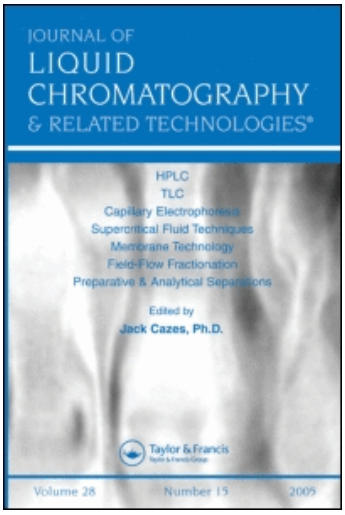

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SEPARATION OF COBALT (III) BIS(ETHYLENEDIAMINE) AMINO ACID COMPLEXES BY REVERSED PHASE HPLC

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ABSTRACT

Methods for the rapid analysis of amino acid cobalt (III) bis(ethylenediamine) complexes by reversed phase high performance liquid chromatography (HPLC) are described with mobile phases containing the pairing ions, p-toluenesulphonate and hexanesulphonate. Under these conditions, the amino acid cobalt (III) bis(ethylenediamine) complexes elute in order of the relative hydrophobicities of the parent amino acids which suggests that the amino acid side chain makes a significant contribution to the retention mechanism. At high sample loadings, these complexes shows a concentration dependent peak splitting effect divergent to that normally experienced with inadequate buffering capacity of the pairing ion reagent.

INTRODUCTION

Over the last several years, reversed phase high performance liquid chromatography (HPLC) has become established as a powerful technique for the rapid separation and analysis of amino acids and peptides. A feature of these advances has been

the development of a variety of novel elution systems which allow adequate control over selectivity and column efficiencies for the separation of these polar amphoteric solutes on microparticulate chemically-bonded hydrocarbonaceous silicas. Our previous experiences [1-3] with these techniques has now led us to investigate the application of similar methods as a means of separating and identifying charged metal complexes of the classical 'Werner' type, particularly as they apply to the monitoring of cobalt (III)-induced synthesis of small peptides [4]. Charged metal complexes of this type have conventionally been separated by ion-exchange or thin layer chromatographic methods [5-7]. It was anticipated that the reversed phase HPLC techniques would allow considerably shorter analysis times, improved resolution and excellent reproducibility if suitable elution systems, particularly those which could exploit pairing ion interactions between the metal complexes and components in the mobile phase, could be developed. In this paper we wish to described the separation of a series of amino acid Co(III) bis (ethylenediamine) complexes, $[Co(en)_2AA]X_2$, under such conditions.

MATERIALS AND METHODS

All solvents were AnalaR grade and purified as reported earlier [8]. Orthophosphoric acid was from May and Baker Ltd., p-toluenesulphonic acid was from BDH, sodium hexanesulphonate was prepared according to published procedures [9]. Water was de-ionised by reverse osmosis and distilled. The bis (ethylenediamine) cobalt (III) chelates of the amino acids were prepared by established methods and the individual diasteriomeric pairs isolated and resolved as described previously [10-13]. All amino acids except for glycine were of the S-configuration.

Optical rotation measurements were carried out on a Perkin Elmer 141 polarimeter using 1dm cells ($\pm$ 0.004^{o}).

All chromatograms were carried out at room temperature (ca. 20^{o}) using isocratic or linear gradient elution conditions with a Waters High Performance Liquid Chromatographic System (two M6000A solvent delivery units, M660 solvent programmer, U6K universal injector) coupled to a M450 variable wavelength UV-visible monitor and a Rikadenki dual channel chart recorder. The μBondapak C_{18} columns (10μm, 30cm x 3.9mm I.D.) were purchased from Waters Associates (N.Z.), Auckland. All columns were equilibrated to new solvent conditions, or following gradient elution to the initial mobile phase conditions, for at least 30min. Flow rate were maintained between 2.0 and 2.5ml min^{-1}, as indicated in the text. Detection of the cobalt complexes was generally at 480nm; in some cases detection was also carried out at 350nm and/or 254nm depending on the nature of the sample and the mobile phase. Sample injections were made with Pressure Lok liquid syringes, Series B110, from Precision Sampling (Baton Rouge, La., U.S.A.).

RESULTS AND DISCUSSION

The pseudo-octahedral bisethylenediamine cobalt (III) complexes of α-amino acid derivatives contain chiral centres about both the metal atom (Δ- or Λ-) and the α-carbon atom of the amino acid (R- or S-). Consequently, these metal complexes exist as diastereoisomers. In order to simplify the analysis, only metal complexes with amino acids of the S-configuration were used, ie. only the diastereoisimeric pairs Δ(S), Λ(S) (Fig.1a,b) were employed. Since these metal complexes exist as cationic 2-plus ions in solution (counterion is often I^{-}, Cl^{-} or ClO_4^{-}), it is not surprising that on octadecylsilica with neat aqueous eluents over a variety of pH

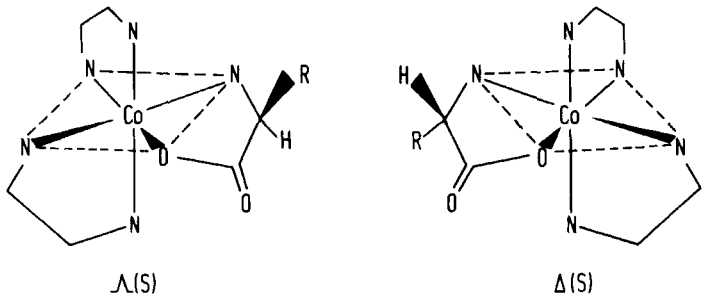

Figure 1.

Two diastereoisomers of the cobalt (III) bisethylene-diamine amino acid complexes.

conditions, they showed no, or little, retention, ie. k´ values <1. In order to obtain satisfactory retention and selectivity recourse was made to the use of low concentrations, of the anionic pairing ions, p-toluente sulphonate and hexane-sulphonate. With chemically bonded alkylsilicas, hydrophobic anionic reagents of this type are known to act as surface active ions as revealed by Freundlich adsorption isotherms [2,8,14-16]. The effect of these reagents on amino acid and peptides retention to alkylsilicas has been discussed [2,3,8] in terms of pairing ion:dynamic liquid-liquid ion-exchange interactions with both hyperbolic and parabolic dependencies of k´ versus pairing ion concentration being evident. A discussion of this concentration dependency, and the manner it can be used to obtain improved selectivity in the separation of a wide range of charged Co(III) complexes will be deferred to later [17]. In the present study 5mM of the pairing ions was chosen for most experiments on the basis of this analysis.

Figure 2 shows a typical separation of the amino acid complexes Δ-$[Co(en)_2AA]^{2+}$ (AA= gly,pro,val,leu, phe) whilst the Table illustrates the effect of pairing ion structure

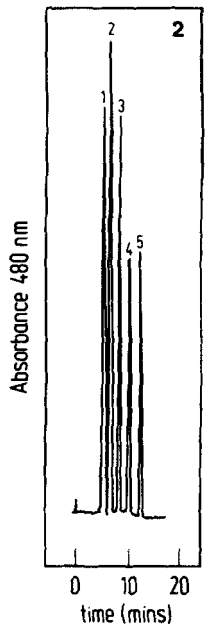

Figure 2.

Gradient elution profile of the Δ[Co(en)$_2$AA]I$_2$ complexes using 5mM p-toluenesulphonate as the pairing ion. The elution order for the complexes was: 1, gly (t_R 5.6min); 2, pro (t_R 6.8min); 3, val (t_R 8.8min); 4, leu (t_R 10.8min); 5, phe (t_R 13.8min). Chromatographic conditions: column, µBondapak C_{18} ; flow rate, 2.5ml min^{-1}; temperature, 18^{o}; elution conditions, 15min. linear gradient from 0 to 100% methanol, containing 5mM p-toluene sulphonate, pH 3.5, sample volume 10µl containing 367 nmol complexes.

on retention. As can be seen, the elution order of the cobalt complexes is the same with both the p-toluenesulphonate and the hexanesulphonate pairing ion system, although to achieve comparable k´ values at a pairing ion concentration of 5mM, the percentage methanol had to be increased approximately ten-fold from 2.5% with the p-toluenesulphonate system to 24% with the hexanesulphonate system. Similar

Table

Comparison of Capacity Factors for the $[Co(en)_2AA]$ Complexes with two Different Pairing Ions.

Co(III) complex AA =	5mM Pairing Ion	
	p-Toluenesulphonate*	Hexanesulphonate†
	k´	k´
-Gly	1.67	1.67
-Ala	1.66	1.83
-Pro	2.11	2.00
-Val	4.00	3.56

* Mobile phase: 2.5% methanol-water, pH 3.5.

† Mobile phase: 24% methanol-water, pH 3.5.

effects have been noted [2,3,8,18] previously with peptide separations under a variety of pairing ion and organic modifier conditions. Compared to traditional methods of analysis, these HPLC separations show excellent control over selectivities, peak shape and short elution times, eg. the above five cobalt (III) complexes could all be eluted reproducibly within 15min of injection. The elution order of these charged metal complexes, eg. k´ Gly<Pro<Val<Leu<Phe, which is the same as that observed [19,20] for the free amino acids when chromatographed on alkylsilicas and similar to that found [21] for these complexes when separated by partition chromatography on cellulose, clearly indicates that there is a preferred orientation of the charged complex with regard to the hydrocarbonaceous stationary phase, such that the amino acid side chain participates significantly in the retention mechanism.

This feature can be exploited for the separation of Δ-S and Λ-S or Δ-R and Λ-R diastereoisomeric mixtures [17]. As anticipated the nature of the cobalt complex salt counterion, $[Co(en)_2AA]X_2$ X = iodide, chloride, perchlorate, did not affect the resolution.

During the course of these studies, we observed an unusual chromatographic effect which is dependent on the association of two (or more) different cationic species and is distinguished from related phenomena normally experienced with distribution coefficient perturbation arising from an inadequate buffering capacity of the pairing ion reagent. Figure 3 summarises these observations. At constant pairing ion conditions but increasing sample loading of two charged cobalt complexes, eg. the Λ-Gly and Λ-Pro complexes, each component peak was progressively split into two (Fig. 3a-d). This peak splitting could also be achieved by lowering the pairing ion concentration at fixed solute concentration but was clearly distinguished from conventional 'overloading' phenomena when the buffer capacity of the pairing ion is inadequate by increasing loadings of only one cobalt-amino acid complex, eg. Λ-Gly complex. In this case the development of a broad asymmetic preview peak, preceding a sharper peak and typical of conventional overloading effects [22-24] was observed. Furthermore the achiral complexes such as $[Co(NH_3)_5X]^{2+}$, X = $CH_3CO_2^-$, $C_2H_5CO_2^-$, showed a similar peak splitting effect as the sample loading was increased.

That this splitting effect must involve an interaction between two (or more) complex ions, the pairing ion, and the stationary phase was demonstrated by the following experiments. Firstly, the Λ-Gly complex was loaded onto the column following the Δ-Val complex after a 10 second delay and 30 second delay (Fig. 4a,b). Peak splitting was evident when both complexes were coinjected or when the Λ-Gly was injected within 10 seconds

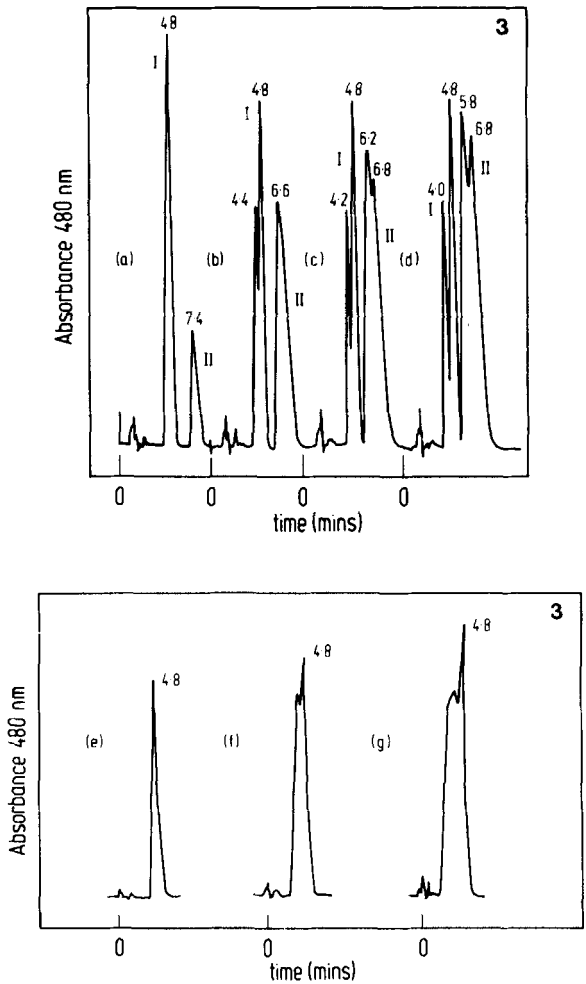

Figure 3.

Effect of increasing sample load with mixtures of the $[Co(en)_2AA]$ complexes, AA = Gly (I) and Pro (II), (a-d) and the individual complex $[Co(en)_2Gly]$ (e-g). Chromatographic conditions: column, μBondapak C_{18}; flow rate, 2.0ml min^{-1}; temperature 18^{o}; mobile phase, 2.5% methanol-water-5mM p-toluene-sulphonate, pH 3.5; sample concentrations: (a-d) Λ-$[Co(en)_2Gly]$ complex 438nmol in 30μl plus Λ-$[Co(en)_2Pro]$ complex at (a) 270 nmol in 5μl, (b) 1080nmol in 20μl, (c) 1620nmol in 30μl (d) 2160nmol in 40μl concentration and (e-g) Λ-$[Co(en)_2Gly]$ complex alone at (e) 591nmol in 30μl, (f) 1182nmol in 60μl and (g) 1970 nmol in 100μl concentration. The t_Rs for the peak maxima are given in minutes.

Figure 4.

Elution profiles obtained by (a) loading the Λ[Co(en)$_2$Gly] complex (1) 10 seconds after the Δ[Co(en)$_2$Val] complex (2) and (b) loading the Λ-[Co(en)$_2$Gly] complex (1) 30 seconds after the Δ-[Co(en)$_2$Val] complex (2) Chromatographic conditions as for Fig.3.

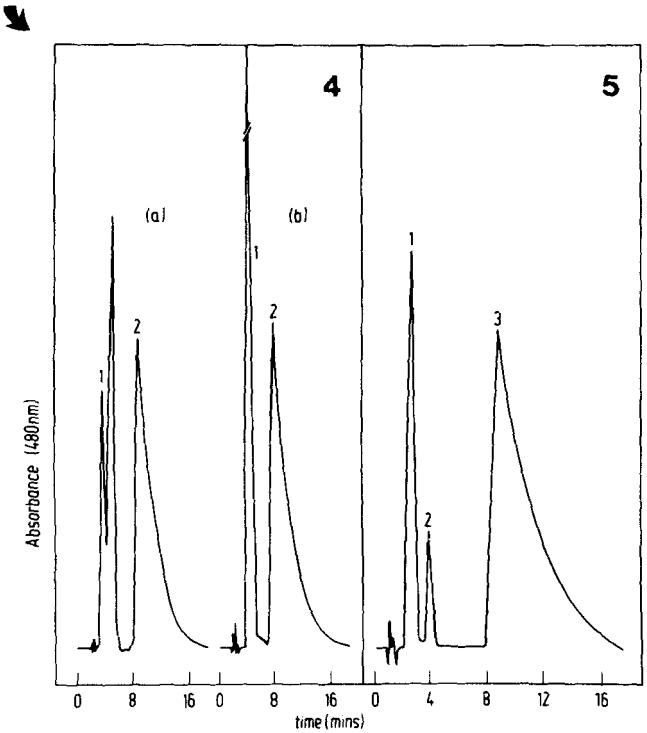

Figure 5.

Elution profile obtained for a mixture of Λ-[Co(en)$_2$Gly] and Δ-[Co(en)$_2$Val] complexes on a μBondapak C_{18} column. Chromatographic conditions as in Fig. 3. After recovery and desalting on Sephadex SP-25, the optical rotations (deg.mol^{-1}.dm^3.cm^{-1}) of the three peak components were: 1, (+ 1.43 ± 0.03) x 10^3; 2, (+ 1.48 ± 0.03) x 10^3; 3, (-2.11 ± 0.04) x 10^3; the values for the optically pure Λ-[Co(en)$_2$Gly] and Δ-[Co(en)$_2$Val] complexes are (+ 1.45 ± 0.03) x 10^3 and (-2.12 ± 0.04) x 10^3 respectively.

of the Δ-Val complex, but did not arise with a longer delay time, despite the fact that the Λ-Gly complex still overtakes the Δ-Val complex on the column. Secondly, it was possible to base-line resolve and recover the peaks for the Λ-Gly complex generated in the presence of the Δ-Val complex (Fig.5). Optical rotation measurements were carried out on these recovered peaks, following desalting on Sephadex SP-25. The optical rotation values obtained were: peak 1, (+ 1.45 ± 0.03) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$; peak 2, (+1.48 ± 0.03) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$; peak 3, (-2.11 ± 0.04) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$. The values for the optically pure Λ-Gly complex and Δ-Val complex are (+ 1.45 ± 0.03) x 10^3 and (-2.12 ± 0.04) x 10^3 $deg.mol^{-1}.dm^3.cm^{-1}$ respectively. This result clearly indicates that both peaks 1 and 2 in Fig. 5 are due solely to the Λ-Gly complex. This was confirmed by re-injecting the recovered peaks under analytical conditions. It thus appears that this type of splitting of individual complexes into multiple peaks on the column arises, in part, due to a co-operative effect between different complexes in the mixture, and is not a property of the loading or the loading zone of an individual complex.

These experiments show that there must be an interaction between two (or more) complex ions which influences their respective distribution ratios, in addition to the normal pairing ion depletion effect observed at high sample concentrations. Under these conditions, interactions between the complex cations and the pairing ion apparently lead to species of different counterion stoichiometry or geometry which have sufficient lifetime on the matrix of the stationary phase to allow separation as discrete entities. This effect can be easily avoided for analytical purposes, eg. for the p-toluenesulphonate-2.5% MeOH mobile phase, peak splitting can be prevented if the solute load (nmoles) to the pairing ion

concentration (mM) ratio is less than 100:1, but could lead to serious misinterpretation with regard to peak assignments, especially when large amounts of these metal complexes are used in preparative studies.

In summary, two aspects of the reversed phase HPLC separation of water soluble Werner type cationic complex ions using mobile phases containing hydrophobic anionic pairing ions have been examined. Conditions for the chromatographic resolution of $[Co(en)_2AA]X_2$ complexes have been developed. These procedures also allow the preparative recovery of individual complexes with, in many cases, elution times under 30min. The application of these techniques to the separation of peptide complexes is under investigation.

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