binding (< 10 %) were observed. Absence of CCK_{36-39} BH-^{125}I binding is compatible

with the weak affinity of this peptide fragment for the CCK receptors of pancreatic acini (8).

All our methodology employed limits the risk of oxidation as is shown by the binding ability of recovered molecules. The time with which the separation is carried out (less than 30 min) most likely avoidssuch problems. Finally, the possible identification of oxidized components during non-iodinated peptide run, gives this method an added advantage (9, 10). All of the data give above show, in addition, that with this chromatographic method the fractions containing a maximum radioactivity are those which present the best biological characteristics, contrary to what is normally found in conventional chromatography (2). Furthermore, these tracers are recognised by antibodies reacting with C-terminal portion of CCK (Prof. J.A. Chayvialle, personal communication). They could be used in radio-immunoassay. $(Thr_{34}, NLeu_{33})$-CCK_{31-39} - BH-^{125}I seems best adapted to this end due to the substitution of oxidisable methonine residues.

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