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# SOLID-PHASE SYNTHESIS OF OXYTOCIN AND ITS ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

I. É. Zel'tser, A. A. Antonov,
A. K Ivanov, V. P. Pakhomov,
and V. N. Karel'skii

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A mixture of peptides containing 30.4% of oxytocin has been obtained by the solid-phase method on the benzhydrylamine resin UR-95. A procedure is given for the quantitative determination of oxytocin in a mixture of peptides.

Synthetic oxytocin is widely used in obstetrics and gynecology, since, in contrast to the preparation obtained from the posterior lobe or the hypophysis, it does not contain undesirable impurities with a hypertensive action.

Work on the synthesis of oxytocin is being carried on in two directions: the classical, or liquid-phase, synthesis and solid-phase synthesis or synthesis on polymeric supports. In spite of the number of advantages of solid-phase synthesis – rapidity and simplicity of the working operations, the possibilities of performing the individual stages of the process under standard conditions, of performing the whole synthesis in one reactor, and of the automation of the process – it has not yet found wide use in our country in, in particular, the development of investigation devoted to mastering the industrial synthesis of peptides. One of the delaying factors in the process of mastering this synthesis is the formation of shortened and other incorrect sequences arising because of the incomplete occurrence of the condensation reactions or because of side reactions.

The quantitative estimation of the desired peptide obtained as the result of synthesis is carried out by determining its biological activity after purification from contaminating auxiliary peptides and salts (by column chromatography, countercurrent distribution, preparative high performance liquid chromatography, etc.). However, this method is fairly laborious and requires large amounts of working time.

The aim of our work was to use the method of high performance liquid chromatography (HPLC) for the quantitative testing of samples of synthetic oxytocin obtained by the solid-phase method. This approach permits an estimation of the influence of the conditions of condensation and of the removal of protective groups on the yield of desired products and the optimization of the process of obtaining the peptide.

Oxytocin was synthesized by the solid-phase method on a sample of UR-95 resin (GDR), which is a commercial product of the copolymerization of styrene with 2% of divinylbenzene containing benzhydrylamine groups (0.7 mmole of $NH_2$ groups per 1 g of resin). The synthesis was performed by the successive growth of the polypeptide chain from the C-end by the carbodiimide method and by the activated-ester method [1]. After the end of synthesis, the peptide was split off from the resin with simultaneous deblocking by the action of liquid HF at 20°C in the presence of anisol [2]. The product after separation from the resin was oxidized in aqueous solution.

The mixture of peptides containing oxytocin was analyzed by reversed phase HPLC (Fig. 1), which permits the separation of peptides of close molecular weights and similar struc-

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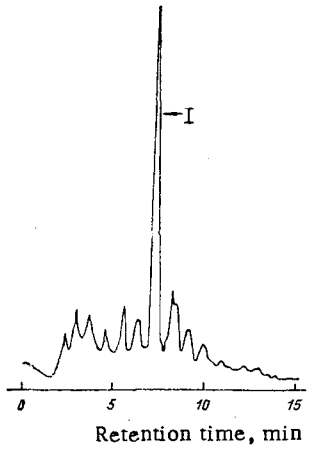


Fig. 1. Chromatogram of the mixture of peptides obtained in the synthesis of oxytocin on UR-95 resin. Conditions of separation: Ultrasphere ODS column (4.6 × 240 mm); eluent: acetonitrile–0.05% solution of trifluoroacetic acid in water (24:76); temperature: 20°C; rate of flow of the eluent: 1 ml/min. I – oxytocin.

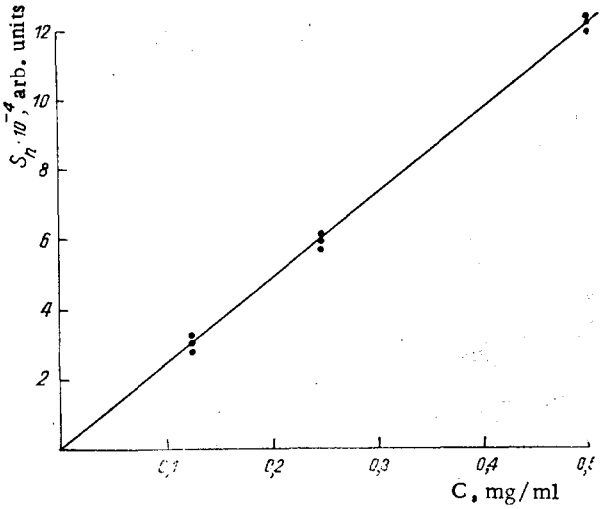


Fig. 2. Dependence of the area of the peak on the chromatogram on the concentration of a standard solution of oxytocin in water.

tures [3]. The chromatographic analysis was performed on an Ultrasphere ODS column using aqueous acetonitrile as eluent. The amount of oxytocin in the mixture obtained was determined from the calibration curve of the dependence of the concentration of a standard sample of oxytocin in solution on the area of the peak on the chromatogram (Fig. 2). The methods give satisfactorily reproducible results correlating with those of tests of biological activity of control samples of oxytocin.

We have investigated the influence of the degree of attachment of the first amino acid of the polymer on the yield of oxytocin. The results obtained are given in Table 1. It was assumed that a decrease in the degree of attachment of the first amino acid through an increase in the volume of the $N^{\alpha}$-protective group should increase the amount of oxytocin in the mixture of peptides obtained after synthesis because of the decrease in the concentration of $NH_2$ groups in the glycyl polymer and, consequently, an increase in their spatial accessibility. However, the concentrations of $NH_2$ groups formed on the

TABLE 1. Synthesis of Oxytocin on UR-95 Resin

| Conditions of synthesis | | Initial resin, g | Peptidyl-polymer after synthesis, g | Conditions of splitting off the peptide from the polymer with liquid HF | | Yield of peptides after splitting off from the resin, % | Peptide obtained from 1 g of resin, mg | Oxytocin in the mixture of peptides, % |
|---|---|---|---|---|---|---|---|---|
| first amino acid | degree of attachment of the amino acid to the polymer, % | | | reaction time, h | temperature, °C | | | |
| 1. BOCGlyOH | 93,5 | 2,0 | 3,30 | 1 | 20 | 78,5 | 430 | 14,7 |
| 2. BOCGlyOH | 93,5 | 2,0 | 3,30 | 2 | 20 | 80,2 | 537 | 14,0 |
| 3. TrtGlyOH | 88,5 | 1,5 | 2,20 | 1 | 22 | 62,2 | 290 | 8,6 |
| 4. BOCLeuGlyOH | 100,0 | 1,5 | 2,75 | 1 | 21 | 62,1 | 518 | 30,4 |

TABLE 2. Program for the Addition of Leu[8], Pro[7], Cys[6], and Ile[3]

| Stage | Reagent | Volume, ml | Time of the operation, min | Number of repetitions |
|---|---|---|---|---|
| 1 | $CH_2Cl_2$ | 20 | 2 | 2 |
| 2 | Condensation: twofold excess of BOC-amino acid in $CH_2Cl_2$; twofold excess of DCC (1 N solution in $CH_2Cl_2$) | 25 | 120 | 1 |
| 3 | $CH_2Cl_2$ | 20 | 2 | 2 |
| 4 | MeOH | 20 | 2 | 2 |
| 5 | $CHCl_3$ | 20 | 2 | 2 |
| 6 | Acylation: 1.1 ml of $Ac_2O$ and 1.6 ml of $Et_3N$ in $CHCl_3$ | 25 | 30 | 1 |
| 7 | $CHCl_3$ | 20 | 2 | 2 |
| 8 | MeOH | 20 | 2 | 2 |
| 9 | $CH_2Cl_2$ | 20 | 2 | 2 |
| 10 | Deblocking: 50% $CF_3COOH$ in $CH_2Cl_2$ | 25 | 5 | 1 |
| | | 25 | 30 | 1 |
| 11 | $CH_2Cl_2$ | 20 | 2 | 2 |
| 12 | MeOH | 20 | 2 | 2 |
| 13 | $CHCl_3$ | 20 | 2 | 2 |
| 14 | 10% solution of $Et_3N$ in $CH_2Cl_2$ | 20 | 5 | 1 |
| | | 20 | 10 | 1 |
| 15 | $CHCl_3$ | 20 | 2 | 2 |
| 16 | $CH_2Cl_2$ | 20 | 2 | 2 |
| 17 | Determination of free $NH_2$ groups [4] | | | |

addition of BOCGlyOH and TrtGlyOH to the polymeric matrix with subsequent deblocking of the $N^{\alpha}$-protective groups were 0.654 and 0.619 mmole/g of resin, and the amounts of oxytocin in the mixtures obtained 14.7 and 8.6%, respectively. Thus, an increase in the volume of the $N^{\alpha}$-protective group of the first amino acid does not lead to the expected result, since the addition of BOCLeuOH to the glycyl-polymer takes place with a yield of 70-76%, determined by Dorman's method [4]. On the addition of the dipeptide BOCLeuGlyOH to the resin, the amount of oxytocin in the mixture of peptides obtained reached 30% (see Table 1); this is connected with the elimination from the scheme of the stage of adding BOCLeuOH, which takes place with a yield that is low for solid-phase synthesis. Furthermore, the addition of the dipeptide BOCLeuGlyOH to the polymeric matrix took place with quantitative yield.

Thus, the amount of oxytocin in the final mixture of peptides depends on the optimum conditions of each stage of the successive growth of the polypeptide chain and not only on the degree of attachment of the first amino acid to the polymeric matrix.

## EXPERIMENTAL

L-Amino acid derivatives were used. The amount of free $NH_2$ groups after the elimination of the Boc protection in each stage was determined by Dorman's method [4].

The mixture of peptides obtained after synthesis was analyzed by the HPLC method. The analysis was performed on a Beckman-Altex instrument fitted with a model 110A pump and a model 210 injector having a 20-μl loop, and also a Uvidec-100 spectrophotometer and a Chromatopac C-RIA integrator. An Ultrasphere ODS column (USA) with dimensions of 4.6 × 250 mm was used. The amount of sample added was 20-40 μg for the materials investigated (20 μl of a 0.1-0.2% solution). The samples for analysis were dissolved in the eluent. The eluent contained acetonitrile (Merck) of "for chromatography" grade and double-distilled water that had been passed through ion-exchange resins. Synthetic oxytocin purified by preparative TLC, with a biological activity of 500 units/mg, was used as standard.

1. Plotting of a Calibration Curve of the Dependence of the Area of the Chromatographic Peak ($S_n$) on the Concentration of Oxytocin. For plotting the calibration curve, solutions were prepared that contained 0.500, 0.250, and 0.125 mg of standard synthetic oxytocin with an activity of 500 units/mg in 1 ml of eluent, which was a mixture of acetonitrile and a 0.05 % solution of trifluoroacetic acid in water (24:76). The solutions prepared were fed to the chromatograph in random order. The analysis of each sample was carried out three times. The areas of the chromatographic peaks corresponding to the oxytocin peak on a chromatogram, expressed in optical density units, were used to plot a graph

TABLE 3. Program for the Addition of $Asn^5$ and $Gln^4$

| Stage | Reagent | Volume, ml | Time of the operation, min | Number of repetitions |
|---|---|---|---|---|
| 1 | DMFA | 20 | 2 | 2 |
| 2 | Condensation: fourfold excess of the p-nitrophenyl ester of a BOC-amino acid in DMFA | 25 | 24 | 1 |
| 3 | DMFA | 20 | 2 | 2 |
| 4 | MeOH | 20 | 2 | 2 |
| 5 | $CHCl_3$ | 20 | 2 | 2 |
| 6–17 | See stages 6-7 in Table 2 | | | |

of the dependence of the area of the chromatographic peak on the concentration of oxytocin in solution (Fig. 2). To check the calibration curve, solutions of synthetic oxytocin of known concentrations were prepared. The result of the analysis of these samples showed that their concentrations corresponded to those obtained for the calibration curve with an error of 10%.

2. Determination of the Concentrations of Oxytocin in Samples Containing Mixtures of Peptides Obtained as the Result of Solid-Phase Synthesis. An accurately weighed sample of the mixture of peptides (10-20 mg) was dissolved in 10 ml of eluent and was analyzed by the HPLC method (for the conditions of analysis, see the caption to Fig. 1). The concentration of oxytocin in solution was determined from the arithmetic mean value of the area of the chromatographic peak (see Fig. 2). The amount of oxytocin in the sample was calculated from the formula

$$\frac{c \times V}{m} \times 100\%,$$

where c is the concentration of oxytocin in the sample, determined from the calibration graph, mg/ml; V is the volume in which the weighed sample was dissolved, ml; and m is the weight of the sample for analysis, mg.

3. Preparation of the Glycyl-Polymer. A. In a reactor for solid-phase synthesis, 2 g of UR-95 resin was washed successively with $CH_2Cl_2$ (20 ml × 2 min), $CHCl_3$ (twice, 20 ml × 2 min), a 10% solution of $Et_2N$ in $CHCl_3$, (twice, 20 ml × 2 min), $CHCl_3$ (20 ml × 2 min), and $CH_2Cl_2$ (twice, 20 ml × 2 min). Then 0.49 g (2.8 mmole) of BOCGlyOH or 0.81 g (2.8 mmole) of BOCLeuGlyOH in 20 ml of $CH_2Cl_2$ was added to the resin, the mixture was stirred for 10 min, after which 2.8 ml of a 1 N solution of DCC in $CH_2Cl_2$ was added. The reaction mixture was stirred at room temperature for 5 h and was left for 12 h, and was then filtered and the resin was washed successively with $CH_2Cl_2$ (twice, 20 ml × 2 min), MeOH (twice, 20 ml × 2 min), and $CHCl_3$ (twice, 20 ml × 2 min). The resin was stirred with 1.1 ml of $Ac_2O$ and 1.6 ml of $Et_3N$ in 20 ml of $CHCl_3$ for 30 min and it was then filtered off and washed successively with $CHCl_3$ for 30 min and it was then filtered off and washed successively with $CHCl_3$ (twice, 20 ml × 2 min), MeOH (twice, 20 ml × 2 min), and $CH_2Cl_2$ (twice, 20 ml × 2 min). Then 25 ml of 50% $CF_3COOH$ in $CH_2Cl_2$ was added to the resin and the mixture was left for 5 min, after which the resin was filtered off and was again treated with 25 ml of 50% $CF_3COOH$ in $CH_2Cl_2$ and the mixture was stirred for 30 min and filtered. Then the resin was washed with $CH_2Cl_2$ (twice, 20 ml × 2 min), MeOH (twice, 20 ml × 2 min), $CHCl_3$ (twice, 20 ml × 2 min), a 10% solution of $Et_3N$ in $CHCl_3$ (20 ml × 5 min and 20 ml × 10 min), $CHCl_3$ (twice, 20 ml × 2 min), and $CH_2Cl_2$ (twice, 20 ml × 2 min). According to Dorman's method [4], the amount of glycine was 0.655 mmole/g of resin, and the amount of leucylglycine 0.7 mmole/g of resin.

B. In a reactor for solid-phase synthesis, 1.5 g of UR-95 resin was activated as described in paragraph A, and then 0.67 g (2.1 mmole) of TrtGlyOH in 25 ml of $CH_2Cl_2$–DMFA (4:1) was added, the mixture was stirred for 10 min, and then 2.1 ml of a 1 N solution of DCC in $CH_2Cl_2$ was run in. The reaction mixture was stirred at room temperature for 5 h and left for 16 h and then filtered and the residue was washed successively with DMFA, MeOH, $CH_2Cl_2$, and $CHCl_3$. Acylation and deblocking were carried out as described in paragraph A. According to Dorman's method [4], the amount of glycine was 0.62 mmole/g of resin.

4. Preparation of (S-Benzyl)cysteinyl(O-benzyl)tyrosinylisoleucylglutaminylasparaginyl-(S-benzyl)cysteinylprolylleucylglycyl-polymer. To the glycyl-polymer were added, successively, $Leu^8$, $Pro^7$, and $Cys^6$ in accordance with the program given in Table 2, $Asn^5$ and $Gln^4$ by the program given in Table 3, and $Ile^3$ by the program given in Table 2. $BOCTyr^2(Bzl)OH$ was added to the corresponding peptidyl-polymer in accordance with the program of Table 2, using a fourfold excess of BOCTyr(Bzl)OH in 20 ml of DMFA, and condensation was carried out with stirring for 6 h and without stirring for 12 h. $BOCCys^1(Bzl)OH$ was added by the program of Table 2 without acylation stages (6-9). The peptidyl-polymer obtained was washed out from the reactor and was dried at 0.1 mm Hg and 40°C to constant weight. The yields of peptidyl-polymer are given in Table 1.

5. Separation of the Peptide from the Resin. Preparation of S,S'-Dihydrooxytocin. The solid-phase reactor was charged with 2 g of the peptidyl-polymer and 1.5 ml of anisole and was cooled to −74°C (dry ice/acetone), and the air was pumped out (twice, for 5 min). After this, 20 ml of liquid HF was condensed into the reactor and the resulting mixture was stirred at 20°C for 15 min plus the time shown in Table 1. Then the HF was evaporated in vacuum over 15 min, and the residue was treated with 30 ml of dry ether, and it was filtered off and washed with ether (3 × 10 ml). Then it was dried at 40°C/0.1 mm Hg. The dry residue was treated with 40 ml of 1 N AcOH, the mixture was stirred for 10 min and was filtered, and the residue was washed with 1 N AcOH (3 × 20 ml) and with $H_2O$ (2 × 25 ml), and the filtrate was lyophilized. This gave 0.64 g of a white powder.

6. Preparation of Oxytocin. The white powder obtained after lyophilization (0.5 g) was dissolved in 0.5 liter of distilled water, and the solution was filtered and was left at room temperature in a flask closed with a polyethylene stopper for 8 days and was then lyophilized. This gave 0.48 g of a white powder which was analyzed by the HPLC method for its oxytocin content (see Table 1).

## SUMMARY

1. A mixture of peptides containing 30.4% of oxytocin has been obtained by the solid-phase method on the benzhydrylamino resin UR-95 (GDR).

2. A method has been developed for the quantitative determination of oxytocin in a mixture of peptides by the HPLC method.

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