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Note

Separation of vasopressin analogues by reversed-phase high-performance liquid chromatography

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The behaviour of neurohypophyseal hormones and their analogues in reversed-phase high-performance liquid chromatography (RP-HPLC) has been the subject of recent investigations. Krummen and Frei[1] were able to separate the pharmaceutical peptides oxytocin, deamino-oxytocin, lysine–vasopressin (LVP), ornithine–vasopressin and 2-phenylalanine–LVP from each other and from by-products and additives by HPLC on octyl- or octadecyl-silica. However, the separation of ornithine–vasopressin from the next higher homologue, LVP, could be effected only at pH 10, a requirement severely affecting the lifetime of the column packing material. A systematic study of the chromatographic properties of oxytocin and seven of its diastereoisomers, excluding only 3-D-isoleucine–oxytocin, was undertaken by Larsen *et al.*[2,3]. With the exception of 6-hemi-D-cystine- and 7-D-proline–oxytocin, all the compounds could be completely separated on an octadecyl-silica support. The influence of solvent, pH and salt concentration was also investigated by both groups. Neurohypophyseal hormones have also been included in studies of more general character, *e.g.*, by Burgus and Rivier[4] and by O'Hare and Nice[5].

For most of the substances studied by Krummen and Frei differences in chromatographic behaviour are indeed expected. At neutral pH the net charge of oxytocin and deamino-oxytocin is one and two units, respectively, lower than those of the other compounds. Elimination of the phenolic hydroxyl group, accomplished by substitution of phenylalanine for tyrosine in position 2, also leads to profound changes in polarity. Even replacement of an amino acid by its D-isomer can be expected to result in a rather drastic change of the properties of a compact cyclic peptide such as oxytocin. Of course not all positions are equally sensitive to configurational changes. Not unexpectedly, the cysteine and tyrosine residues in positions 1 and 2, respectively, were among those most easily affected. Changes in ionic strength or pH were of relatively little importance for the separation, although the order of elution was reversed in some cases when the pH was increased from 4 to 6. However, the choice of organic modifier was found to exert a greater influence on the chromatographic behaviour.

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In the present investigation the separation properties of arginine–vasopressin (AVP) and seven closely related analogues were studied (Table I). The effects of the following structural modifications were compared: (1) elimination of the α-amino group; (2) elongation of the arginine side chain and (3) inversion of the configuration of the basic amino acid. The composition of the mobile phase was varied using 0.05 M ammonium acetate or trifluoroacetate as buffer, with methanol, ethanol or acetonitrile as organic modifier. The stationary phase in all cases was 10-μm octadecyl-silica. The pH region was limited to 2.5–6.5 because of the low stability of the bonded phase below pH 2 and above pH 7. The choice of buffers was further dictated by the desire that these be volatile and removable by lyophilization. Phosphate buffers and HCl–NaCl mixtures were therefore not included.

TABLE I

STRUCTURES OF ARGININE–VASOPRESSIN AND ANALOGUES INVESTIGATED

Har = Homoarginine; Mpa = β-mercaptopropionic acid.

I	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-L-Arg-Gly-NH_2 (1 2 3 4 5 6 7 8 9; disulfide bridge Cys1–Cys6)	
II		L-Har
III		D-Arg
IV		D-Har
V	Mpa	L-Arg
VI	Mpa	L-Har
VII	Mpa	D-Arg
VIII	Mpa	D-Har

EXPERIMENTAL

Reagents

Arginine–vasopressin, D-arginine–vasopressin and their deamino analogues were obtained from Ferring (Malmö, Sweden). Other peptides were prepared in this laboratory[6,7]. Acetonitrile (HPLC grade; Rathburn Chemicals, Walkerburn, Great Britain), methanol (analytical grade; May & Baker, Dagenham, Great Britain) and 95% ethanol (analytical grade; Svensk Sprit, Stockholm, Sweden) were used without further purification. Buffer solutions were 0.05 M in total acid, and were prepared by titration of aqueous acetic or trifluoroacetic acid with concentrated ammonia to the desired pH and dilution to the final volume with Milli-Q reagent grade water (Millipore, Bedford, MA, U.S.A.). Aqueous solutions were passed through a 0.45-μm MF-Millipore filter and were then evacuated for several minutes. Organic solvents were degassed in an ultrasonic bath.

HPLC procedures

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) included two Model 6000A pumps, a Model 660 solvent flow programmer, a Model U6K injector and a Model 450 variable-wavelength detector set at 220 nm. The column (250 × 5 mm I.D.) was packed with 10-μm octadecyl-silica (Nucleosil 10 C_{18}; Macherey, Nagel & Co., Düren, G. F. R.) and was run at room temperature in the

isocratic mode. The flow-rate was 2 ml/min. The peptides were dissolved in water (0.5 mg/ml) and were injected in amounts of 5–10 nmol.

Since the deamino analogues were least affected by changes in the eluent composition, the most retarded peptide, 1-deamino-8-D-homoarginine–vasopressin (VIII), was chosen as the reference. The concentration of organic component in the mobile phase was adjusted so as to produce a k' value of *ca.* 8 for this compound. The capacity factor, k', is defined by

$$k' = \frac{V_R - V_0}{V_0} \quad (1)$$

where V_R is the retention volume of the substance of interest and V_0 the retention volume for an unretarded substance (usually the solvent). The retention data for the compound A are here discussed in terms of the logarithm of the separation factor (selectivity), α, given by

$$\alpha = \frac{V_R - V_0}{V_A - V_0} = \frac{k'_R}{k'_A} \quad (2)$$

where $V_R > V_A$, and consequently

$$\log \alpha = \log k'_R - \log k'_A \quad (3)$$

where R is the reference substance 1-deamino-8-D-homoarginine–vasopressin. The use of $\log \alpha$ permits easy comparison of any two compounds, since:

$$\log \alpha_{AB} = \log \alpha_{RB} - \log \alpha_{RA} \quad (4)$$

RESULTS AND DISCUSSION

The dependence of $\log \alpha$ on pH in different solvent systems is shown in Table II. The order of retention is the same in all the systems investigated, with AVP as the least and 1-deamino-8-D-homoarginine–vasopressin as the most retarded component. The deamino analogues are not affected by pH changes. In fact, for these peptides $\log \alpha$ is quite independent also of the choice of organic component in the mobile phase. Small deviations are observed only with ammonium acetate at pH 6.5. For the compounds carrying an α-amino group, on the other hand, a lower pH value leads to considerably shorter retention times, probably as a consequence of the increasing degree of protonation of this group. The effects are smaller in systems containing trifluoroacetic acid than in those containing acetic acid, which may be due to ion-pair formation with the stronger acid[5]. In acetate buffers the pH dependence is greatly influenced by the nature of the organic co-solvent. The weakest effects are observed with acetonitrile, indicating that this solvent with its higher dipole moment is better able to solvate polar regions of the molecule than are the alcohols[3]. The larger overall difference between amino and deamino analogues in this solvent also supports such a concept.

Changes of $\log \alpha$ related to structural modifications other than deamination are virtually independent of pH as shown by the parallel shifts within the sets of amino

TABLE II

INFLUENCE OF SOLVENT AND BUFFER COMPOSITION ON THE LOGARITHM OF THE SEPARATION FACTOR α

Values of α refer to separation from the most retarded component, 1-deamino-8-D-homoarginine-vasopressin (VIII), *cf.*, text. Column: Nucleosil 10 C_{18} (250 × 5 mm). Eluent: as indicated; total acid concentration, 0.05 *M*; flow-rate, 2 ml/min.

Compound	*Solvent: CH_3OH*					*Solvent: C_2H_5OH*					*Solvent: CH_3CN*				
	CF_3COONH_4 buffer			*CH_3COONH_4 buffer*		*CF_3COONH_4 buffer*			*CH_3COONH_4 buffer*		*CF_3COONH_4 buffer*			*CH_3COONH_4 buffer*	
	pH 2.5	*4.0*	*6.5*	*4.0*	*6.5*	*2.5*	*4.0*	*6.5*	*4.0*	*6.5*	*2.5*	*4.0*	*6.5*	*4.0*	*6.5*
I (L-Arg[8])-VP	0.47	0.44	0.35	0.59	0.33	0.42	0.38	0.34	0.68	0.38	0.51	0.48	0.40	0.50	0.40
II (L-Har[8])-VP	0.45	0.42	0.33	0.58	0.30	0.39	0.37	0.31	0.66	0.32	0.49	0.47	0.37	0.47	0.36
III (D-Arg[8])-VP	0.43	0.40	0.32	0.54	0.26	0.37	0.35	0.29	0.63	0.29	0.49	0.46	0.37	0.47	0.36
IV (D-Har[8])-VP	0.38	0.36	0.27	0.50	0.22	0.32	0.31	0.24	0.59	0.24	0.45	0.42	0.33	0.43	0.31
V (Mpa[1], L-Arg[8])-VP	0.12	0.12	0.12	0.12	0.15	0.12	0.11	0.12	0.13	0.13	0.11	0.11	0.11	0.09	0.12
VI (Mpa[1],L-Har[8])-VP	0.11	0.11	0.11	0.10	0.12	0.10	0.09	0.10	0.11	0.11	0.09	0.09	0.09	0.08	0.10
VII (Mpa[1], D-Arg[8])-VP	0.06	0.05	0.06	0.06	0.07	0.06	0.05	0.06	0.06	0.06	0.05	0.06	0.05	0.04	0.06
VIII (Mpa[1], D-Har[8])-VP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

and deamino compounds. Small deviations from parallelism are observed only in the presence of acetate. Next to deamination, inversion of the configuration of the basic amino acid yields the greatest effects on log α. The largest difference is observed for the deamino analogues, indicating that in those molecules greater portions are made accessible for interaction with the hydrophobic stationary phase. Substitution of homoarginine for arginine produces smaller shifts of log α. However, the effects are more pronounced for the D- than for the L-isomers. In the former, therefore, the side chain of the basic amino acid residue seems to be exposed to the environment to a greater extent than is the case for the peptides containing L-arginine or L-homoarginine.

From the data presented in Table II, ammonium acetate at pH 6.5, admixed with methanol or ethanol, would seem the best choice of mobile phase for separation of the peptides investigated. A typical chromatogram illustrating the influence of the three types of structural modifications in such a solvent mixture is shown in Fig. 1. Of particular interest is the complete separation of D-arginine- and 1-deamino-8-D-arginine–vasopressin from the corresponding homoarginine compounds at this rather low pH value. Acetonitrile, frequently advocated as "the organic modifier" in RP-HPLC of peptides, in this case yields the least satisfactory results.

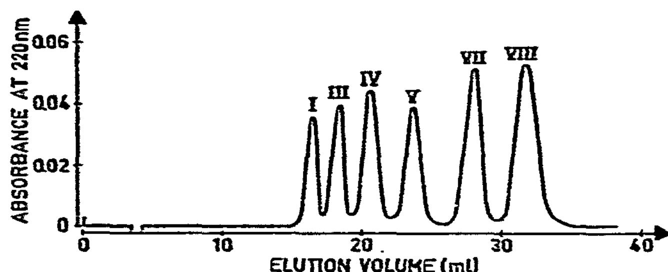

Fig. 1. Chromatogram of vasopressin analogues. Column: Nucleosil 10 C_{18} (250 × 5 mm). Eluent: 0.05 M CH_3COONH_4 (pH 6.5)–CH_3OH (39%, v/v); flow-rate, 2 ml/min. Sample size: 5–10 nmol. For identification of peaks see Table I.

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