Journal of Chromatography, 262 (1983) 411–414
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,808

Note

Side reaction of synthetic peptides during their purification by preparative high-performance liquid chromatography using formate buffers*

R. VIVILLE*, A. SCARSO, J.P. DURIEUX and A. LOFFET
UCB-Bioproducts S.A., Rue Berkendael 68, B-1060 Brussels (Belgium)
(Received February 25th, 1983)

At the present time, besides the usual methods of analysis of synthetic peptides (thin-layer chromatography, amino acid analysis, spectrophotometric methods, etc.), reversed-phase high-performance liquid chromatography (HPLC) is widely used as an accurate method for the determination of their purity. As a consequence, preparative HPLC is sometimes introduced in the purification of synthetic peptides[1–3] as a complementary technique to ion-exchange chromatography, adsorption chromatography or gel filtration.

In this communication the purification of synthetic des Gly^9-NH_2, [Arg^8]-vasopressin (dGAVP) is described. Preparative HPLC was required after silica gel chromatography to prepare the pure octapeptide. The use of a formate buffer (which was the best choice for the analytical separation) gave rise to a chemical modification of the peptide which is described, as well as the reversible reaction which enables us to recover dGAVP.

EXPERIMENTAL

Peptide synthesis

Des Gly-NH_2, [Arg^8]-vasopressin was prepared by the solid phase method of Merrifield[4] on a 1% cross-linked chloromethylpolystyrene esterified to 0.32 mmoles/g with N-α-Boc-Arg (ω-Tos)** by the caesium salt procedure[5]. The synthesis was performed on a Beckman Model 990 automatic peptide synthesizer adapted to large-scale synthesis (up to 100 g of resin). Protected N-α-Boc amino acids used were: Arg (ω-Tos); Pro; Cys (*p*-OMe-Bzl); Asn-ONP; Gln-ONP; Phe; Tyr (2-Br-Z).

The couplings were made with 2.5 equiv. of Boc-amino acid and dicyclohexylcarbodiimide (0.5 *M* in dichloromethane for 240 min, except for Asn and Gln which were coupled as their *p*-nitrophenyl esters). At each stage the Boc group was removed by treatment with 50% trifluoroacetic acid in dichloromethane (30 min), and neutralization was performed with 10% triethylamine in dichloromethane.

The crude peptide was cleaved from the support by HF containing 10% anisole

* Dedicated to Prof. Dr. E. Wünsch on the occasion of his 60th birthday.
** Abbreviations employed: Boc = *tert.*-butyloxycarbonyl; Tos = tosyl; Bzl = benzyl; ONP = *p*-nitrophenyl ester; Z = benzyloxycarbonyl; Me = methyl.

0021-9673/83/$03.00

during 45 min at 0°C[6]. The cyclization through S–S bridge formation was realized by potassium ferricyanide oxidation[7].

Purification

Gel filtration on Sephadex G-10 and G-15 (Pharmacia) was performed with 2 *M* acetic acid as eluent. Lobar® (Merck) Si 60 Type C prepacked columns were used for silica gel chromatography, the eluent system being *n*-butanol–pyridine–acetic acid–water (4:1:1:2) and the flow-rate 60 ml/h. Preparative reversed-phase liquid chromatography was performed on a column (2.6 × 40 cm) filled with 50 g octadecyl silica (Nucleosil, 25–40 μm; Macherey, Nagel & Co.) in triethylammonium formate (1% formic acid), pH 3.5. The elution was performed by a gradient of acetonitrile in the buffer varying from 10 to 20% (1 l of buffer, flow-rate 50 ml/h).

Purification by ion-exchange chromatography was carried out on a carboxymethylcellulose (CMC 52, Whatman) column (5 × 60 cm) with a gradient from 0.05 to 1.75 *M* acetic acid in water (2 l).

Thin-layer chromatography (TLC) on silica gel precoated plates (Merck) was performed in two systems:

A, *n*-butanol–pyridine–acetic acid–water (4:1:1:2); B, ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Spots were visualized with ninhydrin, Pauly, Sakaguchi and chlorine–toluidine reagents.

Amino acid analysis

Peptides were hydrolyzed in 6 *M* hydrochloric acid with 0.1% of phenol at 110°C during 24 h. Their amino acid composition was then determined on an amino acid analyzer, Beckman Multichrom B.

HPLC analysis

Analyses were made on a Varian 5000 liquid chromatograph equipped with a Varian multiwavelength detector and an integration system (Varian CDS 401). An Hibar prepacked column RT 250-4, 4 mm × 25 cm, LiChrosorb RP-18, 10μm (Merck), was used and the eluent consisted of acetonitrile (HPLC grade; Rathburn, Great Britain) in triethylammonium formate (1% formic acid adjusted to pH 3.5 with triethylamine). The acetonitrile concentration varied from 10 to 20% during 20 min; flow-rate 1.5 ml/min, UV detection at 274 nm.

RESULTS AND DISCUSSION

After the cyclization of crude des Gly-NH_2, [Arg[8]]-vasopressin the peptide was separated from polymeric material by gel filtration on Sephadex G-15. It was then submitted to silica gel chromatography (two runs) in the eluent *n*-butanol–pyridine–acetic acid–water (4:1:1:2).

At this stage the product obtained was shjown by TLC (solvents A and B) to be homogeneous and the overall yield was 5% based on the substitution of the polystyrene resin with N-α-Boc-Arg (ω-Tos). Nevertheless the HPLC analysis (Fig. 1a) revealed the presence of two minor impurities eluting after the main peak of dGAVP.

In order to purify further the peptide, 1 g of this product was chromatographed

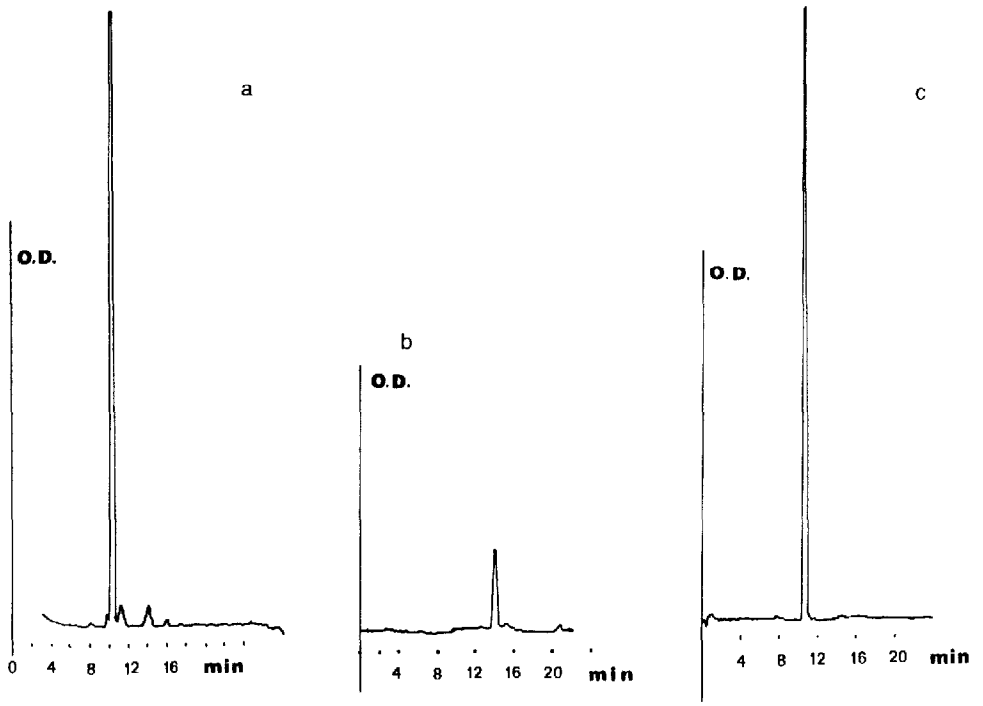

Fig. 1. HPLC analysis of dGAVP (see Experimental for conditions). (a) dGAVP after silica gel chromatography (200 μg; optical density full scale = 0.1 at 274 nm); (b) dGAVP after preparative HPLC and desalting on Sephadex G-10 (20 μg; optical density full scale = 0.2 at 274 nm); (c) pure dGAVP obtained through purification on CM-cellulose and silica gel chromatography (200 μg; optical density full scale = 0.2 at 274 nm).

on octadecyl silica (see Experimental). Fractions characterized by one single HPLC peak were pooled and concentrated by evaporation. The residue was diluted in water, lyophilized twice and desalted on Sephadex G-10. TLC and HPLC analyses indicated that the product was transformed into a less polar compound: higher R_F by TLC in solvent A (see Fig. 2); retention time about 4.3 min greater than before the lyophilization process (Fig. 1b). This product was still visualized by the Pauly, Sakaguchi and chlorine–toluidine reagents but was not visualized with ninhydrin.

This led us to postulate that a N-formylation had occurred during the concentration step due to the presence of a large excess of concentrated formic acid.

As the N-formyl group can be cleaved by 1 *M* hydrochloric acid, we dissolved 170 mg of the modified peptide in 10 ml 1 *M* hydrochloric acid. After 16 h at room temperature the product was desalted through Sephadex G-10. (Reaction for more than 24 h led to some degradation of the peptide.) Seventy-seven milligrams of homogeneous dGAVP were recovered; other fractions from the desalting process contained the peptide still contaminated with salts. This product gave a single HPLC peak, was ninhydrin positive and homogeneous on TLC; the retention time in HPLC and the R_F values in TLC (Fig. 2) were identical with those of an authentic sample of dGAVP.

To avoid reversed-phase chromatography, we have developed another purification scheme based on successive CM-cellulose and silica gel chromatographies. By this procedure we have been able to isolate 1.1 g of homogeneous dGAVP from 4.7 g of material issued from the first Sephadex G-15 chromatography. The HPLC profile of the purified product is shown in Fig. 1c and the amino acid composition (relative content of each amino acid reported to a mean value by amino acid analysis) is: Asx, 0.97; Glx, 1.02; Pro, 1.00; Cys, 1.73; Tyr, 0.98; Phe, 1.01; Arg, 1.00.

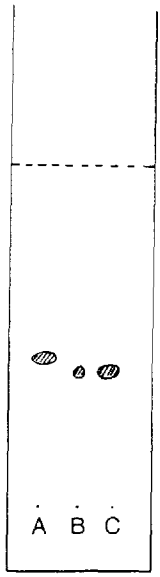

Fig. 2. TLC (solvent system A) of the peptide obtained after preparative HPLC and desalting (A), pure dGAVP (B) and product A after treatment (16 h) with *M* hydrochloric acid (C). Loading 50 μg of peptide. All spots except A were visualized with ninhydrin.

CONCLUSIONS

The choice of the buffer system for preparative HPLC is based on two main parameters: its resolution capacity and its volatility. In our hands, triethylammonium formate seemed a judicious choice because it fulfilled these criteria.

However when using this buffer for the purification of dGAVP it was found that during the concentration step the formic acid present in the solution could react with the free amino group present in the peptide. The resultant N-formylated peptide could be reconverted into the free peptide through reaction with hydrochloric acid.

This observation demonstrates that the usefulness of triethylammonium formate buffer in preparative work is limited, as a simple concentration of eluted fractions (*i.e.*, evaporation of acetonitrile) can lead to the side reaction described.

ACKNOWLEDGEMENTS

This work was supported by grant No. 3853 from the IRSIA. We thank Miss Havelange for typing the manuscript.

REFERENCES

1 O. Schou, P. Larsen, K. Brunfeldt and E.M. Salem, in K. Brunfeldt (Editor), *Peptides 1980*, Scriptor, Copenhagen, 1981, pp. 737–743.
2 T. Kimura, M. Takai, Y. Masui, T. Morikawa and S. Sakakibara, in D.H. Rich and E. Gross (Editors), *Peptides: Synthesis-Structure-Function*, Pierce Chemical, Rockford, IL, 1981, pp. 57 60.
3 A. Scarso, J. Brison, J.P. Durieux and A. Loffet, in K. Brunfeldt (Editor), *Peptides 1980*, Scriptor, Copenhagen, 1981, pp. 321–327.
4 R.B. Merrifield, *J. Amer. Chem. Soc.*, 85 (1963) 2149 2154.
5 B.F. Gisin, *Helv. Chim. Acta*, 56 (1973) 1476 1482.
6 S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and M. Sugihara, *Bull. Chem. Soc. Jap.*, 40 (1967) 2164–2167.
7 E.B. Hope, V.V.S. Murti and V. Du Vigneaud, *J. Biol. Chem.*, 237 (1962) 1563–1566.