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Note

Ion-exchange high-performance liquid chromatographic analysis of the products of the enzymatic degradation of oxytocin

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The cyclic nonapeptide oxytocin, a pituitary gland hormone, is a very important compound in mammalian biology and much attention has been given to its determination. Biological assays are time consuming and lack precision and, therefore, repeated tests are necessary. High-performance liquid chromatography (HPLC) is generally the most suitable method [1–15]; it has become established in pharmaceutical quality control [1,2,7,9–11,13], as the results correlate well with those of the bioassays employing chicken blood pressure, rat uterus contraction [1,2,7] and milk ejection pressure [10,11]. Ion-exchange and reversed-phase separation systems have been utilized with similar analytical quality of the results [10]. Most papers have dealt with the determination of oxytocin in pharmaceutical preparations produced by chemical synthesis or in combined formulations containing ergometrin [10,11].

This paper reports the separation of oxytocin from the products of its enzymatic degradation on a strong cation exchanger. The dependences of the retention on the mobile phase composition, its pH and the organic modifier content were investigated, as was the correlation between the retention and the properties of the displacer salt cations.

EXPERIMENTAL

The test peptides are listed in Table I and were synthesized in the Institute of Organic Chemistry and Biochemistry, Czechoslavak Academy of Sciences [16–19]. All the other chemicals were of analytical-reagent grade from Lachema (Brno, Czechoslovakia), and were used as received.

The HPLC system consisted of an LC-XPD pump, an LC–UV variable-wavelength detector (both from Pye Unicam, Cambridge, U.K.), a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injection valve with a 20-μl loop and a Model TZ 4620 line

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TABLE I

LIST OF THE TEST COMPOUNDS

No.	Compound[a]
1	Asn
2	Gln
3	Gly–NH_2
4	Gln–Asn
5	Leu–Gly
6	Leu–Gly–NH_2
7	(*S*)-(Bzl)–Cys
8	Pro–Leu–Gly–NH_2
9	Ala–Pro–Leu–Gly–NH_2
10	(*S*)-(Bzl)–Cys–Pro–Leu–Gly–NH_2
11	Ile–Gln–Asn–Cys–(Bzl)–Pro–Leu–Gly–NH_2
12	Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2 (oxytocin)

[a] (Bzl) = benzyl.

recorder (Laboratorní Přístroje, Prague, Czechoslovakia).

Two types of column were used: Partisil SCX, 10 μm (25 cm × 4.6 mm I.D.) stainless-steel (Pye Unicam) and Separon HEMA S 1000, 10 μm (15 cm × 3 mm I.D.) glass (Tessek, Prague, Czechoslovakia).

The mobile phase was based on 0.02 *M* aqueous potassium dihydrogenphosphate, the pH of which was regulated by additions of H_3PO_4 and KOH solutions. Various displacer salts (LiCl, NaCl, KCl or $MgCl_2$) and various amounts of methanol were added. The mobile phase was deaerated for 20 min in an ultrasonic bath prior to use.

The column was equilibrated with a particular mobile phase for at least 30 min before commencing the measurements. The work was carried out at laboratory temperature (20 ± 2°C) and all the measurements were repeated at least three times. Stock solutions were prepared by dissolving the test substances in deionized water and were stored in a refrigerator. The working solutions were prepared immediately before use by diluting the stock solutions with the mobile phase. The mobile phase flow-rate was 0.5 ml min^{-1} in all the experiments. The procedure for the enzymatic degradation of oxytocin was described in detail elsewhere [16,20,21].

RESULTS AND DISCUSSION

Two types of column were tested for the separation of the peptides produced by enzymatic cleavage of oxytocin: a strong cation exchanger (Partisil SCX) and a size-exclusion phase (exclusion limit 800–2000 kDa) exhibiting weak hydrophobic interactions (Separon HEMA S 1000). The separation on the HEMA column was poor and could not be improved by varying the mobile phase composition and pH. Alpert and Andrews [14] concluded that reversed-phase HPLC systems are unsuitable for separations of strongly hydrophilic or hydrophobic peptides. Therefore, only the ion-exchange separation was studied further in detail.

Effect of mobile phase pH

This effect was studied over the pH range 4–7 and the log k *vs.* pH dependences for selected solutes are given in Fig. 1. It is obvious that the solute retention is not much influenced. Two opposing effects apparently exist: the dissociation of the acidic functional groups of the cation exchanger increases with increasing pH of the mobile phase and the retention of the protonated solutes should also increase. However, this effect is compensated for by dissociation of the solutes, which contain free amino groups and some of them also $-CONH_2$ groups. These groups are gradually deprotonated with increase in the pH and hence their affinity toward the cation-exchange sites decreases. The result is a slight decrease in the solute retention with increasing pH. The effect of dissociation of the cation exchanger predominates only at low pH values with the solutes containing $-CONH_2$ groups; therefore, the retention increases with increasing pH in this instance (see Fig. 1).

Effect of the type and concentration of the displacer salt

The effect of the displacer salt concentration was studied with KCl and the results are depicted in Fig. 2. It can be seen that the solute retention depends strongly on salt concentration and, in agreement with the theory of ion-exchange, the higher the displacer salt concentration, the smaller are the capacity ratios. The same trend was also observed for the other displacer salts studied, *i.e.*, NaCl, LiCl and $MgCl_2$.

The effect of the type of the displacer salt cation is evident from Fig. 3, in which the logarithms of the solute capacity factors, log k, are plotted against the ratio of the hydrated ion radius to its charge number, r/z. The dependences are linear and the parameters of the regression equation,

$$\log k = a(r/z) + b$$

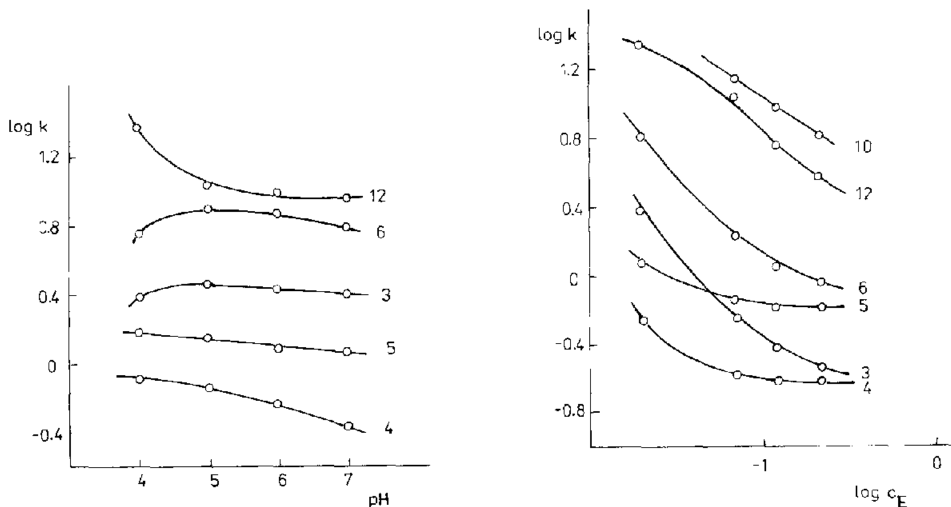

Fig. 1. Dependence of log k on the mobile phase pH. For the solutes see Table I. Mobile phase: 0.02 M KH_2PO_4, pH adjusted with H_3PO_4 or KOH.

Fig. 2. Dependence of log k on log (displacer salt concentration). For the solutes see Table I. Mobile phase: 0.02 M KH_2PO_4 (pH 5.0), various concentrations of KCl.

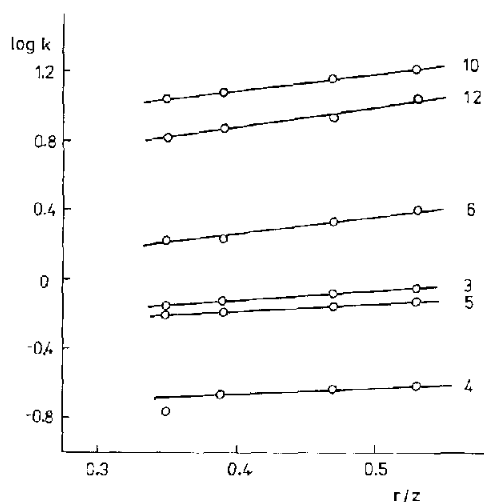

Fig. 3. Dependence of log k on the ratio of the hydrated cation radius r to its charge z. For the solutes see Table I. Mobile phase, 0.02 M KH_2PO_4 (pH 5.0); displacer salt concentration, 0.1 mol l^{-1}.

are listed in Table II. The good correlation verifies the validity of the ion-exchange mechanism for this particular system as the solute electric charge density is the predominant factor in ion-exchange separations.

Effect of organic modifier content

Methanol was used as the organic modifier in the range 0–40% (v/v). The results (Fig. 4) demonstrate that there is an influence on the retention, especially with the higher peptides, with a decrease in the retention with increasing methanol content. This phenomenon is due to two effects: methanol suppresses the protonation of amino acids and peptides and thus decreases their affinity toward the stationary phase

TABLE II

REGRESSION EQUATIONS FOR THE LINEAR RELATIONSHIP BETWEEN LOG k AND THE r/z VALUE OF THE DISPLACING IONS

Displacing ion r/z: Mg^{2+} 3.5, K^+ 3.9, Na^+ 4.7, Li^+ 5.3. Mobile phase, 0.02 M KH_2PO_4 (pH 5.0); concentration of the displacing ion, 0.1 mol l^{-1}.

Compound[a]	Regression equation	Correlation coefficient
3	$\log k = 0.0565\ r/z - 0.3445$	0.9916
4	$\log k = 0.0751\ r/z - 0.9993$	0.8843
5	$\log k = 0.0475\ r/z - 0.3739$	0.9771
6	$\log k = 0.0963\ r/z - 0.1175$	0.9870
8	$\log k = 0.1306\ r/z + 0.68$	0.9997
12	$\log k = 0.1328\ r/z + 0.378$	0.9843

[a] See Table I.

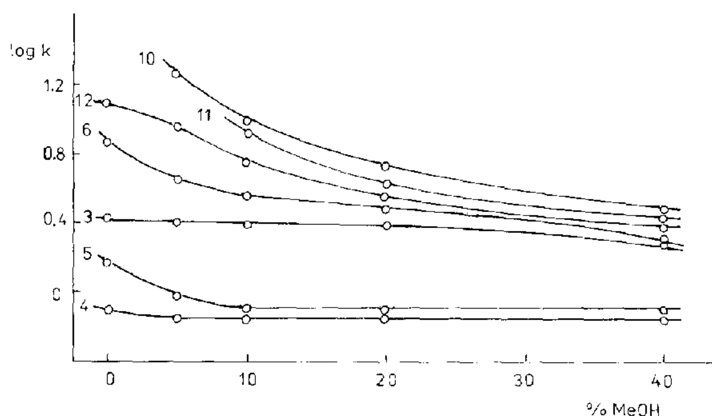

Fig. 4. Dependence of log k on the methanol (MeOH) content in the mobile phase. For the solutes see Table I. Mobile phase: 0.02 M KH_2PO_4 (pH 5.0).

ion-exchange sites, and the solutes, especially the higher peptides, also contain hydrophobic functional groups and hence their solubility in the mobile phase increases with increasing methanol content. In general, the larger the solute molecule, the greater is the effect of the methanol content.

All the above measurements lead to the optimum mobile phase composition 0.02 M KH_2PO_4 (pH 5.0)–methanol (9:1, v/v). The solute capacity factors obtained with this mobile phase are given in Table III and a chromatogram of a model mixture of the solutes under these conditions is shown in Fig. 5. It is clear that some solutes listed in Table III cannot be satisfactorily separated, *e.g.*, groups of substances 1, 2 and 4, 5 and 7 and 8, 9 and 10. However, the enzymatic degradation leads to much simpler mixtures than given in Fig. 5, the composition depending on the enzyme used [16,20–22].

TABLE III

CAPACITY FACTORS AND DETECTION LIMITS FOR THE TEST COMPOUNDS

Compound[a]	Capacity factor	Detection limit (ng in 20 μl)
1	0.61	150
2	0.60	160
4	0.63	18
7	0.79	2.4
5	0.80	37
3	2.63	460
6	3.75	250
12	5.86	18
10	9.4	49
11	8.4	1
8	9.0	240
9	9.0	95

[a] See Table I.

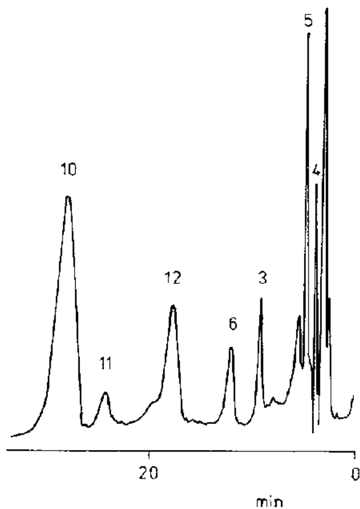

Fig. 5. Separation of the enzymatic degradation products of oxytocin. Column, Partisil SCX; mobile phase, 0.02 *M* KH_2PO_4 (pH 5.0)–methanol (9:1, v/v); flow-rate, 0.5 ml min^{-1}; UV detection at 209 nm. Compounds 3–6 and 10–12 are listed in Table I.

UV photometric detection is difficult with these solutes, as they absorb at very low wavelengths. A wavelength of 209 nm was used in this work and the limits of detection given in Table III demonstrate that the detection sensitivity is not particularly high; tens to hundreds of nanograms of the solutes in the volume injected, 20 μl, can be detected.

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