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Reversed-Phase High-Performance Liquid Chromatography of Diastereoisomeric $[Co(en)_2(AA)]^{+/2+}$ and $[Co(trien)(AA)]^{2+}$ Ions (AA = Amino Acid Anion)

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The reversed-phase HPLC of a variety of diastereoisomeric $[Co(en)_2(AA)]^{+/2+}$ and $[Co(trien)(AA)]^{2+}$ ions (AA = Asp, Glu, Gly, Ala, Thr, Pro, Val, Met, Arg(NO_2), Ile, Tyr, Leu, Phe, Ser(Bzl), Trp, Thr(Bzl), Glu(Bzl), Tyr(Bzl)) are reported. In general, good separations of Δ-*S* and Λ-*S* isomers of the former and α, β_1-*RS,SR*, β_1-*RR,SS*, β_2-*RS,SR*, and β_2-*RR,SS* isomers of the latter (AA = Gly, Ala) are achieved on C_{18}-μ-Bondapak stainless-steel or radial-compression columns using 25 mM toluenesulfonate ion-pairing reagent in water/methanol gradients. Ion-pairing reagents give increasing retention times in the order $As_2(tart)_2^{2-} < Sb_2(tart)_2^{2-} <$ toluenesulfonate < camphorsulfonate < *n*-hexanesulfonate; little or no retention occurs with essentially nonhydrophobic anions (SO_4^{2-}, HPO_4^{2-}, Cl^-). Comparisons of efficiency and capacity are made between the analytical stainless-steel and radial-compression C_{18}-μ-Bondapak columns and high-performance ion-exchange chromatography (Partisil-10SCX column).

Introduction

Although ion-exchange chromatography (IEC) has been extensively used by ourselves and others for separating Δ,Λ-$[Co(en)_2(AA)]^{2+}$ [1-5] and $[Co(trien)(AA)]^{2+}$ [6] diastereoisomers, it is often tedious and inefficient.[7] Its extension to the separation of the related dipeptide complexes Δ,Λ-$[Co(N)_4(AA\text{-}AA'OMe)]^{3+}$ [8] is likewise time consuming and is even less discriminatory. We have recently embarked on a detailed study of the synthesis of small peptides using the $Co(N)_4^{3+}$-active ester method[9] and require a method for rapidly analyzing reaction mixtures. This involves separating $[Co(N)_4(AA)]^{2+}$ and $[Co(N)_4(peptide)]^{3+}$ systems and distinguishing between isomeric couplings.

High-performance liquid chromatography (HPLC) and reversed-phase high-performance liquid chromatography (RP-HPLC) have been extensively used for the separations of amino acids[10] and small peptides[10,11] and, by suitably modifying the stationary[12] or mobile phases,[13] for the resolution of the former. We have recently reported some separations by RP-HPLC of $[Co(en)_2(AA)]^{2+}$ complexes,[14] and Isied has used this technique to characterize a series of $[Co(NH_3)_5(peptide)]^{3+-6+}$ ions,[15] but apart from one report on the separation of Δ,Λ-$[Co(en)_2(Tyr)]^{2+}$ and Δ,Λ-$[Co(en)_2(Asp)]^+$ diasteoisomers by conventional HPLC on silica,[16] there have been no reports on the use of these newer techniques for the separation of diastereoisomeric water-soluble coordination complexes.

We report here results of the RP-HPLC and HPIEC of an extended array of amino acid and protected amino acid complexes in the $[Co(en)_2(AA)]^{+/2+}$ and $[Co(trien)(AA)]^{2+}$ series. In particular, we detail the separations of diastereoisomeric pairs and compare and efficiencies of a variety of ion-pairing reagents and the different column packings and capacities.

Experimental Section

The $[Co(en)_2(AA)]^{+/2+}$ amino acid complexes were prepared by standard methods[3-5,17] or will be reported elsewhere;[18] a similar treatment is given for the $[Co(trien)(AA)]^{2+}$ ions.[6,19,20] All amino acids (except glycine) were of the *S* configuration.

Apparatus. Either a Waters HPLC system (two M6000 A solvent delivery units, M660 solvent programmer, M450 variable-wavelength detector, U6K universal injector) or a Varian 5000 (microprocessor-controlled pump assembly, U6K injector, Varian 50 multiwavelength detector) was used. Chromatograms were recorded on a Varian Model 9176 strip chart recorder.

Columns. HPIEC was carried and on a Partisil-10SCX column (Whatman) and RP-HPLC on either C_{18}-μ-Bondapak columns (Waters; 10 μm, 30 cm × 3.9 mm i.d.) or Radial-PAK cartridges (Waters; 5 μm, 10 cm × 5 mm i.d.; 10 μm, 10 cm × 8 mm i.d.) using a RCM-100 compression module.

Solvents (Mobile Phase) and Sample Preparation. Water/methanol mixtures were used and purified as described.[21] Composition of solvent

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Table I. Comparisons of Capacity Factors (k')[a] for Some $[Co(en)_2(AA)]^{2+}$ Ions Using Different Pairing Anions (pH 3.5; H_2O/CH_3OH Eluent)

AA	eluent % CH_3OH	ion-pairing anion HSA^- [b]	TSA^- [c]	CSA^- [c]	$As_2(tart)_2^{2-}$ [c]	$Sb_2(tart)_2^{2-}$ [c]
Δ,Λ-Gly	2.85		1.36	3.43	0.28	0.28
Δ,Λ-Pro	2.85	3.03	1.42	3.63	0.22	0.30
Λ-Pro	0.95	7.02	5.08	6.64	0.40	0.93
Δ-Pro	0.95	5.29	3.70	4.83	0.40	0.44
Λ-Val	2.85	15.41	7.22	13.9	0.56	0.86
Δ-Val	2.85	19.07	9.41	15.4	0.56	0.86
Λ-Leu	2.85		34.0		0.76	1.95
Δ-Leu	2.85		29.3		0.76	1.39
Λ-Phe	14.25	20.45	8.4	22.1	2.28	6.79
Δ-Phe	14.25	20.45	10.06	22.1	2.76	8.93

[a] See ref 24. [b] 10 mM. [c] 25 mM.

A was 25 mM *p*-tosylate in water at pH 3.50 and solvent B 25 mM *p*-tosylate in 95% $MeOH/H_2O$ at pH 3.50. Programs were as follows (time (min), per cent solvent A): P1 (0, 100; 14, 99; 44, 80; 64, 70); P2 (0, 100; 14, 99; 36, 85; 68, 70; 116, 40; 120, 100); P3 (0, 80; 90, 5); P4 (0, 100; 14, 99; 56, 0) (linear interpolations *or* values as given). Other conditions were as follows: flow rate, 2.0 cm^3 min^{-1}; wavelength for detection, 480 nm; ambient temperature, 18–25 °C for column and 20.0 °C for detector; ion-pair concentration, 25 mM; pH, 3.50; otherwise, as specified.

Solid samples were dissolved in doubly distilled deionized water and filtered through a Swinney filter assembly fitted with a membrane filter (Millipore GSWP 01300; 0.22 μm, 13 mm) before injection.

Procedure. Details are given elsewhere,[21] but the procedure normally consisted of injection of 1–200-μL samples into the injector assembly (Hamilton syringe; 25 μL, 100 μL) with immediate starting of the solvent program. Chromatograms were recorded 0–100 min, 0.005–0.1 AUFS (AUFS = absorbance units full scale).

Results and Discussion

RP-HPLC on C_{18}-Bondapak Columns. Most studies have been with the monomeric C_{18}-bonded phase. This consists of C_{18} alkyl chains chemically attached to silica via dimethylsilyl ether linkages (8–40% loadings).

In the absence of an ion-pairing reagent, cationic Co(III) complexes show no retention on the bonded phase. Even the larger relatively hydrophobic ions such as $[Co(en)_2(Phe)]^{2+}$ and $[Co(en)_2(Trp)]^{2+}$ are eluted close to the solvent front. This indicates that hydrophobic interactions between the ligands and the C_{18} layer are insignificant by comparison with solvation of the ions by the aqueous mobile phase. Addition of small inorganic ions (e.g. Cl^-, SO_4^{2-}, HPO_4^{2-}) has no effect on the retention despite their excellent ion-pairing properties.[2] This must result from an inability of the ion-paired species (e.g. $[Co(en)_2(Phe)]SO_4$) to associate with the stationary phase.

Selective retention is achieved with larger, amphophilic anions. Table I compares the abilities of *n*-hexanesulfonate (HSA^-, a common pairing ion in RP-HPLC[23]), *p*-toluenesulfonate (TSA^-), *d*-camphorsulfonate (CSA^-, a useful "resolving" agent for complex ions), and antimony and arsenic *d*-tartarate ($Sb_2(tart)_2^{2-}$ and $As_2(tart)_2^{2-}$, both useful resolving agents) to retain several $[Co(en)_2(AA)]^{2+}$ ions. Retention increases in the order $As_2(tart)_2^{2-} < Sb_2(tart)_2^{2-} < TSA^- < CSA^- < HSA^-$. Little retention is found with $As_2(tart)_2^{2-}$ or $Sb_2(tart)_2^{2-}$ even though the 2– charge presumably results in a very stable ion pair. CSA^-, containing a bulky alkyl grouping, is better than TSA^- with the aromatic function; clearly the long-chain alkyl anion HSA^- is best. However, enhanced retention does not necessarily result in better resolution of the Δ-*S* and Λ-*S* diastereoisomers. TSA^- is superior to CSA^- and HSA^- in this respect, and it has the added advantages of being relatively inexpensive and easily purified. Although HSA^- and CSA^- are both transparent in the UV region (190–250 nm), TSA^- is not. This property is not required here since Co(III) complexes can be readily detected in the visible region ($\epsilon \approx 50$–200 mol^{-1} dm^3 cm^{-1} at 500 nm).

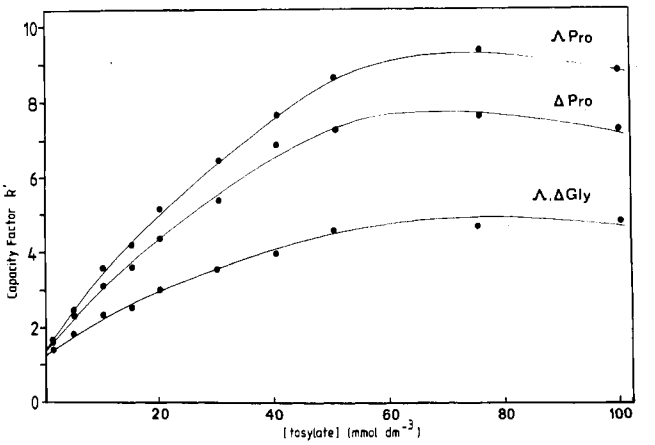

Figure 1. Plot of capacity factor (k')[24] vs. pairing-ion concentration (pH 3.5) for the isomeric Λ- and Δ-$[Co(en)_2(Pro)]^{2+}$ complexes and for Δ,Λ-$[Co(en)_2(Gly)]^{2+}$ (0.95% $MeOH/H_2O$ (isocratic); flow rate 2.0 cm^3 min^{-1}).

The results of Table I suggest that modification of the stationary phase is a very important property of the pairing ion. In this respect, it is interesting to note that the TSA^--modified bonded phase resembles the chemically bonded sulfonated polystyrene structure found in Dowex ion-exchange resins. The dependence of the retention time, t_R,[24] on TSA^- concentration for Δ- and Λ-$[Co(en)_2(Pro)]^{2+}$ and $[Co(en)_2(Gly)]^{2+}$ ions is given in Figure 1. While an increased concentration of TSA^- increases both the retention and selectivity between the diastereoisomers, it also broadens the peaks; the optimum concentration range is 20–30 mM. Curved plots such as those given in Figure 1 have been interpreted by some in terms of ion-pair or ion-exchange retention mechanisms,[25,26] but others[27] have suggested that the situation is rather more complex.

Δ,Λ-$[Co(en)_2(AA)]^{2+}$ Ions. Separations of AA = Gly, Pro, Val, Leu, and Phe complexes (using 5 mM TSA^-) have been described previously.[14] Separation of the Δ-*S*, Λ-*S* diastereoisomeric pairs is given here, and to avoid spurious splittings,[28] 25 mM TSA^- was used. Figure 2a shows that the AA = Gly and Ala complexes can be just separated. In our hands, this could not be achieved by ion-exchange chromatography (Dowex 50W-X2 or Sephadex SP-C25 resins). The Δ-*S* and Λ-*S* diastereoisomers of $[Co(en)_2(Ala)]^{2+}$ were not separated however, and this was the case for a variety of conditions (TSA^-, CSA^-, HSA^-, $Sb_2(tart)_2^{2-}$, $As_2(tart)_2^{2-}$) and also for HPIEC (phosphate pH 6.8; $Sb_2(tart)_2^{2-}$, ClO_4^-, Cl^- eluents);

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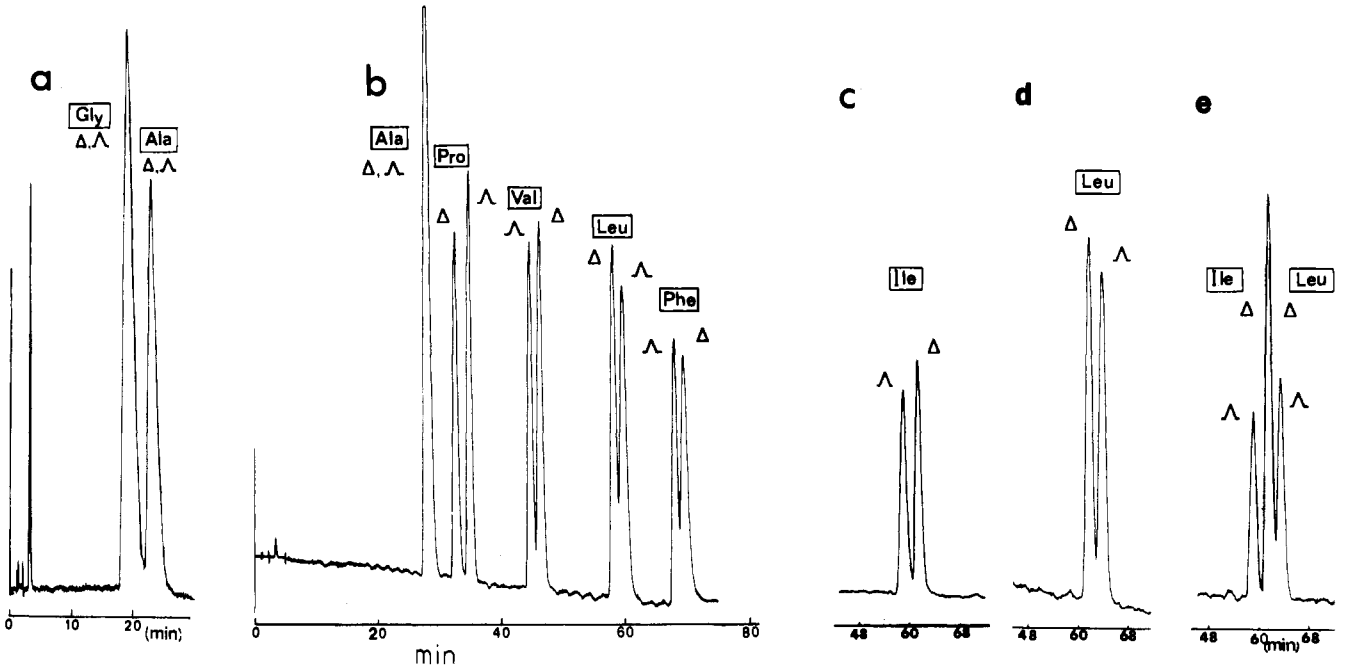

Figure 2. Elution profiles for Δ,Λ-$[Co(en)_2(AA)]^{2+}$ ions (λ 480 nm; AUFS 0.01; gradient P2; flow rate 2.0 cm^3 min^{-1}; chart speed 0.25 cm min^{-1}; 25 mM tosylate, pH 3.5): (a) AA = Gly, Ala (320 nmol, 20 μL); (b) AA = Ala, Pro, Val, Leu, Phe (1280 nmol, 20 μL); (c) AA = Ile (448 nmol, 7.5 μ); (d) AA = Leu (640 nmol, 10 μL); (e) AA = Ile, Leu (960 nmol, 15 μL).

Table II. Order of Increasing Retention Times (t_R) for $[Co(en)_2(AA)]^{+/2+}$ Ions on RP-HPLC

AA	side chain	charge on complex	elution order	hydrophobicity[a]	retention coeff[b]
Asp	$CH_2CO_2^-$	1+	unresolved	−0.02	−0.5
Glu	$CH_2CH_2CO_2^-$	1+	Λ, Δ	−0.07	1.1
Gly	H	2+	unresolved	0.00	0.2
Ala	CH_3	2+	unresolved	0.53	1.0
Thr	$CH(CH_3)OH$	2+	Λ, Δ	−0.26	−0.6
Pro	$CH_2CH_2CH_2$ (ring)	2+	Δ, Λ	1.01	3.1
Val	$CH(CH_3)_2$	2+	Λ, Δ	1.46	4.6
Met	$CH_2CH_2SCH_3$	2+	unresolved	1.08	4.0
Arg(NO_2)	$CH_2CH_2CH_2NHC(NH)NHNO_2$	2+	unresolved		
Ile	$CH(CH_3)CH_2CH_3$	2+	Λ, Δ	1.99	7.0
Tyr	$CH_2C_6H_4OH$	2+	Λ, Δ	1.70	6.7
Leu	$CH_2CH(CH_3)_2$	2+	Δ, Λ	1.99	9.6
Phe	$CH_2C_6H_5$	2+	Λ, Δ	2.24	12.6
Ser(Bzl)	$CH_2OCH_2C_6H_5$	2+	Λ, Δ		
Trp	CH_2(indole)	2+	unresolved	2.31	15.1
Thr(Bzl)	$CH(CH_3)OCH_2C_6H_5$	2+	Λ, Δ		
Glu(Bzl)	$CH_2CH_2CO_2CH_2C_6H_5$	2+	unresolved		
Tyr(Bzl)	$CH_2C_6H_4OCH_2C_6H_5$	2+	Λ, Δ		

[a] Hydrophobicity scale proposed by Rekker.[30] [b] Retention coefficients of AA residues in peptides.[31]

see below. However their separation using a long Dowex 50W-X8 column has been reported.[3]

The diastereoisomers of the Pro, Val, Phe, Leu, and Ile complexes separate (Figure 2b), although the Δ-Leu and Δ-Ile species overlap (Figure 2c). Also, the order, Λ-*S* before Δ-*S*, for AA = Val, Ile, and Phe is reversed for AA = Pro and Leu. Benzylation of side chain CO_2^- and OH groups results in greatly increased retention but may destroy Δ-*S*, Λ-*S* discrimination. The AA = Thr(Bzl), Tyr(Bzl), and Ser(Bzl) complexes show similar or enhanced discriminations compared to their unprotected cogeners, but Δ,Λ-$[Co(en)_2(Glu(Bzl))]^{2+}$ is unresolved. HPLC of an extended series, AA = Trp, Arg(NO_2), Met, and Asp, did not succeed in resolving these species. The polar functional groups decrease both t_R and discrimination between the Δ-*S* and Λ-*S* forms. Thus, for AA = Asp, Met, Arg(NO_2), Trp, and Glu(Bzl) complexes, the capacity of the side chain to H bond to the aqueous mobile phase clearly discriminates against selective binding to the nonpolar stationary phase. The AA = Glu complex is however an exception to this rule; the reason for this may reside in preferred intramolecular H binding of the carboxylate to the amino group of the ethylenediamine chelate.[29]

A summary of the elution order is given in Table II, with the Δ-*S* and Λ-*S* assignments being based on comparisons with authentic optically pure materials or, in cases where these were not available, on collections of a sufficient number of bands to allow the sign of rotation to be observed. The elution order is consistent with the hydrophobicity order proposed by Rekker[30] with the exception of AA = Thr. The same trend, and exception, is found with retention coefficients obtained by analyzing the contributions of amino acid fragments and terminal groups in a series of 100 peptides of varying sequence and comparing these to the retention time of the peptides on a C_{18}-RP column (CH_3CN/H_2O, 0.1% H_3PO_4, $H_2PO_4^-$).[31]

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Table III. Order of Diastereoisomer Separation for $[Co(en)_2(AA)]^{+/2+}$ on IEC

AA	column support	eluting reagent	elution order	ref
Ala	Dowex 50W-X2	NaCl	Λ, Δ	*a*
MeAla	Dowex 50W-X2	phosphate (pH 6.8)	Δ, Λ	*b*
Pro	Dowex 50W-X2	NaCl; phosphate (pH 6.8)	Λ, Δ	*a, b*
Cys(Me)	Dowex 50W-X2	NaCl	Λ, Δ	*a*
(homo)Ser	Dowex 50W-X2	NaCl	Λ, Δ	*a*
Glu	Dowex 50W-X2	NaCl; 0.2 M $NaClO_4$	Λ, Δ	*a, c, d*
Asn	Dowex 50W-X2	NaCl	Λ, Δ	*a, e*
Asp	Dowex 50W-X2	NaCl	Λ, Δ	*a, d, e*
Cys	Sephadex SP-C25	$Sb_2(tart)_2^{2-}$	Λ, Δ	*f*
Gly	Sephadex SP-C25	$Sb_2(tart)_2^{2-}$	Λ, Δ	*g*
Phe	Dowex 50W-X2	$Sb_2(tart)_2^{2-}$; $tart^-$	Λ, Δ	*h*
Thr	Dowex 50W-X2	NaCl	Λ, Δ	*i*
Leu	Dowex 50W-X2	NaCl	Δ, Λ	*a*
Gln	Dowex 50W-X2	NaCl	Δ, Λ	*a, e*

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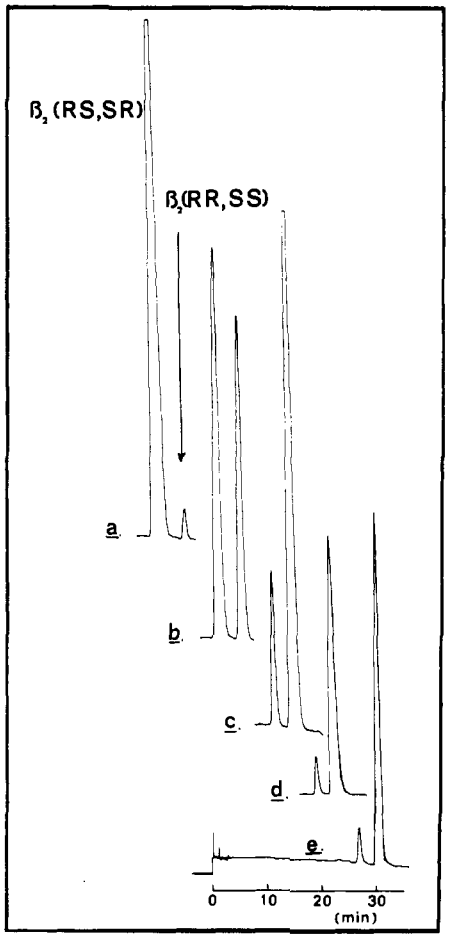

Figure 3. Chromatograms of β_2-(*RS,SR*)-$[Co(trien)(Gly)]^{2+}$ during mutarotation to the β_2-*RR,SS* isomer at pH 6.0 in water (512 nmol, 8 μL; λ 480 nm; gradient P2; flow rate 2.0 cm^3 min^{-1}; chart speed 0.25 cm min^{-1}; 25 mM tosylate, pH 3.5): (a) 1 h; (b) 1 day; (c) 4 days; (d) 8 days; (e) 9 days. AUFS: (a–c) 0.02; (d, e) 0.05.

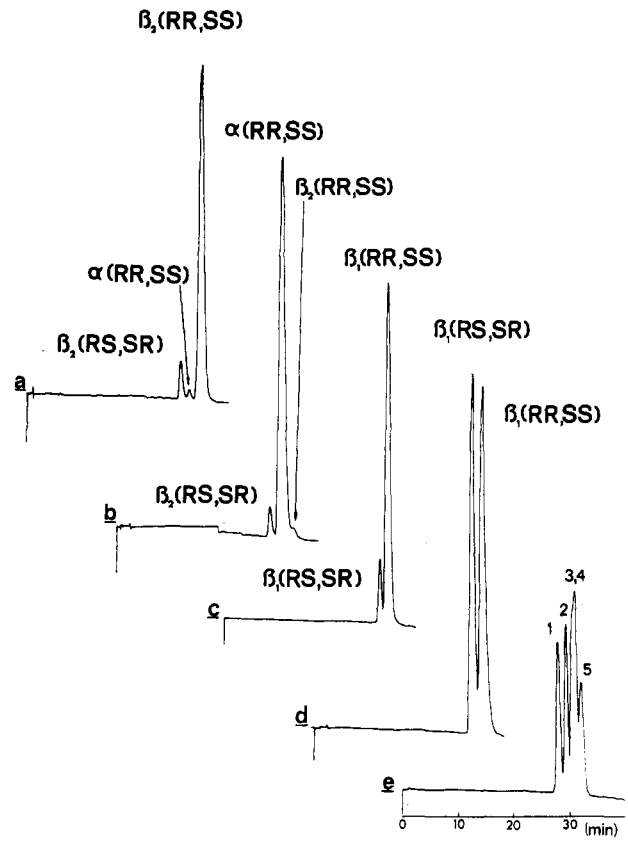

Figure 4. Elution profiles for $[Co(trien)(Gly)]^{2+}$ isomers (AUFS 0.02; gradient P2; λ 480 nm; chart speed 0.25 cm min^{-1}; pH 3.5; 25 mM tosylate): (a) β_2-*RR,SS* (288 nmol, 6 μL); (b) α-*RR,SS* (384 nmol, 6 μL); (c) β_1-*RR,SS* (380 nmol, 6 μL); (d) β_1-*RR,SS* isomer following mu taratation at pH 13 (2 min) and adjustment to pH 6 (600 nmol, 6 μL); (e) mixture of the five isomers (1) β_2-*RS,SR*, (2) β_1-*RS,SR*, (3) α-*RR,SS*, (4) β_1-*RR,SS*, and (5) β_2-*RR,SR* (288 nmol, 6 μL).

The data illustrate the importance of the side-chain structure in determining t_R for the $[Co(en)_2(AA)]^{2+}$ complexes; the same order is obtained for unbound amino acids on a C_{18}-RP column.[10]

Discrimination between the Δ-*S* and Λ-*S* isomers is clean and in many cases complete. With the exception of AA = Pro and Leu, Λ-*S* precedes Δ-*S*. The reasons for this remain obscure. For example, reversal occurs with AA = Leu and Ile where the side chains differ only in the position of a methyl group. The same order is found in IEC (and HPIEC, see below) except that the AA = Pro complex now follows the normal order, Λ-*S* preceding Δ-*S*. Clearly, the two mechanisms of retention are not identical. Table III lists IEC data for $[Co(en)_2(AA)]^{+/2+}$ complexes. There is some evidence to suggest that Λ isomers form stronger ion pairs with appropriate anions in the mobile phase, thereby decreasing their interaction with the ion-exchange resin.[32] However, such a proposal would predict the reverse order for RP-HPLC since it is the ion pair that associates with the stationary phase. Obviously the situation is complex, and it is interesting to note that the order observed by Warner and Legg[16] for AA = Tyr and Asp on silica (polar stationary phase) using a relatively nonpolar eluent (70/30 isopropyl alcohol, 2 M $Et_3NH_2CO_3$, pH 9.0) is opposite to that found here and that found by RP-HPLC and IEC.

$[Co(trien)(AA)]^{2+}$ Ions. Five enantiometric pairs of diastereoisomers are possible for $[Co(trien)(Gly)]^{2+}$ and ten diastereoisomeric pairs for complexes with asymmetric amino acids.[33] However, the potential of RP-HPLC to unravel this

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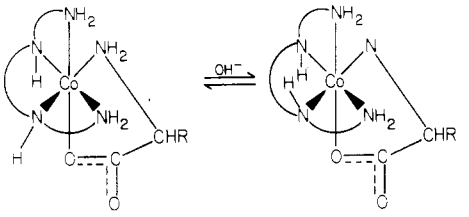
complicated array is demonstrated in Figures 3 and 4. Figure 3 depicts mutarotation about the "planar" N center of racemic β_2-*RS,SR*-[Co(trien)(Gly)]$^{2+}$ in water (pH 6.0, ~18 °C). The *RS,SR* and *RR,SS* isomers are clearly separated, the



Λ-*RS* Λ-*RR*

half-life for the interconversion can be estimated at ~100 h, and the final equilibrium [*RR,SS*]/[*RS,SR*] ≃ 10. These are in excellent agreement with the calculated rate under the conditions (3.9×10^{-6} s^{-1}) and equilibrium constant (9.0).[34] Figure 4a–c gives chromatograms of the "pure" β_2, α, and β_1 configurational isomers, but traces of other isomers can be clearly seen. Figure 4d gives the chromatogram of the fully mutarotated β_1-(*RR,SS*)-[Co(trien)(Gly)]$^{2+}$ isomer after 1 min (pH 13), with an equilibrium constant in this case of ~1. Figure 5e (supplementary material) gives the chromatogram of a mixture of the five conformational plus configurational isomers with an order of elution β_2-*RS,SR*, β_1-*RS,SR*, α-*RR,SS*, β_1-*RR,SS*, and β_2-*RR,SS* with some overlap between the last three. Previously IEC has been used to separate β_2-*RS,SR*/β_2-*RR,SS* and β_1-*RS,SR*/β_1-*RR,SS* pairs, but long columns and over 24 h of elution were required in each case;[6] the mixture of isomers has never been separated by IEC (or HPIEC). It is interesting to note that the *RS,SR* conformation of the triethylenetetramine quadridentate elutes before the *RR,SS* conformer and that this conformational property is at least as important as the configurational one of orientation of the glycinate chelate.

The above positive assignments of the [Co(trien)(Gly)]$^{2+}$ isomers allowed a preparative mixture of [Co(trien)(Ala)]$^{2+}$ to be analyzed. Treatment of [Co(trien)(OH)OH$_2$]$^{2+}$ with alanine methyl ester gave the product (after crystallization as the I$^-$ salt) given in Figure 5a (cf. supplementary material). By analogy with the glycinate system, the preparative method is expected to contain largely the β_1 isomer[33] and no α isomer.[35] Following mutarotation at pH ~11, the chromatogram given by Figure 5b (supplementary material) resembles that for the [Co(trien)(Gly)]$^{2+}$ mixture (Figure 4e) with the exception that the α isomer is missing. The similarity of the chromatogram in Figure 5b to that of Figure 4e attests to the correctness of the assignment for the alaninate system and also implies that the additional complexity introduced by the asymmetry of alanine does not influence the separations. This latter observation is in agreement with the inability to separate the Δ-*S* and Λ-*S* [Co(en)$_2$(Ala)]$^{2+}$ diastereoisomers noted above.

Figure 6 (cf. supplementary material) gives the elution profile of a number of β-[Co(trien)(AA)]$^{2+}$ ions AA = Ala, Pro, Val, Leu, Phe, and Ser(Bzl), with the general order being the same as that for the [Co(en)$_2$(AA)]$^{2+}$ series. The configurational assignments however are not clear at this time, except that α isomers are not likely to be present.[35]

Comparisons of RP-HPLC and Radial-Compression RP-HPLC. HPIEC using a Partisil 10-SCX column (sulfonic acid groups bonded to Partisil silica) and aqueous phosphate buffers

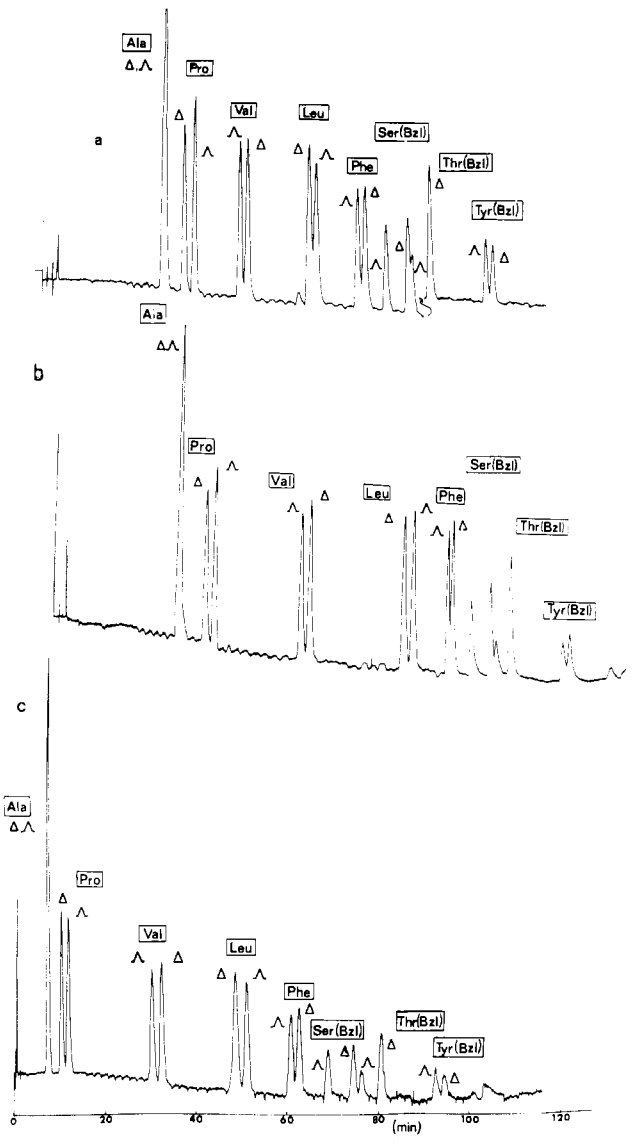


Figure 7. Comparisons of 30-cm stainless-steel analytical C_{18}-Bondapak column and C_{18} radial-compression column (RCC) using elution profiles of Δ,Λ-[Co(en)$_2$(AA)]$^{2+}$ complexes where AA = Ala, Pro, Val, Leu, Phe, Ser(Bzl), Thr(Bzl), Tyr(Bzl) (gradient P2; λ 480 nm; AUFS 0.01; chart speed 0.25 cm min^{-1}): (a) stainless-steel column, 1000 nmol, 20 μL (25 mM tosylate, pH 3.5; gradient P2; flow rate 2.0 cm^3 min^{-1}); (b) RCC, 1000 nmol, 20 μL (25 mM tosylate, 0.25% Et$_3$N, pH 3.5, gradient P2; flow rate 2.0 cm^3 min^{-1}); (c) RCC, 3000 nmol, 60 μL (25 mM tosylate, 0.25% Et$_3$N, pH 3.5; gradient P2; flow rate 8.0 cm^3 min^{-1}).

(isocratic, 1 M, pH 4.2–6.45) gave, in general, poor results. Elution times are comparable to, or in some cases shorter than, those obtained by RP-HPLC, and discrimination between the Δ-*S* and Λ-*S* isomers is not as good. Other runs using phosphate at pH 6.8 (a good eluent in IEC), Cl$^-$, ClO$_4^-$, Sb$_2$(tart)$_2^{2-}$, and the bromocamphorsulfonate ion confirmed that this method gave broader peaks and poorer resolutions of diastereoisomeric systems.

Use of a radial-compression RP-HPLC system speeded up the separations and allowed much larger amounts to be loaded without loss of resolution. Figure 7a,b compares the normal analytical stainless-steel column with the RC column under identical conditions. Similar separations between Δ-*S* and Λ-*S* isomers occur with similar retentions for AA = Ala and Pro but somewhat longer retentions on the RCC for AA = Val, Leu, Phe, Ser(Bzl), Thr(Bzl), and Tyr(Bzl). However, Figure 7c gives a chromatogram run at 4 times the speed (8.0 cm^3 min^{-1} vs. 2.0 cm^3 min^{-1}) and at 3 times the loading. Apart

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from the more rapid elution, the chromatograms are very similar, with no obvious decrease in resolution at the faster flow rate. The loading factor was further examined, and Figure 8a (supplementary material) shows the effect of increasing the loading of $[Co(en)_2(Pro)]^{2+}$ on the normal analytical C_{18} column. Good peak resolution is maintained until 2500 nmol when the leading edge of the Δ ion becomes split into two. This effect has been described previously.[27] Further loading results in further splitting followed by peak broadening at 5120 nmol. With use of the RC column, good peak shape is maintained up to 25 600 nmol (14 mg) (Figure 8b (supplementary material)). A further increase results in peak broadening and a shorter retention time. Hence, for these complexes up to 10 times the loading can be accommodated with the RC column. Also, the peak splitting effect observed when the normal analytical column was used has been eliminated.

The maximum loading factor is also dependent on the retention time. Whereas only 2500 nmol of $[Co(en)_2(Pro)]^{2+}$ could be loaded onto the analytical C_{18} column before peak splitting occurred, as much as 6400 nmol of Δ,Λ-$[Co(en)_2(Ser(Bzl))]^{2+}$ could be loaded (Figure 8c) (supplementary material)).

Supplementary Material Available: Figures 5, 6, and 8, displaying chromatograms of β-$[Co(trien)(Ala)]^{2+}$ isomers, of a series of $[Co(trien)(AA)]^{2+}$ complex ions, and of the effect of increased sample loadings for $[Co(en)_2(Pro)]^{2+}$ and $[Co(en)_2(Ser(Bzl))]^{2+}$ (4 pages). Ordering information is given on any current masthead page.

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Generalized Molecular Orbital Calculations on the Ground and Ionic States of (η^4-Cyclobutadiene)tricarbonyliron(0)

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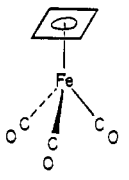
Generalized molecular orbital calculations with configuration interaction are reported for $(\eta^4\text{-}C_4H_4)Fe(CO)_3$ and several of its low-lying ionic states. The results provide insight into the cyclobutadiene–metal bonding and suggest rather strong polarization of the six electrons in the cyclobutadiene–metal bond toward cyclobutadiene. Calculations on the ionic states resolve the longstanding discrepancy between the experimental assignments and the results of ab initio calculations. Differential electron correlation is shown to be important in determining the order of the low-lying ionic states.

Introduction

Cyclobutadiene (Cbd), which has intrigued chemists for more than a century, has been extensively studied theoretically, even though it has never been isolated experimentally.[1] Yet, these calculations have sought to establish the nature of both the ground-state geometry of this species (whether square or rectangular) and its electronic structure (whether singlet or triplet).[2] In 1956 Longuet-Higgins and Orgel suggested that Cbd might be stabilized by and isolated in complexes with transition-metal fragments, of which tricarbonyliron(0) was a likely candidate.[3] Over the next decade a number of transition-metal complexes containing substituted Cbd ligands were prepared[4] before Pettit and co-workers isolated the species proposed by Longuet-Higgins and Orgel, (η^4-cyclobutadiene)tricarbonyliron(0) (**1**).[5]

Although a solid-state structure of this complex has never been performed, a gas-phase electron diffraction study indicated that the Cbd ligand was square planar (D_{4h} symmetry)



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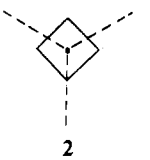

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and periplanar to the $Fe(CO)_3$ fragment.[6b] A crystal structure on the tetraphenyl derivative of **1** revealed several interesting features: (1) the complex had the eclipsed conformation, **2**; (2) the C–C bond lengths were divided into two unequal sets, making the Cbd slightly diamond shaped; (3) the $Fe\text{–}C_{Cbd}$ distances were unequal; (4) the phenyl substitutes were displaced out of the Cbd plane away from the metal.[6a] These structural distortions were shown to be consequences of the interaction of the $Fe(CO)_3$ d_π orbitals with the Cbd p_π orbitals.[7]

Cyclobutadiene is considered to be antiaromatic according to the Hückel rules as it has only four π electrons.[8] However, when complexed to metal fragments—in particular, to $Fe(CO)_3$—it exhibits extraordinary reactivity of an electrophilic nature, much as benzene (an aromatic species) does.[9] This would indicate that **1** undergoes an internal transfer of electron density from the $Fe(CO)_3$ fragment to the Cbd ligand, which is then subject to attack by electrophiles.[10] That this might be a correct analysis has been demonstrated by the mass spectra of these compounds, which show the clear presence

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