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Note

Reversed-phase high-performance liquid chromatography of dermorphins, opiate-like peptides from amphibian skins

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Methanol extracts of fresh skins of *Phyllomedusa sauvagei*, a South American frog, contain, in addition to peptides belonging to the bradykinin, caerulein, tachykinin, bombesin and sauvagine families, a novel peptide possessing exceptionally intense and long-lasting peripheral and central opiate-like actions[1,2]. On account of its origin and properties, this peptide has been given the name dermorphin (acronym from derm and morphine). Parallel research carried out on skin extracts of different Phyllomedusinae frogs has demonstrated the presence of other dermorphin-like peptides[3], the amino acid sequences of which are reported in Fig. 1. With the exception of the NH_2-terminal tyrosine residue and the hydrophobic character of the amino acid chain, dermorphins have little in common with the known enkephalin–endorphin sequences[4,5]. On the contrary, they are more closely related to β-casomorphin[6], an opioid peptide isolated from β-casein.

Dermorphin	H-*Tyr*-Ala-*Phe*-Gly-Tyr-*Pro*-Ser-NH_2
Deamidated dermorphin	H-*Tyr*-Ala-*Phe*-Gly-Tyr-*Pro*-Ser-OH
Hyp^6-dermorphin	H-*Tyr*-Ala-*Phe*-Gly-Tyr-Hyp-Ser-NH_2
Deamidated Hyp^6-dermorphin	H-*Tyr*-Ala-*Phe*-Gly-Tyr-Hyp-Ser-OH
Met-enkephalin	H-*Tyr*-Gly-Gly-Phe-Met-OH
β-Endorphin	H-*Tyr*-Gly-Gly-Phe-Met-Thr-Ser-...
β-Casomorphin	H-*Tyr*-Pro-*Phe*-Pro-Gly-*Pro*-Ile-OH

Fig. 1. Amino acid sequence of dermorphins and other opiate-like peptides. Identical residues are italicized. Ala = D-Ala; Hyp = *trans*-4-hydroxyproline.

To our knowledge, dermorphins offer the first surprising example of peptides from Vertebrata containing a D-amino acid residue, which is of crucial importance for the biological activity[1].

In this paper we describe the use of reversed-phase high-performance liquid chromatography (RP-HPLC) with isocratic elution for the separation of the different dermorphins on C_8 or C_{18} bonded silica. The peak resolutions and retention times of these compounds are discussed and correlated with their amino acid sequences.

EXPERIMENTAL

Reversed-phase high-performance liquid chromatography was carried out at constant pressure, using a DuPont Model 841 apparatus with a detector operating at

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a fixed wavelength of 254 nm. The organic solvents used were of HPLC grade (Merck, Darmstadt, G.F.R.).

L-Ala^2-dermorphin was synthesized in our Peptide Department. The natural compounds were isolated as previously reported from the methanol extracts of the skins of South American Phyllomedusinae frogs (*Phyll. sauvagei*, *Phyll. rhodei*)[2,3]. Their purity was tested by thin-layer chromatography (TLC), Edman degradation and field desorption mass spectrometry. All other materials were of analytical-reagent grade.

The samples for chromatography were freshly dissolved in the mobile phase and injected via a Rheodyne septumless valve with a 10-μl loop on to a μBondapak C_{18} column (300 × 3.9 mm I.D.; Waters Assoc., Hartford, Great Britain) or on to a Nucleosil 10 C_8 column (250 × 4 mm I.D.; Macherey, Nagel & Co., Düren, G.F.R.). All separations were performed isocratically at room temperature (*ca.* 25°C).

Further experimental details are given in the legends to the figures. The organic solvents chosen for this study represented those commonly used in RP-HPLC because of their miscibility with water and low UV cut-off.

RESULTS AND DISCUSSION

The isocratic elution profile of the four dermorphins isolated from the skins of *Phyll. sauvagei* and *Phyll. rhodei** is shown in Fig. 2. The order of elution of these

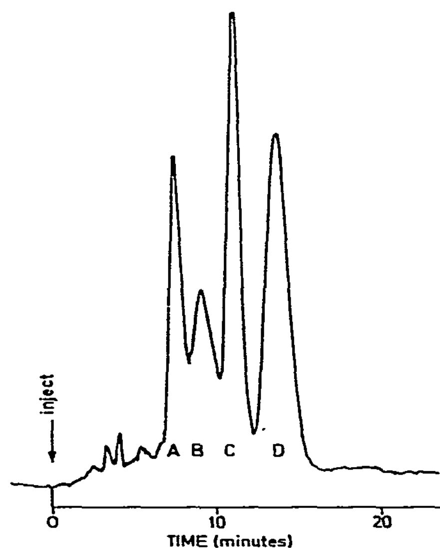

Fig. 2. Elution pattern from HPLC of dermorphins. Chromatographic conditions: column, μBondapak C_{18}; mobile phase, methanol–0.01 *M* sodium acetate solution (1:1); flow-rate, 0.7 ml/min; chart speed, 5 mm/min. Sample size, *ca.* 10 μg of each peptide (0.04 a.u.f.s.). A = Deamidated Hyp^6-dermorphin; B = deamidated dermorphin; C = Hyp^6-dermorphin; D = dermorphin.

* Curiously the methanol extracts of the skins of *Phyll. burmeisteri*, captured near Rio de Janeiro, contain only dermorphin. Hyp^6-dermorphin is not present, probably because of the absence of a specific prolyl hydroxylase[7,8].

peptides is in accordance with the decreasing polarity from deamidated Hyp^6-dermorphin to dermorphin, as previously observed in TLC on silica gel plates (solvent system: *n*-butanol–glacial acetic acid–water, 4:1:1).

The resolving power is shown by the ability to separate very closely related peptides: dermorphin differs from Hyp^6-dermorphin by a single Pro–Hyp replacement by post-translational modification; this structural variation is sufficient to yield differences in the retention times. A similar observation is also valid for the corresponding deamidated heptapeptides, which elute faster from the column probably because their carboxyl groups are in a dissociated form at the pH of the mobile phase (about 6.6). In addition, this chromatographic system also allows stereoisomers to be separated: dermorphin and its L-Ala^2 synthetic analogue show different retention times of 14 and 12 min, as shown in Fig. 3. The decrease in the retention time of the L-Ala^2 diastereoisomer of dermorphin would imply a different spatial orientation of the molecule. As suggested by Larsen *et al.*[9] for the synthetic analogues of oxytocin, the substitution D-Ala → L-Ala in the molecule causes the resulting diastereoisomer to be significantly more polar than the native active peptide. Nevertheless, it would be necessary to consider other factors affecting the separation of these two compounds, *viz.*, their interaction with (i) the organic solvent, (ii) the water or (iii) the octadecylsilica in the stationary phase.

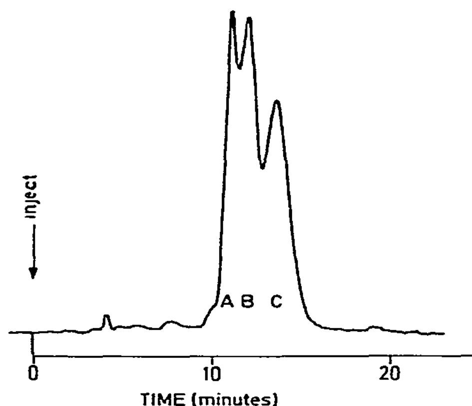

Fig. 3. Elution pattern from HPLC of dermorphin (C), L-Ala^2-dermorphin (B) and Hyp^6-dermorphin (A). Conditions as in Fig. 2.

To optimize the separation of the four natural dermorphins, the mobile phase was modified by the use of the ternary system constituted by methanol–acetonitrile–0.01 *M* sodium acetate solution (16:20:64) (Fig. 4). The separation among dermorphin, Hyp^6-dermorphin and deamidated dermorphin is increased and narrower peaks are obtained; however, the presence of acetonitrile in the mobile phase modifies the chromatographic behaviour of the synthetic compound L-Ala^2-dermorphin, which elutes from the column with the same retention time as Hyp^6-dermorphin.

Replacement of sodium acetate with ammonium acetate does not change the elution profile of dermorphins substantially (Fig. 5). Although the separation using sodium acetate–methanol–acetonitrile is better than that using ammonium acetate–methanol–acetonitrile (better resolution between deamidated dermorphin and Hyp^6-

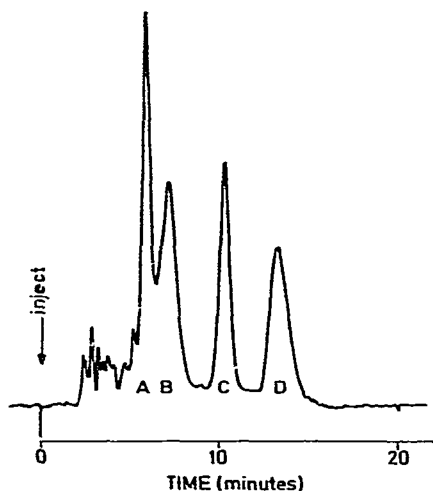

Fig. 4. Elution pattern from HPLC of dermorphins. Chromatographic conditions: column, μBondapak C_{18}; mobile phase, methanol–acetonitrile–0.01 *M* sodium acetate solution (16:20:64); flow-rate, 0.8 ml/min; chart speed, 5 mm/min. A = Deamidated Hyp^6-dermorphin; B = deamidated dermorphin; C = Hyp^6-dermorphin; D = dermorphin. Hyp^6-dermorphin and L-Ala^2-dermorphin co-elute.

dermorphin and narrower peaks), the latter mobile phase is preferred for preparative purposes, because it is very volatile. The recovery of these peptides from the C_{18} column, under the conditions described above (see Fig. 5), is greater than 80% based on amino acid analysis. Good reproducibility of the retention time for multiple injections of mixtures of these natural peptides is observed. Tailing peaks in the elution profile are obtained when a lower concentration of ammonium acetate is used.

No change in the retention order of the natural dermorphins is observed when using a spherically shaped packing (Nucleosil 10 C_8 column) with acetonitrile–meth-

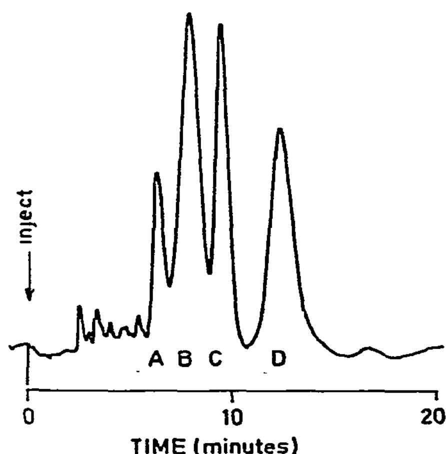

Fig. 5. Elution pattern from HPLC of dermorphins. Conditions as in Fig. 4, except mobile phase: methanol–acetonitrile–0.02 *M* ammonium acetate solution (16:20:64) (pH 6.6). A = Deamidated Hyp^6-dermorphin; B = deamidated dermorphin; C = Hyp^6-dermorphin; D = dermorphin.

anol–sodium acetate as the eluent. As can be seen in Fig. 6, the two deamidated dermorphins elute from the column with a lower retention time than that previously obtained (compare Figs. 6 and 4), probably because of the shortness of the column with a concomitant reduction in the void volume. In addition, better resolution is obtained between Hyp[6]-dermorphin (retention time 12.8 min) and dermorphin (retention time 17.6 min). These data will probably be useful for semi-preparative purposes (possibility of injecting a large volume of material without peak overlapping). In our experience, the octadecylsilyl-bonded stationary phase, more hydrophobic than C_8, shows a greater retention for small peptides, as reported by Majors[10].

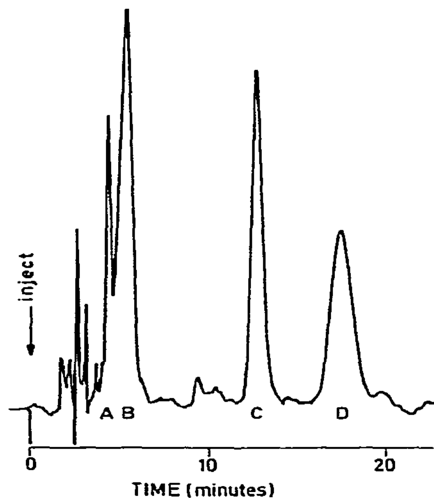

Fig. 6. Elution pattern from HPLC of natural dermorphins on a Nucleosil 10 C_8 column. Conditions as in Fig. 4. A = Deamidated Hyp[6]-dermorphin; B = deamidated dermorphin; C = Hyp[6]-dermorphin; D = dermorphin. Hyp[6]-dermorphin and L-Ala[2]-dermorphin co-elute.

For this reason, we do not know at present if the octyl groups in the hydrocarbon phase, in conjunction with the totally spherical packing, can explain the elution profile observed for dermorphins on Nucleosil 10 C_8, as reported in Fig. 6. Therefore, this preliminary approach to the use of this column will be investigated more fully in the near future.

CONCLUSION

The data reported here demonstrate that RP-HPLC using octyl- or octadecylsilylsilica columns is suitable for the resolution of dermorphins. In particular, the methods described are able to separate rapidly Hyp[6]-dermorphin from dermorphin. The isolation of these two heptapeptides, as previously reported[2,3], has been carried out using chromatography on a silica gel column, but this purification procedure is a difficult and timeconsuming step, because several recyclings are necessary in order to obtain a sufficiently pure sample of Hyp[6]-dermorphin. In our experience, it is not possible to purify dermorphins from the methanol extracts of amphibian skins directly by HPLC, because of the relatively small amounts of these peptides present

together with large amounts of other biological substances. A similar observation has been reported by Brantl *et al.*[11] and Henschen *et al.*[6] during the isolation of β-casomorphin.

HPLC could perhaps be useful for separating active peptides during the final steps of a purification procedure. Concerning the potential of the methods described here for preparative purposes, we think that the methanol–acetonitrile–ammonium acetate eluent (see legend to Fig. 5) is suitable for the purification of dermorphins because the solvents employed are easily removed *in vacuo* or by lyophilization without the risk of the individual compounds undergoing a change in structure.

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