Journal of Chromatography, 391 (1979) 391–397

CHROM. 11,944

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INTERMEDIATES IN THE OXYTOCIN SYNTHESIS*

F. NACHTMANN
Biochemie GmbH, A-6250 Kundl (Austria)
(First received March 22nd, 1979; revised manuscript received April 25th, 1979)

SUMMARY

A high-performance liquid chromatographic method for the determination of peptides formed during the synthesis of oxytocin, is described. No derivatization is necessary; all peptides are detected in UV at 215 nm. The limits of detection are in the ng range, both for free and protected peptides. Reproducibilities of 2–3% relative standard deviation were obtained. The simplicity, speed and selectivity of the separations render this technique suitable to in-process control of oxytocin synthesis.

INTRODUCTION

In peptide synthesis, many steps must be taken to protect functional groups before the coupling of amino acids, as well as for their subsequent liberation. However, in the absence of a 100% yield for these reactions, impurities in peptides, similar to the main product, will occur. There is a need for chromatographic techniques capable of determining qualitatively and quantitatively closely related peptides. A review of all techniques available up until 1972, such as thin-layer chromatography (TLC), paper chromatography (PC), gas chromatography (GC) or electrophoresis, was published by Rosmus and Deyl[1]. TLC and PC permit rapid qualitative results, but exact quantitative determinations are hardly possible. GC can only be applied after derivatization, and is therefore not suitable for routine analysis. Thus, many attempts in column liquid chromatography have been made during the last few years. Selective peptide separations are possible with classical anion exchangers: however, this a very time-consuming method[2,3]. Important improvements have been made since the development of chemically modified stationary phases for high-performance liquid chromatography (HPLC). Different authors describe separations of peptides and proteins without previous derivatization[4–19]; most of these separations are carried out by reversed-phase HPLC.

In this paper it is shown that reversed-phase HPLC is a powerful tool for the determination of free, as well as, of protected peptides that are formed during the

* This paper was presented at *Euroanalysis III, August 20–25, 1978, Dublin.*

synthesis of oxytocin. This is the basis for the optimization and in-process control of the complete synthesis.

EXPERIMENTAL

Materials

All solvents used were of analytical grade quality (E. Merck, Darmstadt, G.F.R.). The peptides were provided by Sanabo (Vienna, Austria). Their structures are given in Table I. As a stationary phase for the HPLC separations LiChrosorb RP-8 (E. Merck), 5 μm particle size, was used. Steel columns, 15 cm × 3.2 mm I.D., were packed by a slurry technique[20].

TABLE I

STRUCTURE OF THE INVESTIGATED SUBSTANCES
CbO = Carbobenzoxy.

Structure	*Compound*
CbO-Pro	CbO-proline
CbO-Leu-Gly-ethyl ester	CbO-dipeptide ester
CbO-Pro-Leu-Gly-ethyl ester	CbO-tripeptide ester
CbO-S-benzyl-Cys-Pro-Leu-Gly-NH_2	CbO-tetrapeptideamide
CbO-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	CbO-pentapeptideamide
Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	Pentapeptideamide
CbO-Gln-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	CbO-hexapeptideamide
Gln-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	Hexapeptideamide
PyroGln-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	Pyroglutamine-hexapeptide amide
CbO-Gln-ONP	CbO-Gln-nitrophenyl ester
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	Nonapeptideamide
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	Oxytocin

Apparatus

A Hewlett-Packard 1010B liquid chromatograph, equipped with a Rheodyne 905-42 loop injector (injection volume 20 μl) was used for all separations. For detection a Perkin-Elmer LC 55 and a Schoeffel SF 770 spectrophotometer were used. The detection wavelength was 215 nm. All separations were performed isocratically at room temperature (20–22°).

A Laboratory Data System 3353 (Hewlett-Packard) was used for the evaluation of the data. Before injection, solid samples were dissolved in the mobile phase or methanol. Liquid samples (*e.g.* reaction solutions, mother liquors) can be injected after dilution to a suitable concentration with the mobile phase.

RESULTS AND DISCUSSION

The aim of the investigation was to develop a simple method for the determination of the most important intermediates in oxytocin synthesis. Therefore only one column was used and only isocratic elution was applied for all analyses. A column, length 15 cm, filled with the smallest particles commercially available (5 μm) was sufficient for an optimal separation of the relevant peptides.

Qualitative separations

For peptide separations reversed-phase materials with a carbon chain of 8 or 18 carbon atoms, proved to be suitable[7,8,16–18]. Our experiments showed no significant difference in the selectivity of LiChrosorb RP-8 (C_8 carbon chain) and LiChrosorb RP-18 (C_{18} carbon chain). All further investigations were performed with LiChrosorb RP-8.

For the mobile phase, mixtures of sodium phosphate buffer and acetonitrile

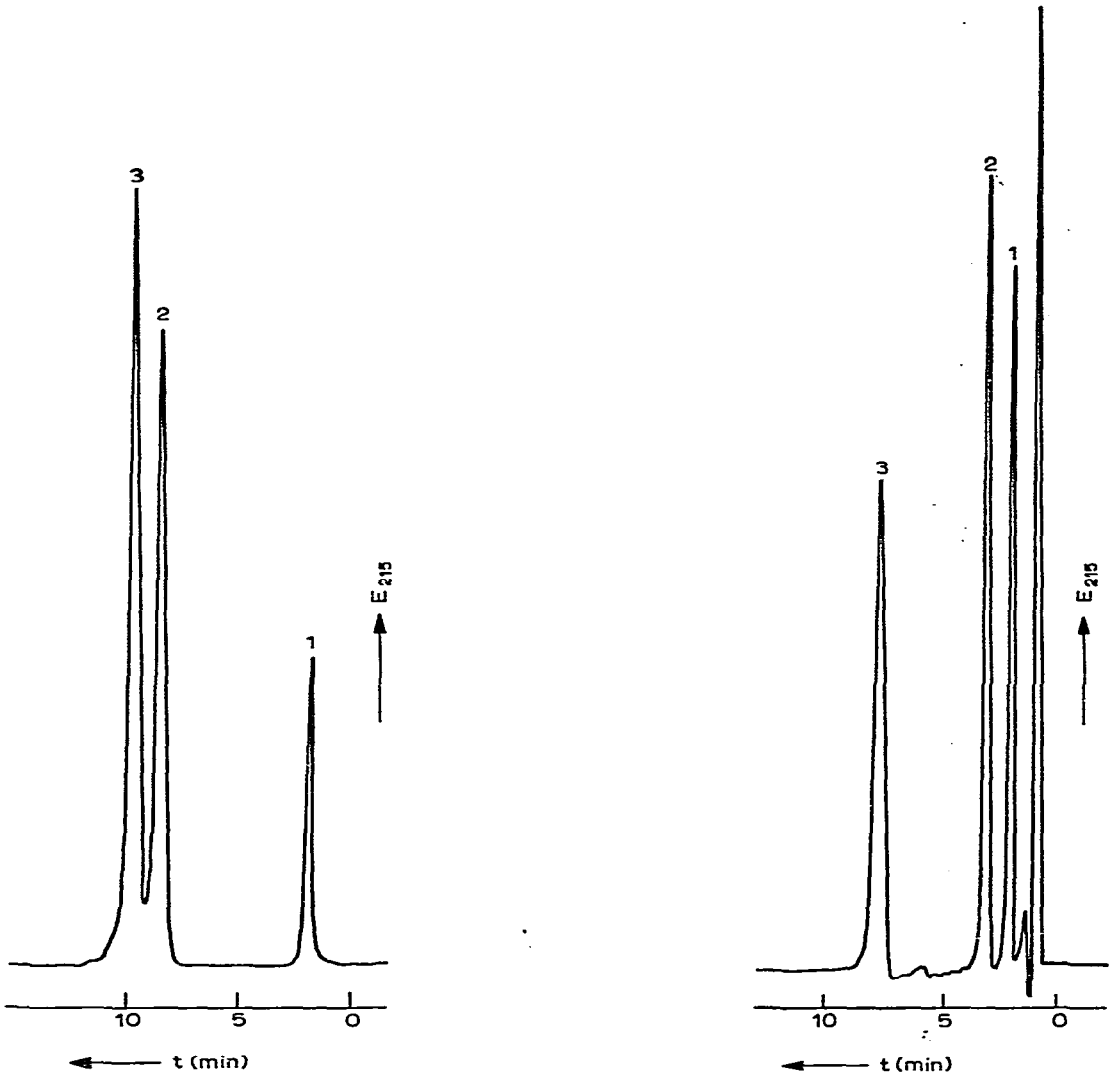

Fig. 1. Separation of CbO-Pro (1), CbO-Leu-Gly-ethyl ester (2) and CbO-Pro-Leu-Gly-ethyl ester (3). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: phosphate buffer (0.015 *M*, pH 7.0)–acetonitrile (60:40); pressure, 120 bar; flow-rate, 0.7 ml/min. Detector: Perkin-Elmer LC 55.

Fig. 2. Separation of CbO-hexapeptideamide (1), CbO-pentapeptideamide (2) and CbO-tetrapeptideamide (3). Column, mobile phase and detector as in Fig. 1. Pressure, 200 bar; flow-rate, 1.5 ml/min.

were found to be the most suitable. The use of triethylammonium phosphate[17], or other ion-pairing agents, were of no advantage to the following separations.

Fig. 1 shows the separation of a mixture containing a carbobenzoxy-protected amino acid and a protected dipeptide ester. They are the basis for the synthesis of the third component, the protected tripeptide ester. The three compounds are eluted in order of their molecular weight. Unmarked peaks are solvent peaks or unknown impurities.

In the following synthesis a CbO-tetrapeptideamide, a CbO-pentapeptideamide and a CbO-hexapeptideamide are produced. These three peptides can be separated without difficulties, as shown in Fig. 2. The elution order, compared to Fig. 1, has changed. The molecule with the highest molecular weight elutes first, the smallest molecule (CbO-tetrapeptideamide) last.

The lengthening of the peptide chain by one amino acid can be studied using a similar mobile phase. An example is given in Fig. 3. Free pentapeptideamide is reacted with CbO-glutamine-nitrophenyl ester to give CbO-hexapeptideamide. For optimal control of the reaction it should be possible to monitor the concentration of all reactants in the same run. As can be seen from Fig. 3, a separation of the relevant compounds is possible within a few minutes. Additionally, CbO-pentapeptideamide that may be present as an impurity, is detectable. For a quantitative determination the substances must be dissolved in the mobile phase, because the free pentapeptideamide is eluted shortly after the dead volume time. In practice, signals from reaction solvents overlap with the signal of the free pentapeptideamide. In most cases, how-

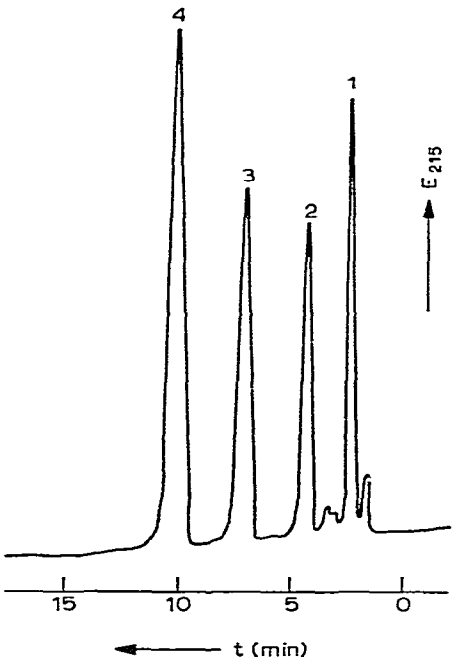

Fig. 3. Separation of pentapeptideamide (1), CbO-hexapeptideamide (2), CbO-pentapeptideamide (3) and CbO-Gln-ONP (4). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: phosphate buffer (0.015 *M*, pH 7.0)–acetonitrile (70:40); pressure, 130 bar; flow-rate, 0.6 ml/min. Detector: Perkin-Elmer LC 55.

ever, it proved to be sufficient to monitor the increase of the amount of CbO-hexapeptideamide that is parallel to the decrease of the amount of free pentapeptideamide.

Peptides containing glutamine easily form pyroglutamine peptides by an intramolecular cyclisation of glutamine, especially in acidic media. Therefore a determination of pyroglutamine peptides is of great interest. An example of such a separation is shown in Fig. 4. Free pentapeptideamide, free hexapeptideamide, which is synthesized after coupling of glutamine, and pyroglutamine-hexapeptideamide, that can be formed as a by-product, are clearly separated.

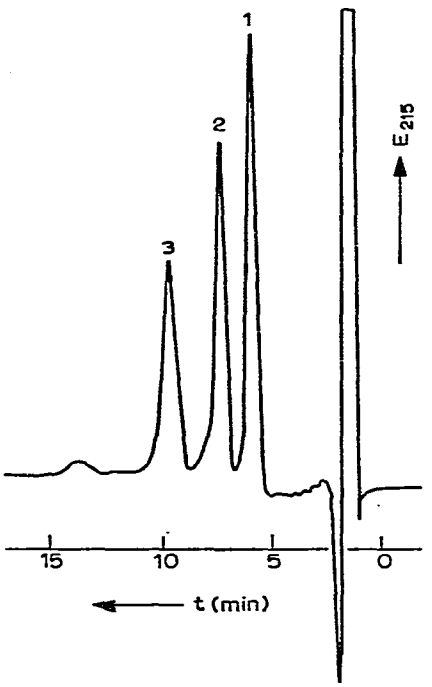

Fig. 4. Separation of hexapeptideamide (1), pentapeptideamide (2) and pyroglutamine-hexapeptideamide (3). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: phosphate buffer (0.015 *M*, pH 3.0)–acetonitrile (78:22); pressure, 175 bar; flow-rate, 0.9 ml/min. Detector: Schoeffel SF 770.

The good selectivity of the technique is demonstrated by the separation of oxytocin and its preliminary stage, the reduced nonapeptideamide (Fig. 5). The two compounds differ only in their oxidation stage. The cysteine groups of the reduced nonapeptideamide are oxidized to cystine in oxytocin (Table I). As can be seen in Fig. 5, the pH value has a strong influence on the selectvity of this separation. Whereas at pH 3.0 a baseline separation is possible (A), at pH 7.0 no suitable separation can be achieved (B). Some precautions must be taken to allow for the detection of nonapeptideamide in its reduced form; all solvents necessary in preparing the sample solution or the mobile phase, must be treated with nitrogen before use. Nonapeptideamide must be stored under nitrogen; otherwise oxidation takes place.

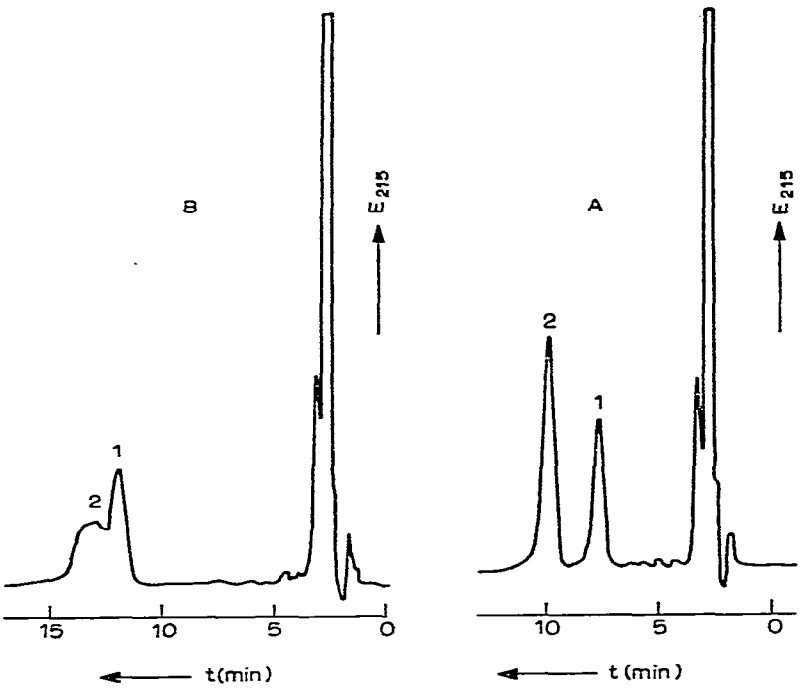

Fig. 5. Separation of oxytocin (1) and nonapeptideamide (2). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: (A) phosphate buffer (0.015 *M*, pH 3.0)–acetonitrile (82:18); (B) phosphate buffer (0.015 *M*, pH 7.0)–acetonitrile (82:18); pressure, 140 bar; flow-rate, 0.7 ml/min. Detector: Perkin-Elmer LC 55.

Quantitative determinations

As detection wavelength, 215 nm was chosen. This is near the UV-absorption maximum of all tested peptides and CbO-peptides, and thus extends the detection limits to the ng range. The results of a study of 4 peptides are summarized in Table II. All values are based on the evaluation of peak areas. The correlation coefficients were determined for 5 injections in a concentration range of 3–300 μg peptide/ml. For this range, a linear calibration curve is obtained. The reproducibilities were calculated for 7 determinations at the concentrations given in Table II. The higher standard deviation of CbO-tetrapeptideamide is caused by its high retention time (Fig. 2). The form of the eluted peak is not ideal under these conditions, so that peak integration

TABLE II

QUANTITATIVE DETERMINATION OF 4 PEPTIDES

Peptide	*r**	*Relative S.D. (%)*	*Detection limit*		*Conditions*
			μg/ml	*ng*	
CbO-tetrapeptideamide	0.999	3.7 (33 μg/ml)	0.84	42	Fig. 2
CbO-pentapeptideamide	0.999	1.8 (18 μg/ml)	0.42	21	Fig. 2
CbO-hexapeptideamide	0.999	1.9 (13 μg/ml)	0.34	17	Fig. 2
Hexapeptideamide	0.998	2.2 (50 μg/ml)	1.00	50	Fig. 4

* Regression coefficient.

is difficult. A less polar mobile phase gives a lower retention time, and leads to a standard deviation comparable to those for the other compounds (Table II).

The main reasons for the different detection limits of CbO-tetrapeptideamide, CbO-pentapeptideamide and CbO-hexapeptideamide can also be found in the different retention times of the substances. The detection limits of CbO-peptides and free peptides do not differ significantly ,as can be seen by comparing the values of CbO-hexapeptideamide and free hexapeptideamide.

CONCLUSIONS

With the example of oxytocin synthesis, it has been shown that free and protected peptides can be determined simultaneously, simply and quickly by using isocratic reversed-phase liquid chromatography. The method has been applied in routine analysis for more than half a year. The use of this technique for controlling the synthesis of other peptides of similar form, should be possible.

ACKNOWLEDGEMENT

I wish to thank Mr. E. Riemer for his helpful discussions, as well as for the synthesis of all peptides tested.

REFERENCES

1 J. Rosmus and Z. Deyl, *J. Chromatogr.*, 70 (1972) 221–239.
2 C. Haworth, *J. Chromatogr.*, 67 (1972) 315–323.
3 H. Frank and H. Hagenmaier, *J. Chromatogr.*, 106 (1975) 461–464.
4 S. Ho Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 125 (1976) 103–114.
5 R. Burgus and J. Rivier, *Pept., Proc. Eur. Pept. Symp., 14th*, (1976) 85–94.
6 W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *FEBS Lett.*, 72 (1976) 139–141.
7 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27–36.
8 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429–436.
9 J. J. Hansen, T. Greibrokk, B. L. Currie, K. N.-G. Johansson and K. Folkers, *J. Chromatogr.*, 135 (1977) 155–164.
10 E. J. Kikta, Jr. and E. Grushka, *J. Chromatogr.*, 135 (1977) 367–376.
11 W. Mönch and W. Dehnen, *J. Chromatogr.*, 140 (1977) 260–262.
12 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299–309.
13 J. Molnár and C. Horváth, *J. Chromatogr.*, 142 (1977) 623–640.
14 J. T. M. Bakkum, H. C. Beyerman, P. Hoogerhout, C. Olieman and D. Voskamp, *Rec. Trav. Chim. Pays-Bas*, 96 (1977) 301–306.
15 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415–418.
16 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241–254.
17 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343–366.
18 B. Larsen, V. Viswanatha, S. Y. Chang and J. Hruby, *J. Chromatogr. Sci.*, 16 (1978) 207–210.
19 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377–384.
20 M. Martin and G. Guiochon, *Chromatographia*, 10 (1977) 194–204.