Journal of Chromatography, 186 (1979) 677–682

CHROM. 12,123

LIGAND-EXCHANGE CHROMATOGRAPHY OF SMALL PEPTIDES ON COPPER(II)-MODIFIED SILICA GEL: APPLICATION TO THE STUDY OF THE ENZYMATIC DEGRADATION OF METHIONINE-ENKEPHALIN

F. GUYON, A. FOUCAULT and M. CAUDE

Laboratoire de Chimie Analytique de l'Ecole Supérieure de Physique et de Chimie Industrielles de Paris, 10 rue Vauquelin 75231 Paris Cédex 05 (France)

SUMMARY

A copper(II)-modified silica gel has been used for the high-performance liquid chromatography of small peptides, isomeric dipeptides and glycine oligomers. This packing combines the high selectivity of the ligand-exchange procedure with the high column efficiency obtained with microparticles of silica (7 μm).

The capabilities of this new packing are shown by the separation of fragments of methionine-enkephalin, which is a neuroendocrine pentapeptide (Tyr–Gly–Gly–Phe–Met). Two isocratic systems have been used in conjunction, the first for the separation of Met-enkephalin tetrapeptides, tripeptides and Phe-Met, and the second for that of dipeptides and amino acids.

These systems allowed the separation of all possible fragments of Met-enkephalin with an analysis time of about 15 min. It is then easy to observe the appearance and evolution of peptidic fragments following enzymatic degradation. Moreover, direct injection of the enzymatic media can be performed without storage of the frozen samples.

INTRODUCTION

The copper(II)-modified silica gel has proven to be a suitable stationary phase for ligand-exchange chromatography because it combines the high efficiency and good mechanical properties of small silica particles with the high selectivity of the ligand-exchange process[1,2].

We have shown previously[2] that the stability of the modified silica-based material against reaction with alkaline media is much more dependent on the amount of water present than on the ammonia concentration of the mobile phase.

With the Cu(II)-modified silica gel as the stationary phase and water–acetonitrile–ammonia mixtures as the mobile phase, the capacity factor for a given solute is governed by both its hydrophobicity and its ability to form complexes with the coordination site of the stationary phase.

We report here that ligand-exchange chromatography is a suitable method for the analysis of small peptides, particularly hydrophilic peptides, for which reversed-phase chromatography fails[3]. An application of biological interest is described,

namely the enzymatic degradation of methionine-enkephalin (Met-enkephalin), a neuroendocrine pentapeptide (Tyr–Gly–Gly–Phe–Met)*. Ligand-exchange chromatography with fine particles of copper(II)-modified silica gel allows the separation of all possible fragments of Met-enkephalin with an analysis time of about 15 min.

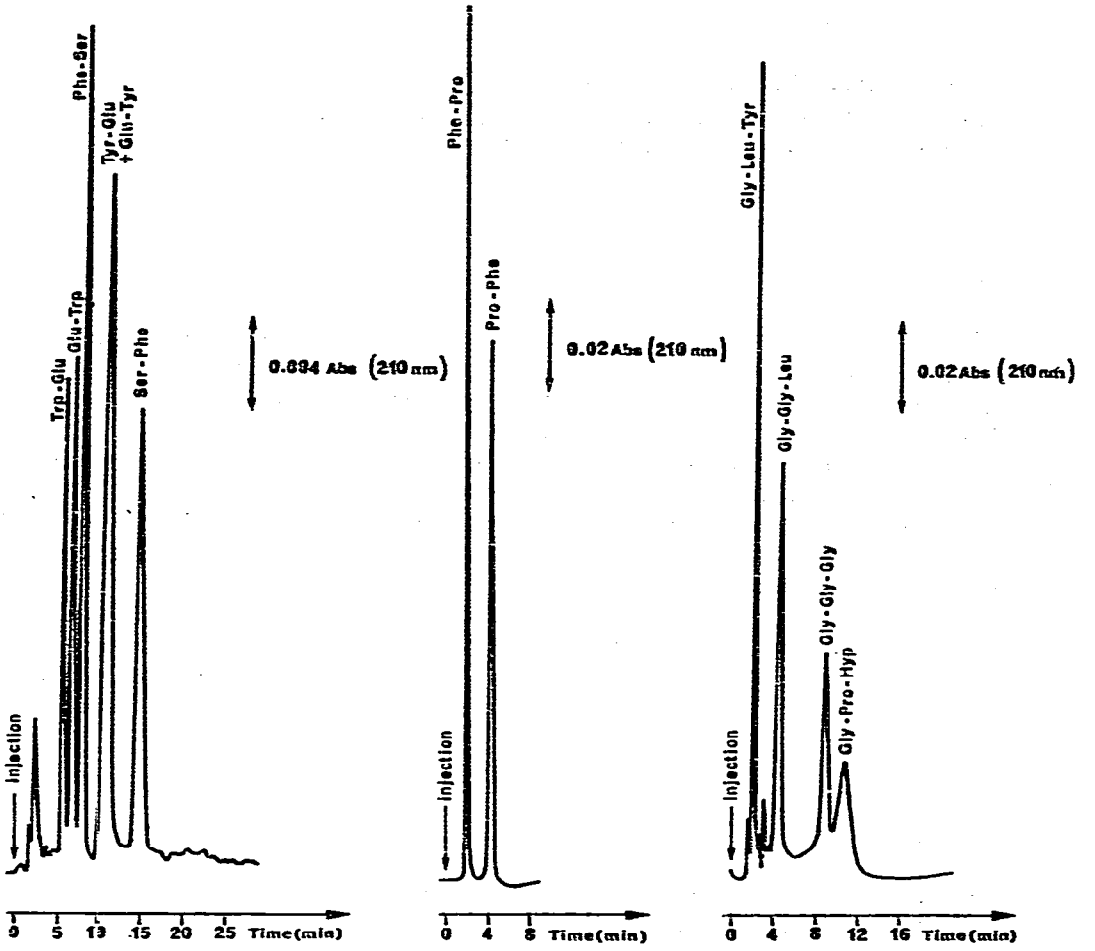

Fig. 1. Separation of some dipeptides by ligand-exchange chromatography. Column: 20 × 0.48 cm I.D. Packing: Cu(II)-modified Partisil 5. Mobile phase: water–acetonitrile (13:87) + NH_3 (0.125 *M*). Flow-rate: 1 ml·min^{-1}. Detection: UV at 210 nm.

Fig. 2. Rapid separation of isomeric dipeptides. Conditions as in Fig. 1 except mobile phase = water–acetonitrile (21:79) + NH_3 (0.205 *M*).

Fig. 3. Example of separation of some hydrophilic tripeptides by ligand-exchange chromatography. Conditions as in Fig. 1 except mobile phase = water–acetonitrile (22:78) + NH_3 (0.22 *M*).

* This is part of a joint study[4] undertaken with the Laboratory of Pr. B. P. Roques (UER des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire, 75006 Paris, France) and the Laboratory of Pr. J. C. Schwartz (Unité 109 de l'INSERM, 2 ter rue d'Alésia, 75014 Paris, France).

Direct injection of enzymatic media can then be performed without storage of frozen samples.

EXPERIMENTAL

Chromatographic apparatus

Liquid chromatography was performed using a Varian Model 8510 (Varian, Palo Alto, Calif., U.S.A.) and/or an Altex Model 110 pump (Altex, Berkeley, Calif., U.S.A.). Typical sample sizes of 2–5 μl were introduced with a 5-μl Hamilton syringe via a stop-flow injector. The injector was a Varichrom (Varian) and/or an LDC Spectro-Monitor II (Laboratory Data Control, Riviera Beach, Fla., U.S.A.), both equipped with a cell of volume 8 μl. The wavelength was usually 210 nm. The chromatographic columns were not thermostated.

Mobile phases

The mobile phases were mixtures of doubly distilled water, acetonitrile (Spectrosol; SDS, Valdonne-Peypin, France) and ammonia (Normapur; Prolabo, Paris, France).

Chemicals and packings

Experiments were performed using Partisil 5 silica gel of mean particle size 7 μm (Whatman, Clifton, N.J., U.S.A.). Peptides were obtained from Serva (Heidelberg, G.F.R.), or were kindly supplied by UER des Sciences pharmaceutiques et biologiques, Département de Chimie Organique, Paris, France. Other chemicals were obtained from Merck (Darmstadt, G.F.R.) and Prolabo.

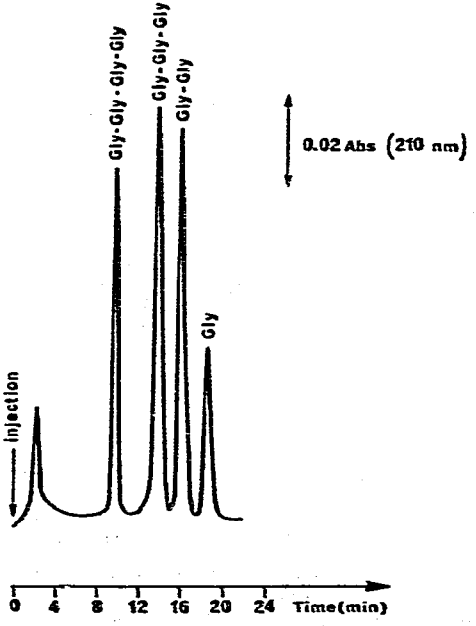

Fig. 4. Separation of glycine oligomers. Conditions as in Fig. 2.

Cu(II)-modified silica gel columns

The chromatographic columns were prepared as described previously[2]. Stainless-steel columns (20 × 0.48 cm I.D.) were packed by the slurry technique with pure Partisil 5. An aqueous solution of copper(II) sulphate (10^{-2} or $2 \cdot 10^{-2}$ *M*) and ammonia (1 *M*) was then allowed to percolate through the column until equilibrium was reached [when copper(II) ions appeared in the effluent]. The column was then fitted on the chromatograph and equilibrated with the mobile phase.

RESULTS AND DISCUSSION

Separation of peptides, including hydrophilic peptides

Typical chromatograms are shown in Figs. 1–4. It can be seen that isomeric dipeptides are easily separated, as they are with other chromatographic methods[3,5,6]. As has been shown for amino acids[2], independent variations of the water and ammonia concentrations in the mobile phase permit the easy regulation of the capacity

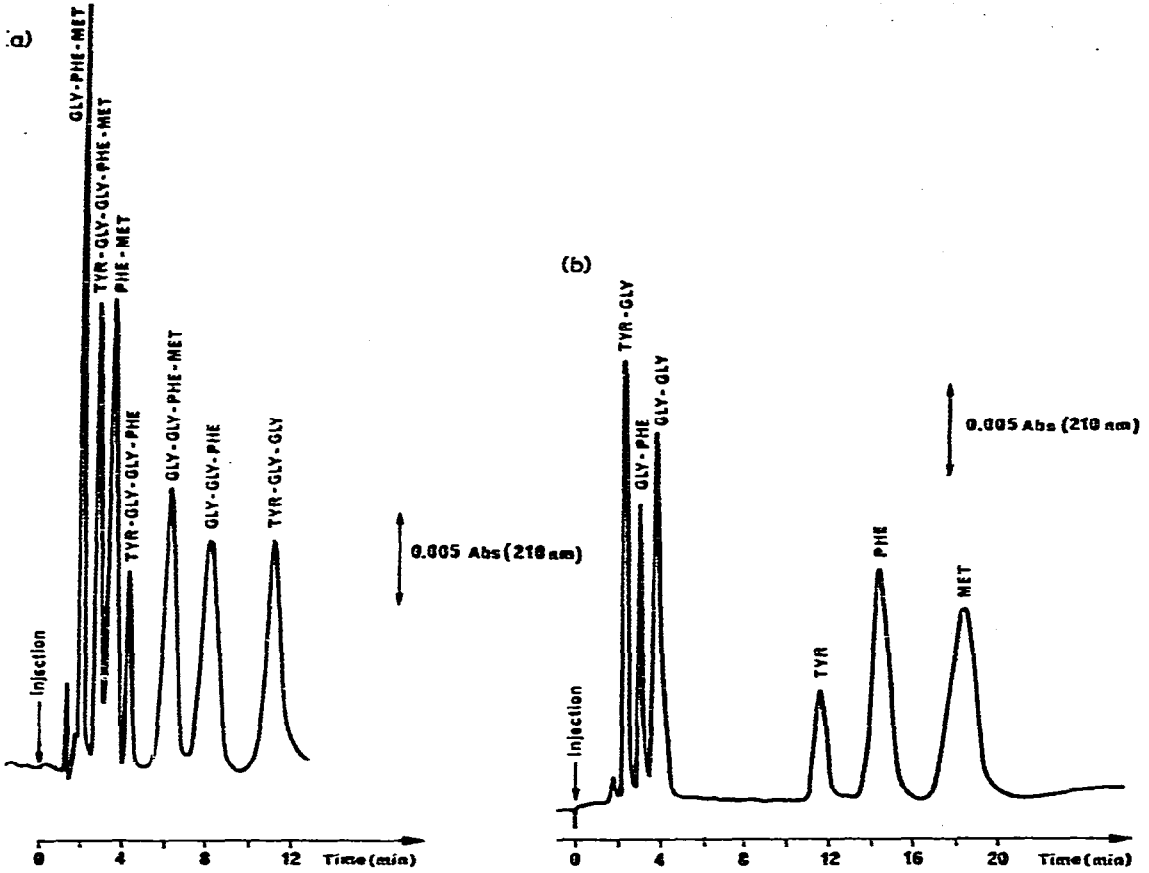

Fig. 5. Separation of the possible degradation fragments of Met-enkephalin by two isocratic runs. Column: 20 × 0.48 cm I.D. Packing: Cu(II)-modified Partisil 5. Mobile phase: (a) water–acetonitrile (13:87) + NH_3 (0.125 *M*); (b) water–acetonitrile (70:30) + NH_3 (0.2 *M*). Flow-rate: 1.5 ml·min^{-1}. ΔP: (a) 50 atm; (b) 100 atm. Detection: UV at 210 nm.

factor for peptides. Fig. 4 clearly indicates the combined ligand-exchange and normal-phase partition mechanism for the separation of the oligomers of glycine. The elution order is the opposite of that found by Molnár and Horváth[3] for the oligomers of alanine with a hydrophobic stationary phase. This elution order is due to a decrease in both the hydrophilicity and the complex formation constant[7] of peptides with increasing number of glycine residues. It should be noted that hydrophilic peptides are easily retarded with the copper(II)-modified silica gel.

Enzymatic degradation of Met-enkephalin

The enzymatic degradation of Met-enkephalin was monitored by the ligand-exchange chromatographic procedure.

It is well known that enkephalins, which are pentapeptides, are neurotransmitters acting through the opiate receptors[8]. A knowledge of the inactivation process for these peptides would provide a better understanding of the regulation of their action. The degradation of these peptides in various brain extracts has already been studied by several methods, such as thin-layer chromatography[9], ion-exchange chromatography with an amino acid analyser[10], adsorption on Porapak Q beads[11] and reversed-phase chromatography[12,13], but only a few of these fragments were identified.

With our method, all degradation products are monitored by the use of two isocratic systems with an analysis time of about 15 min. The first was for the separation of the Met-enkephalin, tetrapeptides, tripeptides and Phe-Met and the second

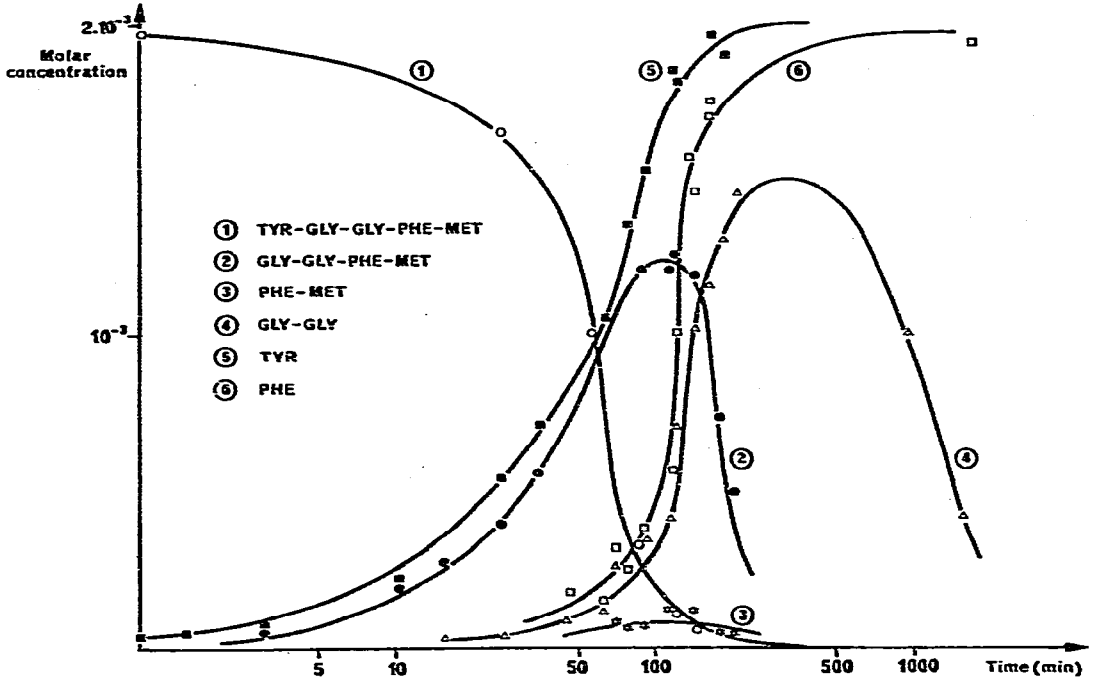

Fig. 6. Evolution of Met-enkephalin and fragmental peptides in a typical brain enzymatic preparation[4].

for that of dipeptides and amino acids (Fig. 5). It is then easy to observe the appearance and evolution of peptidic fragments following enzymatic degradation. Direct injection of a 5-μl sample from the enzymatic media was performed without storage of frozen samples[14]. A typical example of Met-enkephalin degradation with a brain enzymatic preparation is shown in Fig. 6.

CONCLUSION

The high selectivity of the ligand-exchange procedure combined with the high efficiency obtained with the silica microparticles make ligand-exchange chromatography as efficient as other modern methods and provide a useful means for the rapid separation of amino acids and peptides. The simplicity of the preparation of the metal-modified silica gel columns and the variety of applications should make the method increasingly popular.

ACKNOWLEDGEMENTS

We are indebted to A. Guyon for her enthusiastic and fruitful collaboration related to the study of Met-enkephalin, Professor R. Rosset for helpful discussions and P. Sassiat for skillful assistance with the experimental work. We are grateful to Dr. M. C. Fournier-Zaluski and Dr. G. Gacel for the gift of synthesized peptidic fragments of Met-enkephalin.

REFERENCES

1 M. Caude and A. Foucault, *Anal. Chem.*, 51 (1979) 459.
2 A. Foucault, M. Caude and L. Oliveros, *International Symposium on Column Liquid Chromatography, Boston, 1979.*
3 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
4 A. Guyon, B. P. Roques, F. Guyon, A. Foucault, R. Perbrisot and J. C. Schwartz, to be published.
5 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299.
6 J. Crommen, B. Fransson and G. Schill, *J. Chromatogr.*, 142 (1977) 283.
7 *Stability Constants of Metal Ion Complexes*, Special Publications Nos. 17 and 25, Chemical Society, London, 1971.
8 T. W. Smith, J. Hughes, H. W. Kosterlitz and R. P. Sosa, in H. W. Kosterlitz (Ed.), *Opiates and Endogenous Opioid Peptides*, Elsevier-North Holland Biochemical Press, Amsterdam, 1976, pp. 57–62.
9 J. M. Hambrook, B. A. Morgan, M. J. Rance and C. F. C. Smith, *Nature (London)*, 262 (1977) 782.
10 N. Marks, A. Grynbaum and A. Neidle, *Biochem. Biophys. Res. Commun.*, 74 (1977) 1552.
11 B. Malfroy, J. P. Swerts, A. Guyon and B. P. Roques, *Nature (London)*, 276 (1978) 523.
12 J. L. Meek, H. Y. T. Yang and E. Costa, *Neuropharmacology*, 16 (1977) 151.
13 F. B. Craves, P. Y. Law, C. A. Hunt and H. H. Loh, *J. Pharmacol. Exp. Ther.*, 206 (1978) 492.
14 J. A. Feldman, M. L. Cohn and D. Blair, *J. Liquid Chromatogr.*, 1 (1978) 833.