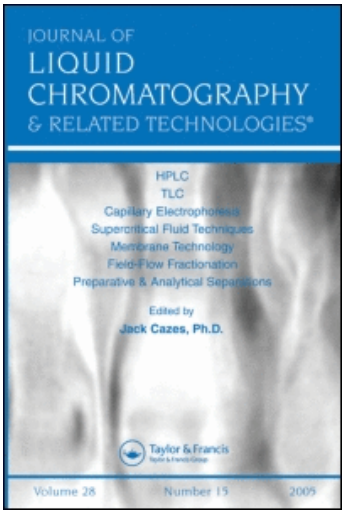



**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Peptide Formation in the Presence of Metal Ion Protecting Groups: III. The Separation of [$(NH_3)_5$Co(III)-] Amino Acids and Peptides by Reverse Phase High Pressure Liquid Chromatography

Stephan S. Isied[a]; John Lyon[a]; Asbed Vassilian[a]; Greg Worosila[a]
[a] Department of Chemistry, Rutgers, The State University of New Jersey, New Brunswick, New Jersey

**To cite this Article** Isied, Stephan S. , Lyon, John , Vassilian, Asbed and Worosila, Greg(1982) 'Peptide Formation in the Presence of Metal Ion Protecting Groups: III. The Separation of [$(NH_3)_5$Co(III)-] Amino Acids and Peptides by Reverse Phase High Pressure Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 5: 3, 537 — 547
**To link to this Article: DOI:** 10.1080/01483918208066912
**URL:** http://dx.doi.org/10.1080/01483918208066912

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(3), 537-547 (1982)

# PEPTIDE FORMATION IN THE PRESENCE OF METAL ION PROTECTING GROUPS. III. THE SEPARATION OF [$(NH_3)_5Co(III)$-] AMINO ACIDS AND PEPTIDES BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

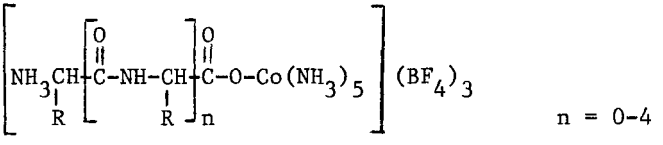
Stephan S. Isied*, John Lyon, Asbed Vassilian, and Greg Worosila

Department of Chemistry,
Rutgers, The State University of New Jersey,
New Brunswick, New Jersey 08903



## ABSTRACT

Pentaammine cobalt(III) amino acid and peptide complexes of the type

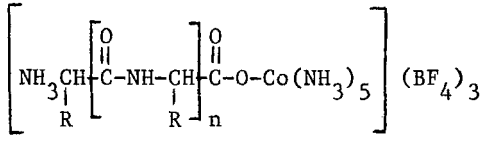


n = 0-4

can be separated rapidly under mild conditions by high pressure liquid chromatography (HPLC) using octadecyl silane derivatized columns (RP-18) with 0.2% sodium trifluoroacetate (NaTFA) in aqueous-methanol at pH 2.5. The retention time of these cobalt(III) complexes is a function of the hydrophobicity of the amino acid or peptide ligand and the total charge on the complex. In the trifluoroacetate media used for these separations, the hydrophobic ligand and the number of trifluoroacetate counter ions on the cobalt complex contribute to the selective elution of these complexes.

## INTRODUCTION

A number of cobalt(III) complexes of amino acids and peptides of the general formula



n = 0-4

(I)

have been synthesized using $[(NH_3)_5Co(III)-]$ as a carboxyl protecting group (1,2). In our earlier work we have used ion exchange (3) and gel filtration methods to purify the metal peptide complexes synthesized. These methods, although successful at times, were extremely time consuming especially when used at every step of the synthesis.

With the development of reverse phase high pressure liquid chromatography (HPLC) for the analysis of polar and charged molecules we became interested in developing methods to separate these metal-peptide complexes (I) using HPLC. The advantages of reverse phase HPLC over conventional ion exchange techniques for the separation of charged complexes are a) the speed of the analysis (ca few minutes), and b) the mild conditions used (ca pH 3, weakly acidic aqueous-organic solutions). Recently a number of chromatographic methods for the analysis of a variety of related cobalt(III) complexes using high pressure liquid chromatography (4-6) have been reported.

In this paper we present systematic methods that we have developed for the separation of $[(NH_3)_5Co(III)-]$ amino acid and peptide complexes (2), with hydrophobic and hydrophilic side chains, using reverse phase high pressure liquid chromatography. The cobalt peptide complexes have been synthesized by stepwise

peptide formation using $[(NH_3)_5Co(III)-]$ as a C-terminal protecting group (2).

## RESULTS AND DISCUSSION

A number of methods using different salt media in methanol-water and acetonitrile-water solvents were attempted for the separation of the $[(NH_3)_5Co(III)-]$ amino acid and peptide complexes. In most cases broad unresolved peaks were obtained. In the following section some of these early methods are presented. These methods were found to be of only limited use. The separation method that we have adopted for the separation of cobalt(III) amino acid and peptide complexes is discussed under a separate heading.

All the separations have been obtained using octadecyl-silane derivatized silica gel columns ( μ-Bondapak, 10 μ, Waters Assoc.) with the radial compression module.



### I. Early Methods

Ion Pairing with Sodium Heptanesulfonic Acid (NaHSA). At 0.1-0.2% NaHSA in aqueous-methanol the complexes $[(NH_3)_5Co(III)-L]$ where L = Gly, GlyGly, and GlyPhe were selectively eluted from the column with $[PheGlyCo(NH_3)_5]^{3+}$ retaining the most and $[GlyCo(NH_3)_5]^{3+}$ retaining the least. A similar separation for $[ProProCo(NH_3)_5]^{3+}$ and $[ProCo(NH_3)_5]^{3+}$ resulted in sharp peaks with longer retention times for $[ProProCo(NH_3)_5]^{3+}$. When this solvent system was used for other more hydrophobic cobalt peptides, e.g. $[PheLeuCo(NH_3)_5]^{3+}$, broad unresolved peaks were obtained. Addition of ammonium acetate ($NH_4OAc$) (1-2%) to NaHSA in aqueous-methanol extended the usefulness of this system, but here again, broad unresolved peaks were obtained for hydrophobic di- and tripeptides.

II. A General Method for the Separation of Hydrophobic and Hydrophilic Cobalt Peptide Complexes .

There are a number of reports on the separation of peptides on reverse phase high pressure liquid chromatography using trifluoroacetatic acid in aqueous organic solvents (7-10). By using trifluoroacetate anion (TFA) as a counter ion for the tripositively charged cobalt complexes, with, for example, 0.2% HTFA (pH adjusted to 2.5 with NaOH) in methanol-water mixtures varying from 20-60% methanol, we have been able to obtain good separation for a number of $[(NH_3)_5Co(III)$-amino acid] and $[(NH_3)_5Co(III)$-peptide] complexes of closely related structures. We have been able to resolve $[(NH_3)_5Co(OH_2)]^{3+}$ (2.6 min) from $[GlyCo(NH_3)_5]^{3+}$ (3.1 min); $[GlyGlyCo(NH_3)_5]^{3+}$ (3.2 min) from $[PheGlyCo(NH_3)_5]^{3+}$ (5.9 min) and $[ProProCo(NH_3)_5]^{3+}$ (6.3 min) with 20% methanol-water in 0.2% HTFA (pH 2.5) at a flow rate of 2.0 ml/min.

A large number of cobalt peptide complexes have been separated using this trifluoroacetate aqueous-methanol solvent system. Some representative examples will be shown here. Figure 1 shows the facile separation of $[(NH_3)_5Co(OH_2)]^{3+}$ from $[MetCo(NH_3)_5]^{3+}$ ( Met = L-methionine).
This separation allows one to monitor the formation of $[MetCo(NH_3)_5]^{3+}$ from $[(NH_3)_5Co(OH_2)]^{3+}$ and methionine (eq 1)(2).

$$[(NH_3)_5Co(OH_2)]^{3+} + Met \longrightarrow [MetCo(NH_3)_5]^{3+} \qquad (1)$$

For a series of $[(NH_3)_5Co(III)$-] complexes with neutral amino acids, the retention time was found to be proportional to the hydrophobicity of the amino acid side chain. The complex $[PheCo(NH_3)_5]^{3+}$ retained more than $[ProCo(NH_3)_5]^{3+}$, which in turn retained more than $[GlyCo(NH_3)_5]^{3+}$, however, the differences were small (ca fractions of a minute) when using

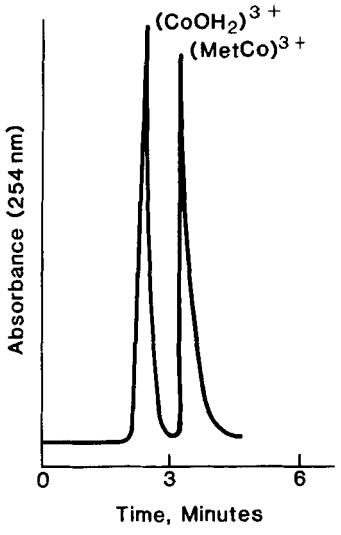



FIGURE 1.

Chromatographic Separation of $[(NH_3)_5Co(OH_2)]^{3+}$ from $[MetCo(NH_3)_5]^{3+}$. (0.2% TFA, pH 2.5, 20% methanol-water, 2 ml/min).



0.2% TFA , pH 2.5, 20% aqueous-methanol. No further separation of these $[(NH_3)_5Co$-amino acid] complexes was investigated.

For the cobalt(III)-peptide complexes, the retention time increased with the hydrophobicity of the peptide side chains. For example, $[ProProCo(NH_3)_5]^{3+}$ (6.3 min) retained significantly longer than $[GlyGlyCo(NH_3)_5]^{3+}$ (3.2 min). Figure 2 shows the retention time of two different cobalt peptide complexes and the effect on the retention time of increasing the methanol concentration from 20 to 40%. In 40% methanol the retention time of the two peptide complexes decreased.

The biologically active pentapeptides Leu-enkephalin (TyrGlyGlyPheLeu ) and Met-enkephalin ( TyrGlyGlyPheMet ) were synthesized by seqential peptide formation on $[(NH_3)_5Co(III)$-]

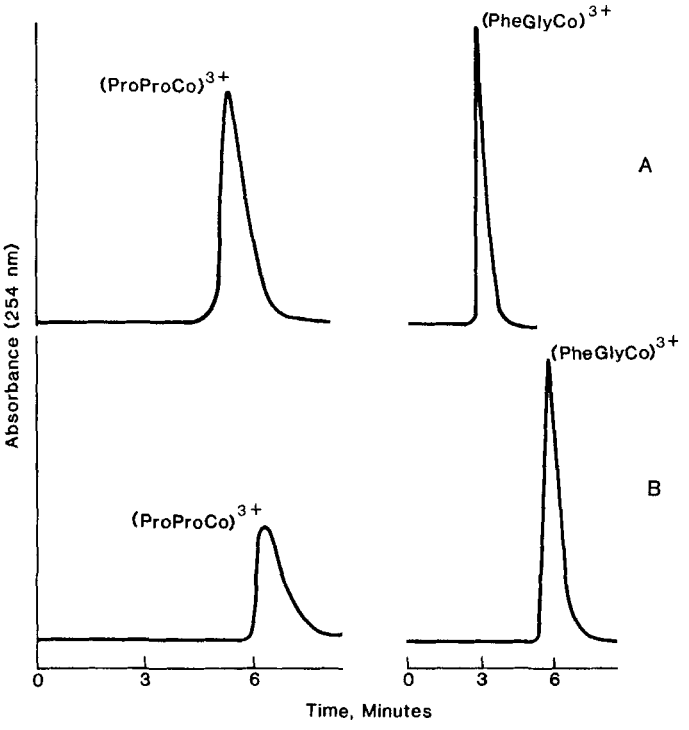



FIGURE 2.

Chromatograms of $[ProProCo(NH_3)_5]^{3+}$ and $[PheGlyCo(NH_3)_5]^{3+}$ in 40% methanol-water (A) and in 20% methanol-water (B). (Both in 0.2% TFA, pH 2.5, 2 ml/min).

and the fragments were subjected to amino acid analysis (2). The retention times of the peptide complexes increased with increasing number of amino acid residues (2). Figures 3a and 3b show a comparison between the retention times of the free peptides and the cobalt-peptides. It should be noted that in the trifluoroacetate solvent system used, the retention times of the cobalt-peptide complexes are longer than those of the free peptides.

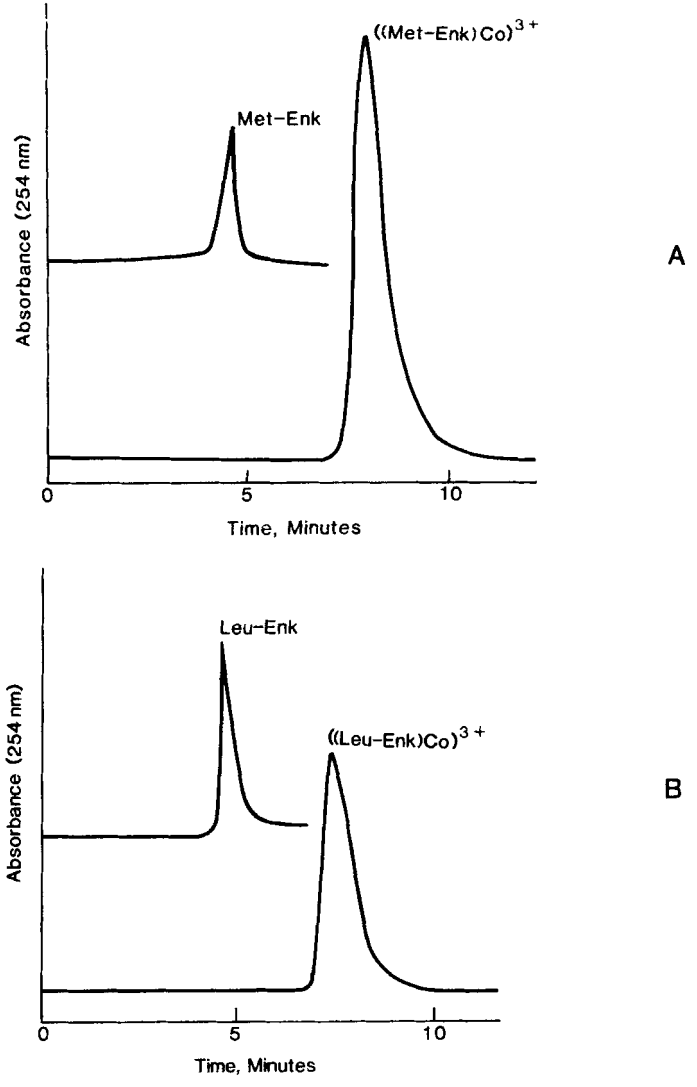



FIGURE 3.

Chromatograms of Free Met-enkephalin (Met-Enk) and its Corresponding $[(NH_3)_5Co(III)-]$ Complex (A) and Free Leu-Leu-enkephalin (Leu-Enk) and its Corresponding $[(NH_3)_5Co(III)-]$ Complex (B). (in 40% methanol-water, 0.2% TFA, pH 2.5, 2 ml/min). (Co = $[(NH_3)_5Co(III)-]$ )

The trifluoroacetate solvent system was also used for the separation of hydrophilic peptide sequences during the synthesis of the tetrapeptide complex $[HisGlyHisGlyCo(NH_3)_5]^{5+}$. Figure 4 shows the chromatographic separation of the intermediates in the synthesis of $[HisGlyHisGlyCo(NH_3)_5]^{5+}$ (in a synthetic mixture prepared from all the pure components). The fragments $[GlyCo(NH_3)_5]^{3+}$, $[HisGlyCo(NH_3)_5]^{4+}$, $[GlyHisGlyCo(NH_3)_5]^{4+}$, and $[HisGlyHisGlyCo(NH_3)_5]^{5+}$ are well resolved. These highly charged species can be well resolved on reverse phase HPLC by

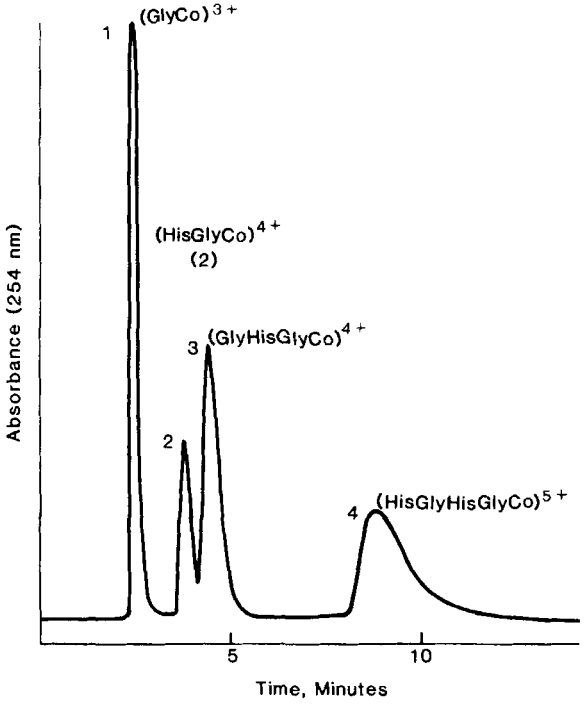



FIGURE 4.

Chromatographic Separation of the Peptide Fragments in the Synthesis of $[HisGlyHisGlyCo(NH_3)_5]^{5+}$. (40% methanol-water, 0.2% TFA, pH 2.5, 2 ml/min). (Co = $[(NH_3)_5Co(III)-]$ ).

ion pairing with trifluoroacetate. It is noteworthy that the separation of a highly charged complex such as $[HisGlyHisGlyCo(NH_3)_5]^{5+}$ from its homologs is very time consuming by classical ion exchange techniques. In the present separation (Figure 4) the ion pairing with five trifluoroacetate anions increases the retention time of the cobalt-peptide complex and makes the separation of the tetrapeptide complex from the di- and tri-peptide complexes possible in a short period of time (ca minutes).

## Retention Times of $[(NH_3)_5Co(III)-]$ Amino Acid and Peptide Complexes

In the trifluoroacetate solvent system (0.2% TFA, pH 2.5, aqueous-methanol) the retention time of the Co(III) complexes depends on at least two factors: a) the hydrophobicity of the amino acid or peptide bound to cobalt(III), and b) the ion pairing of the cobalt complexes as trifluoroacetate salts. The cobalt amino acid or peptide complexes with chloride, perchlorate, or fluoroborate counter ions do not retain on these $C_{18}$ columns. Most of the free amino acids also do not retain appreciably with this solvent system. The interaction between the derivatized silica gel support and the $[(NH_3)_5Co(III)-]$ amino acid or peptide complexes involves both the hydrophobic amino acid or peptide moiety and the total number of trifluoroacetate counter ions with the cobalt complex as a result of its charge. The combination of these two factors contribute to the selective retention times that these cobalt(III) complexes exhibit. The separation techniques used in this study are not unique to Co(III) and should also prove useful to the separation of amine complexes of other charged, substitution-inert metal ions such as Rh, Ru, Ir, and Os complexes. In general the combination of the overall charge of the complex and the

hydrophobicity of the ligands on metal complexes can result in their rapid and selective elution from derivatized silica gel columns.

## SUMMARY

The retention time of the pentaammine cobalt(III) amino acid or peptide complexes on reverse phase $C_{18}$ columns is a function of two factors: a) the hydrophobicity of the sixth ligand (amino acid or peptide) bound to cobalt and b) the charge on the Co(III) complex which is ion paired to the trifluoroacetate counter ion.

## EXPERIMENTAL

### Materials and Methods

All solvents were HPLC grade (purchased from Baker Chemical). House distilled water was further purified by passing it through organic purifiers and ion exchangers (Barnsted System). All chromatography was carried out at room temperature using isocratic elution conditions on a Waters HPLC system (two M 6000 A pumps, M 660 solvent programmer, Model 440 UV detector and a RCM-100 radial compression module) and a Perkin Elmer LC 75 variable wavelength detector. Radial-Pak $C_{18}$ columns ( 8 mm ID x 10 cm) (Waters) were used for all the analyses. The flow rate was maintained at 1-3 ml/min. Detection of the cobalt-peptides was done at 254 nm and detection of the free peptides with aromatic side chains was also done at 254 nm. The syntheses of all the cobalt amino acid and peptide complexes are described in references 1 and 2.

## ACKNOWLEDGEMENTS

This work is supported in part by Grant GM 263-24 from the U.S. Public Health Service and by the Charles and Johanna Busch Memorial Fund(Rutgers). S. Isied is also the recipient of a

Public Health Service Research Career Development Award (5 KO4 Am 0735 ).

## REFERENCES

1. S. S. Isied and C. Kuehn, J. Am. Chem. Soc 100, 6752 (1978).
2. S. Isied, A. Vassilian, and J. Lyon, manuscript submitted to J. Am. Chem. Soc.
3. G. H. Searle, Austr. J. Chem. 30, 2625 (1977).
4. a) B. D. Warner and J. I. Legg, Inorg. Chem. 20, 1625 (1981). b) ibid, 18, 1839 (1979).
5. D. A. Buckingham, C. R. Clark, R. Tasker, and M. Hearn, J. Liquid Chrom. 4, 689-700 (1981).
6. a) G. G. Strazza and C. M. Polcaro, J. Chromatogr. 147, 516 (1978). b) P. Heizmann and K. Bullschmiter, J. Chromatogr. 137, 153 (1977).
7. B. Currie, J. Chang and R. Cooley, J. Liquid Chromatogr. 3, 513-27 (1980).
8. C. Bishop, L. Meyer, D. Harding, W. Hancock, and M. Hearn, J. Liquid Chromatogr. 4, 661-680 (1981).
9. C. E. Dunlap, S. Gentleman, and L. Lowney, J. Chromatogr.160, 191-8 (1978).
10. D. Voskamp, C. Olieman, and H. Beyerman, Recueil, J. Roy. Neth. Chem. Soc. 99, 105 (1980) and references therein.