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Note

Use of perfluorated carboxylic acids in the separation of metabolites of vasopressin prior to radioimmunoassay

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During the past few years much information has appeared about the distribution of vasopressinergic fibres in the brain [1–5], and the receptors have been located [6–10]. These findings, together with the demonstration that vasopressin (AVP) can be released by stimulation [11] and that degrading enzymes are present in tissue homogenates [12], suggest a transmitter role for the peptide in the brain [13].

Most studies aimed at evaluating the functional properties of the vasopressinergic nervous system use variations in the tissue concentration of the whole molecule as a measure of altered activity. However, it is possible that variations in the rate of peptide synthesis may mask a variation in its rate of degradation. In order to get a more precise index of activity, the accumulation rate of AVP metabolites should be measured.

Another reason for an assay of AVP metabolites is that behavioural studies have shown that pGlu4-Cyt6-AVP (4–9) is 1000 times more potent than the parent molecule. Since specific receptors for this fragment have been demonstrated [13] it is likely that the fragment constitutes a functional principle in brain. An assay able to measure changes in the tissue concentration of pGlu4-Cyt6-AVP (4–9) would thus be of value for investigating this possibility.

The present study was initiated to investigate AVP turnover. Several fractionation procedures were examined using reversed-phase fast protein liquid chromatography (RP-FPLC) with perfluorated carboxylic acids as ion-pairing agents.

The results show that the acids used give a highly efficient separation of fragments differing in only one amino acid. It is shown that standards containing picomolar amounts can be recovered successfully.

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EXPERIMENTAL

Fast protein liquid chromatography

FPLC (by Pharmacia) is a concept that differs from high-performance liquid chromatography essentially in the structure of the silica. Because the silica is very homogenous, pre-column pressures are low. The PEP-RPC™ HR-5/5 column (Pharmacia, Laboratory Separation Division, Uppsala, Sweden) was used. This is a silica-based reversed-phase material grafted with C_2 and C_{18} alkyl chains with a pore size of 100 Å and a particle size of 6 μm. The polar solvent (A) was 0.2–10 m*M* of either pentafluoropropionic acid (PFPA) or heptafluorobutyric acid (HFBA) in Millipore-filtered water. The non-polar solvent (B) used was 80% methanol (LiChrosolv quality, KEBO Lab., Spånga, Sweden) with the same acid concentrations as in A. A linear gradient was run from 0 to 100% B with a flow-rate of 1.0 ml/min.

The chromatographic behaviour of AVP (1–9) and of a number of fragments that possibly occur in the brain and plasma were studied using the aforementioned ion-pairing acids. The fragments used were: Cyt^6-AVP (3–9), Cyt^6-AVP (5–9), Cyt^6-AVP (2–8), Cyt^6-AVP (5–8), Cyt^6-AVP (6–8), $pGlu^4$-Cyt^6-AVP (4–8), all kindly supplied by Organon (Oss, The Netherlands), and $pGlu^4$-Cyt^6-AVP (4–9) (Sigma, St. Louis, MO, U.S.A.).

Radioimmunoassay

Prior to radioimmunassay (RIA), standards containing 100 pmol/cross-reactivity ratio/l of the C-terminal intact peptides were added to the column with 0.8 m*M* HFBA in both A and B. Fractions of 1 ml were collected from 7 to 24 ml through the column. The collected fractions were freeze-dried in a Savant Speed-vac centrifuge (Techtum Instrument, Umeå, Sweden).

The RIA procedure was performed essentially as described by Möhring and Möhring [14]. The incubation mixture was made up of 50 μl of [^{125}I]AVP, 200 μl of antiserum A8 (Ferring, Malmö, Sweden, final dilution 1:800 000) 200 μl of standard ranging from 1 to 250 p*M* or 200 μl of sample. Incubations were carried out for 48 h at 4 °C followed by the separation of bound and free radioactivity by the addition of 1 ml of plasma-coated charcoal. The assay diluent was phosphate buffer (pH 7.5) containing 0.2% neomycin sulphate and 0.1% bovine serum albumin. Freeze-dried samples were reconstituted in 1 ml of phosphate buffer, and the assay was performed in duplicate.

RESULTS

When crude extracts of samples are injected, the bulk of the unretained material elutes in the first 5 ml passing the column. This must be kept in mind when the results are considered.

As shown in Figs. 1 and 2, PFPA did not retain the most hydrophilic peptides adequately to reach the 5-min criterion, except when the highest concentrations were used. The separation of peptides was good, however, which makes this acid useful when cleaner samples are injected.

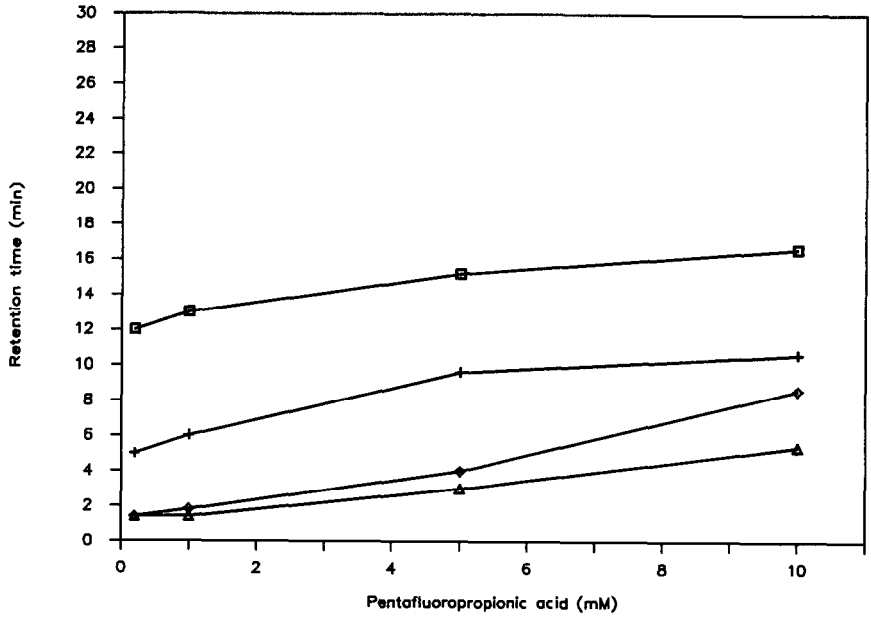

Fig. 1. Retention times of desglycinamide fragments of arginine-vasopressin on a reversed-phase PEP-RPC™ HR-5/5 FPLC column with a C_2-C_{18} stationary phase. Equal concentrations of the ion-pairing acid pentafluoropropionic acid were used in both the aqueous phase (Millipore-filtered quality) and the organic phase (methanol of LiChrosolv quality). (□) AVP (2-8); (+) AVP (4-8); (◇) AVP (5-8); (△) AVP (6-8).

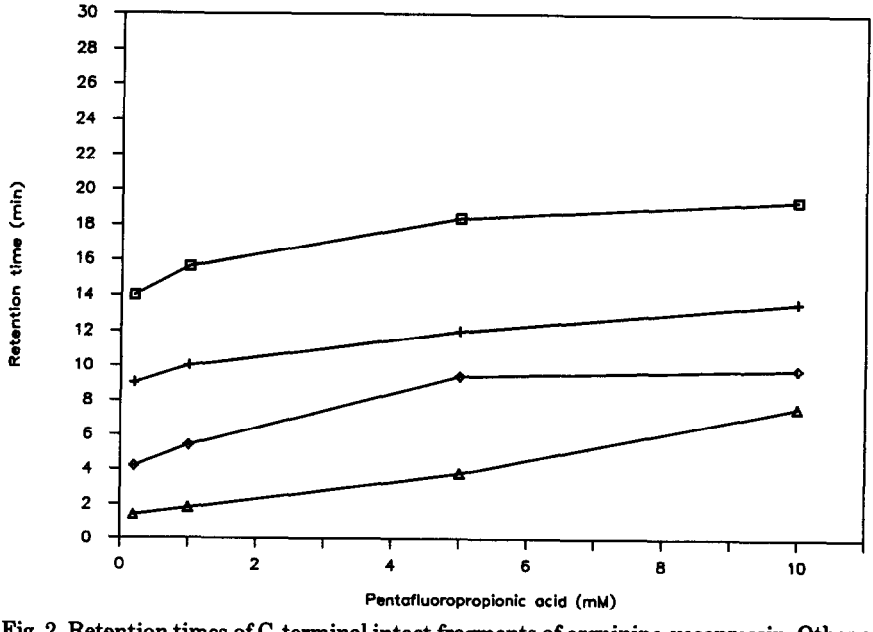

Fig. 2. Retention times of C-terminal intact fragments of argninine-vasopressin. Other conditions as in Fig. 1. (□) AVP (1-9); (+) AVP (3-9); (◇) AVP (4-9); (△) AVP (5-9).

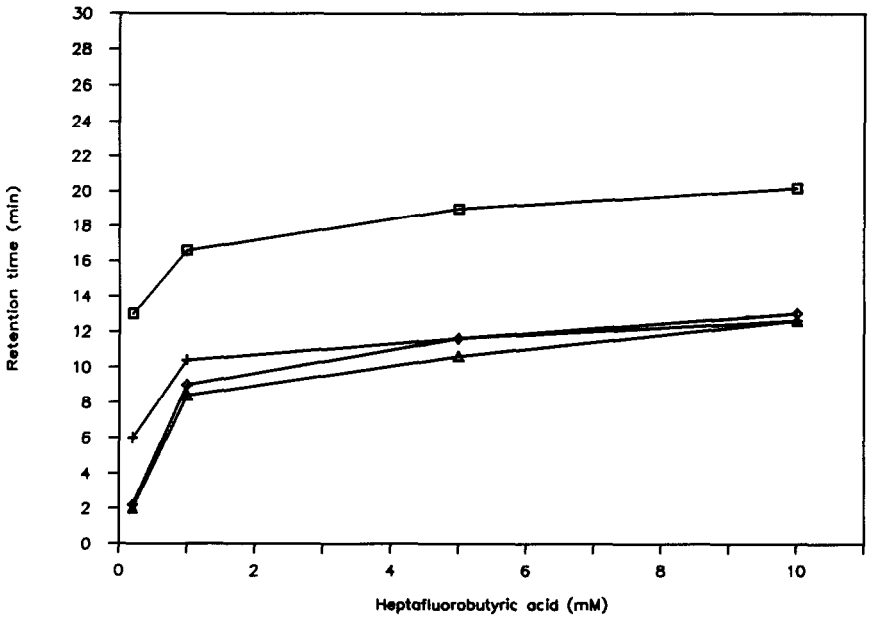

Fig. 3. Retention times of desglycinamide fragments of arginine-vasopressin with heptafluorobutyric acid as the ion-pairing agent. Other conditions as in Fig. 1. (□) AVP (2-8); (+) AVP (4-8); (◇) AVP (5-8); (△) AVP (6-8).

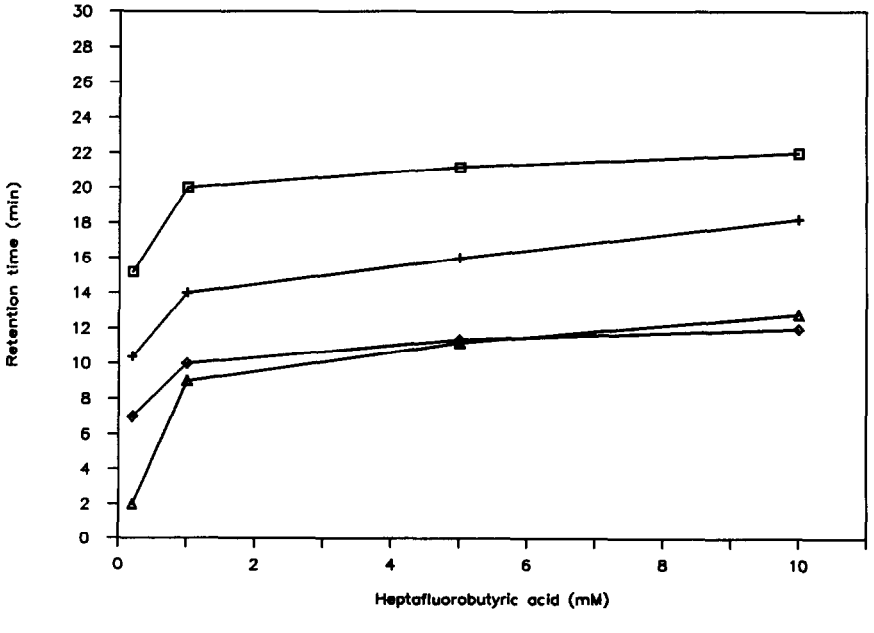

Fig. 4. Retention times of C-terminal intact fragments of arginine-vasopressin with the use of heptafluorobutyric acid as the ion-pairing agent. Other conditions as in Fig. 1. (□) AVP (1-9); (+) AVP (3-9); (◇) AVP (4-9); (△) AVP (5-9).

TABLE I

CROSS-REACTIVITY WITH AVP AT 50% INHIBITION OF BINDING OF OXYTOCIN AND FRAGMENTS OF THE VASOPRESSIN MOLECULE

Analogue	Cross-reactivity (%)
AVP	100.0
Cyt^6-AVP (3-9)	57.5
pGlu^4-Cyt^{6}AVP (4-9)	57.5
Cyt^6-AVP (5-9)	21.8
Cyt^6-AVP (2-8)	<0.045
pGlu^4-Cyt^6-AVP (4-8)	<0.045
Cyt^6-AVP (5-8)	<0.045
Cyt^6-AVP (6-8)	<0.045
Oxytocin	<0.045

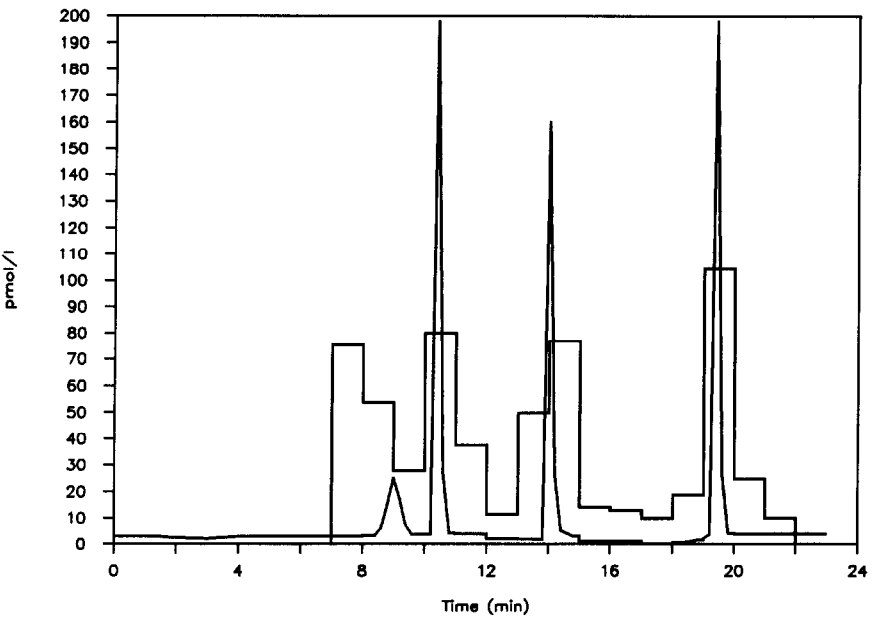

Fig. 5. Sample chromatogram of C-terminal intact fragments (5 μg of each) of arginine-vasopressin with 0.8 m*M* heptafluorobutyric acid in both the aqueous phase (Millipore-filtered quality) and the organic phase (methanol of LiChrosolv quality). Superimposed is the recovery of a standard containing 100 pmol/cross-reactivity ratio/l of each substance.

HFBA on the other hand (Figs. 3 and 4) retained all the peptides adequately when used in concentrations above 0.5 m*M*. The separation was inferior to PFPA.

The antibody used is directed toward AVP and cross-reacts with fragments as shown in Table I. Fig. 5 shows the recovery of synthetic C-terminal intact peptides, together with the elution pattern of 5 μg of these detected at 214 nm.

DISCUSSION

Reversed-phase chromatography is widely used to separate closely related peptides. The possibility of injecting crude tissue extracts directly into the column using volatile buffers enables efficient handling of the samples before RIA. The use of ion-pairing perfluorated carboxylic acids to separate closely related peptides is a well established technique [15,16]. These acids make the hydrophilic amino acids fully charged to associate with the hydrophobic anions. This tends to increase the affinity of the peptides for the reversed-phase support and to increase both retention time and resolution.

Studies during the past few years in which HPLC methods have been used to isolate vasopressin from biological extracts fall mainly into two groups, according to the technique employed. The use of trifluoroacetic acid (TFA) seems to be the more widespread [17–20]: ammonium acetate has been used by Chauvet et al. [21] and Burbach and co-workers [22–24]. In our hands, the most hydrophilic fragments were lost when 0.1% TFA was used as the ion-pairing agent. Ammonium acetate on the other hand seemed to make the fragments hydrophobic enough to be retained on the C_{18} solid phase. Although the ion-pairing capacity of the perfluorated acids HFBA and PFPA is known [15,16], these acids have been neglected.

The method described in this paper shows that these acids make possible the separation of fragments of AVP when used in adequate concentrations. The method is rapid, simple and yields a high recovery of small amounts of the fragments.

The main problem is retaining the most hydrophilic peptides to avoid contamination by unretained material. To improve further the retention of these peptides, the acid concentration in the aqueous buffer may be two or three times the concentration in the organic buffer. Since most hydrophobic peptides are rather insensitive to changes in the acid concentration, such a technique will mainly affect the retention time of the hydrophilic peptides.

HFBA must be preferred to PFPA since a concentration of only 0.8 m*M* is sufficient to retain all C-terminal intact peptides, and 2 m*M* to retain all desglycinamides. The low acid concentrations are beneficial for the chromatographic and freeze-drying equipment.

Studies on the functional properties of AVP in the central nervous system (CNS) are apparently directed toward measuring changes in tissue concentrations of the whole molecule. What is measured is presumably the pools of vasopressin stored in terminals ready to be released on stimulation. The transmitter must be inactivated after release to allow repolarization of the postsynaptic cell. The amount of metabolites would therefore be an index of activity. Notably, only one group has dealt with the analysis of the turnover of AVP [12,13]. Burbach and co-workers have studied which metabolites are formed by homogenates of brain tissue. They have thus initiated what may become of great importance for the understanding of vasopressinergic functions in the CNS. The use of antibodies directed to one or the other end of the molecule in conjunction with efficient

separation of fragments with high-performance liquid chromatography/FPLC makes possible the quantification of fragments in tissue and thus provides an index of turnover of the parent molecule.

Without direct comparison it is impossible to say whether the method presented here has any advantages over the one used by Burbach and co-workers [12,13], who used 10 m*M* ammonium acetate (pH 4.15) and 0.15% acetic acid in methanol. The present method seems, however, to be faster (the retention time for AVP (1–9) was 19 min, compared with 32 min in the method used by Burbach and co-workers) without loss of selectivity between fragments. The possibility of obtaining more information about vasopressinergic mechanisms has thus been increased by the development of another selective fractionation procedure.

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