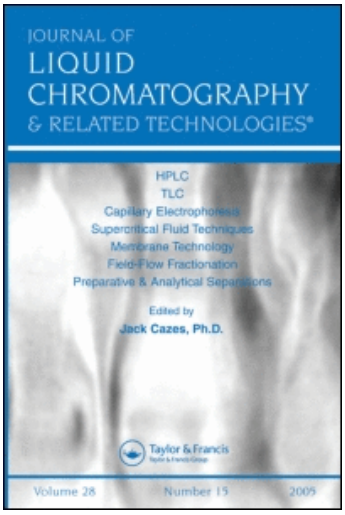

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D. Fourmy[a]; L. Pradayrol[a]; A. Ribet[a]
[a] INSERM U 151 CHU de Rangueil, TOULOUSE CEDEX, FRANCE

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SEPARATION OF THE SECRETIN-GLUCAGON FAMILY PEPTIDES BY RP-HPLC IN TRIETHYLAMMONIUM PHOSPHATE BUFFERS

D. FOURMY, L. PRADAYROL, A. RIBET

INSERM U 151 CHU de Rangueil 31054 TOULOUSE CEDEX F R A N C E

ABSTRACT

Four preparations of hormonal polypeptides related to the secretin-glucagon family (secretin, glucagon, vasoactive intestinal polypeptide, gastric inhibitory polypeptide) and a preparation of pancreatic polypeptide are analyzed by reverse-phase HPLC on a μ-Bondapack column in triethylammonium phosphate buffers. The chromatographic results reveal the presence of impurities in natural peptides (VIP, GIP) and in synthetic secretin. Moreover, an important relative difference in apparent hydrophobic interactions is showed between VIP and the others peptides investigated. When a mixture of these five polypeptides is chromatographed in a 7 minute concave gradient buffer of 26 % to 31 % acetonitrile, VIP, GIP, S, G and PP are successively eluted in a total time of less than 20 minutes. This elution sequence does not fit with predicted retention times calculated according to the method proposed by Meek (1).

INTRODUCTION

HPLC has already been widely used in the field of polypeptide biochemistry, as one of the steps in the purification of natural molecules (2, 3, 4) or as a means of separating the different molecular forms of a given polypeptide after tissue ex-

traction (5, 6). Another wide spread use of HPLC concerns the estimation of the purity and the state of synthetically or naturally prepared peptides prior to their biological activity studies (7, 8). Finally, recent application of HPLC has been introduced for the purification of iodinated polypeptides used in biological studies (interaction with membrane receptors, intracellular mechanisms) and as radioactive tracers in radioimmunoassay (9, 10, 11).

In all the areas of application mentioned above, RP-HPLC offers valuable advantages over conventional chromatographic techniques (ion exchange, affinity, gel permeation, etc). It has, in particular, a high resolution ability and is also capable of dealing with very small quantities of peptides.

We will now illustrate the separation of five hormonal peptides from the digestive system by RP-HPLC : secretin (S), vasoactive intestinal polypeptide (VIP), gastric inhibitory polypeptide (GIP), glucagon (G) and pancreatic polypeptide (PP). The first four peptides were chosen for their close primary structure similarities. In fact, porcine pancreatic glucagon (12) has 14 amino-acids homologous to that of secretin (13), 7 with VIP (14) and 15 with GIP (15). The pancreatic peptide is less structurally related to these peptides ; yet, its behavior in RP-HPLC, compared to that of glucagon is interesting, since it is during one of the final purification steps of pancreatic

glucagon and insulin, that Chance (16) and Kimmel (17) showed its existence and achieve its isolation.

EXPERIMENTAL

Peptides

Synthetic secretin was a gift of Hoffman-Laroche Laboratories (Basle, Switzerland). Synthetic glucagon and bovine pancreatic polypeptide were purchased respectively from Novo Industries Pharmaceutiques (Paris, France) and Bachem (Basle, Switzerland). Natural vasoactive intestinal polypeptide and gastric inhibitory polypeptide were gifts of professors V. Mutt and J.C. Brown from Karolinska Institutet (Stockholm, Sweden).

Chemicals

All reagents were of analytical grade. Triethylamine puriss and acetonitrile for spectroscopy were supplied by Fluka AG (Buchs, Switzerland). Orthophosphoric acid GR was purchased from Merck (Darmstadt,RFA). Water was deionized and then distilled in glass.

Samples preparation

Lyophilized peptides were dissolved in the mobile phase buffer used for HPLC at a concentration of about 0.1 µg/µl.

HPLC

HPLC was performed on a µ-Bondapak C-18 column (0.39 x 30 cm) at room temperature. The mobil phase was composed of a triethylammonium phosphate (TEAP) buffer 0.25 N, pH = 3.5 (18)

combined with acetonitrile. The Waters Associates liquid chromatograph consisted of a U6K injector, two 6000 A pumps, a model 660 solvent programmer, a Schoeffel model 770 multiwave length detector and an Omniscribe chart recorder.

Analytical HPLC of each peptide was run isocratically in a TEAP + acetonitrile buffer in which the ratio of acetonitrile was suitable for a good separation (table I). The chromatographic separation of a mixture of all peptides was performed isocratically in a TEAP/Acetonitrile 74/26 buffer and in a 7 minute gradient elution buffer of 26 % to 31 % acetonitrile.

Flow rate and back pressure were respectively 2 ml/min and 1500 PSI. Detection of the eluted substances was done by absorption at 210 nm. The presence of impurities was estimated by calculating the relative area (r.a.) of peaks with respect to the main component.

RESULTS

1) Chromatographic analysis of each peptide

The composition of the buffer used for isocratic runs was determined in order to provide a sufficient retention and maximum resolution on the C-18 column. The results of the chromatographic analysis of the five peptides are summarized in Table 1. For the five peptide preparations analyzed, the presence of the peptide in the major peak was verified in vivo or in vitro by biological activity tests.

TABLE 1

Isocratic chromatographic analysis of five peptides. Column : μ-Bondapack G-18 (0.39" x 30 cm). Mobile phase TEAP 0.25 N pH = 3.5 + CH_3 CN. Flow rate : 2 ml/min. Back pressure = 1500 PSI. Chart speed : 0.5 cm/min. UV detection at 210 nm.

Peptides	% CH_3CN	retention times (min)			Relative area (o.d. 210 nm)	
		major peak P	Secondary peaks 1	2	1/P	2/P
VIP	25	4.5	3.3		0.21	
GIP	28	8.8	3.8	6.0	0.22	0.35
S	30	6.5	4.6		0.20	
G	30	7.5				
PP	32	5.6				

VIP in a 25 % CH_3CN buffer is eluted as a major peak (t_R = 4.5 min) preceded by an impurity peak (r.a: 0.21), eluted at 3.3 min. The GIP preparation is resolved in a 28 % CH_3CN buffer and showed three peaks : a major peak at 8.8 min, and two other important peaks at 3.8 and 6.0 minutes respectively with an r.a. of 0.22 and 0.35. The secretin preparation is eluted in a 30 % CH_3CN buffer as a major peak at 6.5 min. and a secondary peak (r.a. = 0.20) at 4.6 min.

Glucagon and the pancreatic polypeptide are each eluted as single peaks, respectively at 7.5 min and 5.6 min. These experi-

ments showed the presence of impurities in natural peptides as well as in synthetic secretin. Moreover, an important relative difference in apparent hydrophobic interactions is revealed between VIP and the other peptides investigated.

2) Chromatographic separation of the VIP, GIP, S, G and PP mixture

a) Isocratic conditions

The presence of VIP in the mixture, weakly hydrophobic in comparison to the four other peptides, as was shown earlier, imposed upon us the choice of a buffer containing 27 % CH_3CN, which is the limiting value permitting the retention of VIP on the column.

In this buffer, VIP, GIP, S and G peptides are eluted respectively at 2.5, 13.2, 32, and 52 minutes, as large individual peaks. The PP is too strongly retained to elute, since no observable U.V. peak at 210 nm is found.

b) Acetonitrile gradient

Figure 1 shows the chromatogram obtained after injection of the five peptide mixture during a concave gradient run : 26 % to 31 % CH_3CN. Under these conditions, the retention times correspond to the following major peaks : VIP : 2.8 min ; GIP : 10.6 min ; S : 12.6 min, G : 16 min, PP : 18 min. It must be noted that the five peptides, elution and separation under isocratic conditions are extremely difficult ; here with the acetonitrile gradient they are perfectly separated. In addition,

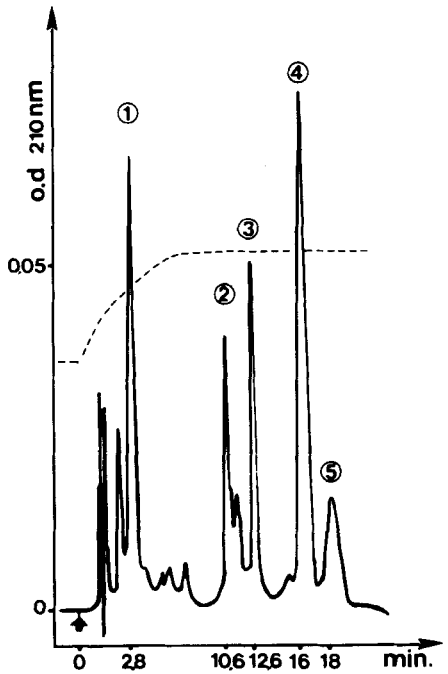

FIGURE 1

RP-HPLC Chromatography of : ① : VIP : 4 µg + ②GIP : 3 µg + ③ secretin : 4 µg + ④: Glucagon : 5 µg + ⑤ : pp : 2 µg.
Colum : µ-Bondapack C-18 (0.39 x 30 cm). Mobil phase : TEAP 0.25 N pH = 3.5 + CH_3CN. Gradient : run 5, 26 % to 31 % CH_3CN in 7 min. Flow rate : 2 ml/min. Back pressure = 1500 PSI. Chart speed : 0.5 cm/min. UV detection at 210 nm.

the impurities revealed during each peptide run are resolved as well defined and distinct species of the major peaks.

DISCUSSION

The present chromatographic study shows that the separation of the four peptides belonging to the secretin glucagon

family and that of the pancreatic polypeptide is possible in a TEAP/CH_3CN system on a C-18 column.

The separation, attained here in less than 20 minutes, of the five peptides which are very closely related to each other biochemically, demonstrates the power of RP-HPLC. The elution order of the peptides (VIP, GIP, S, G, PP) does not correlate with the predicted retention times calculated by summing the retention coefficients for each amino-acid contained in the peptides. The retention times calculated by the method proposed by Meek (1) are : VIP : 55.1 ; GIP : 100 ; S : 33.6 ; G : 58.2 ; PP : 73 minutes. The calculated retention times for S, G, PP are in quite good agreement with the experimental values observed in our study. On the other hand however, unexpected low interactions for VIP and GIP have been found. If for GIP the higher molecular weight of this peptides could account for the discrepancy between calculated and experimental retention times, the striking VIP behavior is much more difficult to explain. But, at least two different causes may be argued. First, the basicity of VIP compared to that of the others peptides which might be underestimated as experimental retentions were observed at pH = 3.5 while calculations referred to pH 2.1. Second, restricted conformations of this peptide may shield some residues from interacting with the C-18 column.

RP-HPLC is also useful for verifying the state of peptide preparations ; these purity controls are run for quantities of

2 to 5 µg in less than 15 minutes. The control of initial peptide preparations and their state after being stored, is mandatory prior to any physiological studies and requires the ever increasing use of RP-HPLC ; this type of chromatography in addition, has permitted the normalization of the synthetic preparations of peptides. The hydrophobic properties on which RP-HPLC analysis is based appears complementary to other properties (size, charge etc...) on which are based chromatographic techniques during the early stages of natural molecule purification.

The example of GIP illustrates well the contribution of RP-HPLC in the field of peptide purification from natural origins. In fact, it is only recently that the nature of a contaminant in the GIP preparation has been elucitated by the combined use of HPLC and sequential analytical techniques (19). This contaminant was determined to be a 3-32 fragment of GIP most likely to be the component eluting at 6.0 minutes in the 28 % CH_3CN buffer (see table).

In a general way, RP-HPLC rightfully wins its place in the final steps of natural peptide preparations (20). In this area, the possibility of analytical runs with small quantities of raw material make HPLC a valuable tool for establishing extraction methodologies of for the elucidation of a variety of molecular forms of a peptide from an extract. In this last case, the technique can be coupled with a specific test, such as radioimmunoassay to the eluted molecules. The measure of the

bioactivity coming from the eluent can also be used as a means for the identification of the chromatographed molecular species. By eluting in a minute amount in a concentrate form, they maintain their bioactivity which can be directly measured without a lyophyilization step.

The use of an acetonitrile gradient system results in a better resolution of the different peaks ; and for the same run, the possibility of eluting different hydrophobic species permit the total elution of molecules retained on the column by increasing the percentage of CH_3CN to very high levels.

In conclusion, the power of RP-HPLC as illustrated in this article is fast becoming a valuable tool for physiological studies : the stability and the study of the degradation processes of hormonal peptides have recently been undertaken using HPLC (21). An extension of this technique can be foreseen in two different ways : the characterization and the measurement of peptides, for which radio-immunoassay is readly accessible from raw tissue extracts ; and the study of the outcome of hormonal peptides in cell culture media.

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