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A Versatile, High-Resolution Thin-Layer Chromatographic System for the Analytical and Preparative Separation of Complexes and Amino Acid Ligands

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An effective thin-layer chromatographic (TLC) technique has been developed for the separation of cobalt(III) geometrical isomers and diastereomers and various derivatized amino acid and peptide ligands. A single solvent system containing isopropyl alcohol and a triethylammonium bicarbonate buffer is employed to achieve both analytical and preparative separations. The technique is more rapid and sometimes exhibits better resolution than conventional column ion-exchange chromatography. One-step purifications of compounds which have required tedious purification procedures have also been achieved. Isomers possessing aromatic functional groups which strongly adsorbed to the polystyrene matrix of ion-exchange resins can be separated easily by this TLC method.

Introduction

Conventional open-column ion-exchange chromatography is a well-established technique for the purification of Co(III) coordination complexes as well as separation of their isomeric and diastereomeric forms.[1,2] Previously this was the only chromatographic technique which provided adequate resolution and capacity and was applicable to an extensive variety of complexes. However, long elution times (sometimes months) and tedious desalting procedures were often necessary.[3] Additionally the presence of aromatic functional groups frequently caused irreversible adsorption of complexes to the polystyrene portion of the ion-exchange resin.

The current research in our laboratory has been directed toward the synthesis and characterization of Co(III) complexes of aromatic amino acids and azo dye ligands which serve as structural analogues of certain biological systems. Incorporation of Co(III) into proteins as a structural probe and modifier of inherent biological function[4,5] has necessitated the development of more rapid high-resolution chromatographic techniques. Preparative high-pressure liquid chromatography (high-pressure LC) appeared to be the solution to our separation problems; therefore efforts were directed at developing or adapting already existing TLC solvent systems. The bewildering variety of solvent systems available for TLC (almost one for every Co(III) compound chromatographed) did not simplify matters, though in specific instances excellent separations have been achieved.[6]

In the course of these investigations a *single* high-resolution TLC solvent system was developed which has been used to separate a variety of Co(III) complexes, their geometrical and diastereomeric forms; amino acid ligands; peptides; diazotized amino acids and peptides; and azo dye–metal complexes; both analytically and preparatively.

Experimental Section

Materials. Carbon dioxide used in preparation of the buffer was purchased from Liquid Air Inc. Technical grade triethylamine was obtained from Eastman and was distilled before use. Distilled isopropyl alcohol was used for the LC studies. The preparations of the complexes used in this paper are referenced in the tables. All other chemicals were at least reagent grade.

Thin-Layer Chromatography. Analytical Procedure. Developments were carried out in either 6.5 cm diameter × 22.5 cm cylindrical or 10 × 25 × 30 cm rectangular chambers equilibrated with the buffer at least 1 h before use. Analytical separations were performed on Merk 5532 aluminum-backed plates or Whatman/Quantum K1 glass-backed plates. Elution orders were always identical on either type of plate, and R_f's were similar for the two types of plates, though the K1 plates occasionally exhibited better resolution.

Samples of 1–2 μL of 0.1–10 mM solutions were spotted, with 1.5 mm o.d. glass capillary tubes drawn to a 0.5-mm tip, in a single aliquot. All compounds were run at least in triplicate and developed for 10 or 20 cm. Mixtures of the isomers of a compound exhibited R_f's identical with those obtained for the pure isomers. Coordination complexes were either visually detected or sprayed with a 20% ammonium sulfide solution for visualization. Complexes containing the primary amine functionality were detected with ninhydrin or fluram sprays obtained from Whatman. The Pauly spray was used to detect imidazole functionality. Visualized plates were preserved with Krylon clear spray varnish. Those plates that could not be stored easily in a notebook were photocopied[7] and the copy was retained.

Preparative Procedure. Developments were carried out in a 10 × 25 × 30 cm rectangular tank equilibrated with the solvent system at least 1 h before use. Two 20 cm × 20 cm × 1.5 mm plates were run simultaneously in the tank and the used solvent system was discarded. Whatman/Quantum PR1F plates were used, or plates were produced in the following manner: Glass plates (20 × 20 cm), recovered from used commercial plates, were carefully washed in hot detergent solution and rinsed thoroughly with cold tap water, deionized water, and then distilled water. Gloves were worn to prevent finger oils from adhering to the plate. Plates were dried between layers of paper towels. For the preparation of five plates, 100 g of Merk Silica 60 for preparative TLC was completely suspended in 260 mL of distilled H_2O in a 400-mL polyethylene beaker. The beaker was placed in a bell jar and degassed for 1 h. The slurry was then poured into a Camag 21200 TLC applicator set for 1.5-mm thickness. The poured plates were placed on a rack and allowed to air-dry in a closed cabinet for 3 days without disturbance. Plates were not activated in any other manner before use. These plates exhibited resolution and loadability comparable to those of commercial plates.

Samples were applied in as small a volume as possible. Usually 1 mL of a 100–500 mg/mL solution was applied to within 1 cm of either edge of the plate with the spreader shown in Figure 1. The sample was drawn into a 1-mL disposable syringe and a small amount of air admitted in order to make room for the volume displaced by the capillary tube. The bent capillary was inserted and when the syringe was held horizontally, the sample flowed automatically to the tip. The assembly was then taped to the slanted block at a height such that the capillary tip was just touching the plate. The capillary tip should not be substantially lower than the capillary end or rapid siphoning of the sample onto the plate will occur making loading difficult. Once accomplished the block was butted against the bottom edge of the TLC plate and moved slowly back and forth, ensuring sample application parallel to the edge of the plate. The dimensions of the block determined the dimensions of the capillary. Samples applied in this manner were allowed to air-dry for 30 min before development. If volumes larger than 1 mL were spotted, 15–30 min was allowed between applications in order to permit the layer to dry. This reduced broadening (bands 0.5 → 1 cm are desirable) and prevented damage to the relatively fragile layer.

Plates were developed in a single pass and usually required 8–10 h for a full 20-cm movement of the solvent front. With suitable drying time between runs it was possible to develop a plate several times for compounds with very low R_f's. Plates were air-dried in a hood at room temperature for 2–4 h. The desired bands were scraped off with a razor blade and the silica suspended several times in H_2O or H_2O plus TEABC (20 mL to 1 mL, see below for preparation of TEABC) and centrifuged. The supernatants were pooled and rotovaped to dryness at 35–40 °C. During some separations residual solvent remained; however, rotovaping from H_2O or H_2O/methanol (1:1)

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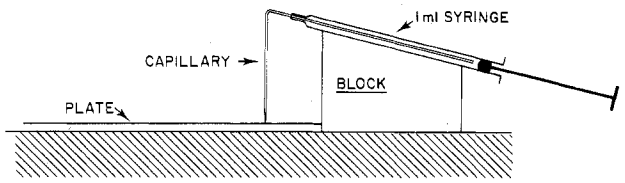

Figure 1. Preparative TLC sample applicator.

usually removed the excess solvent. Anionic counterions were replaced in many metal complexes with CO_3^{2-}; therefore, if necessary ion-exchange chromatography could be undertaken in the workup or acid could be added to replace CO_3^{2-} with the desired anion.

Preparation of the Triethylammonium Bicarbonate Buffer. The triethylammonium bicarbonate (TEABC) buffer was prepared in a hood by slowly bubbling CO_2 though a vigorously stirred solution of 680 mL of distilled triethylamine and 1720 mL of H_2O until all the triethylamine was taken up into the aqueous phase. This occurred in 3–5 h depending upon the rate of CO_2 addition. Then an aliquot of the solution was cooled to room temperature and its pH determined. The bubbling was continued until the pH reached 9.5. Further neutralization of the buffer (pH <9.0) rendered the buffer ineffective for TLC. This buffer solution was stable for several months under refrigeration.

Preparation of the Solvent System. For TLC the TEABC buffer described above was thoroughly mixed in volume to volume ratio with reagent grade isopropyl alcohol to form the desired composition of the IS–TEA solvent system (see Results and Discussion) and used immediately. The solvent system was stable under refrigeration for several months.

Results and Discussion

The Solvent System. Practical experience has shown that it is advantageous to buffer solvent systems designed to separate highly polar compounds. In TLC separations, pyridine/acetic acid has been used extensively for this purpose;[8] however, pyridine absorbs strongly in the UV region making it impossible to extrapolate TLC results to analytical or preparative LC where UV detection is required. Pyridine can also be difficult to remove during workup procedures. Most importantly, better selectivity and control of retention has been obtained with the solvent system employed in this study.

Triethylammonium bicarbonate was used as a buffer in the solvent system since it exhibited high resolving power, transparency in the UV region, and easy volatility under vacuum near room temperature. The latter characteristic was a distinct advantage over the pyridine solvent system since it greatly facilitated purification of the compounds chromatographed. This permits isolation of compounds in a short period of time as opposed to conventional ion-exchange chromatography where large volumes of salt solution must be removed from eluted compounds. Isopropyl alcohol is the other component of the solvent system. Though there were other solvents with similar solvent strengths ($\epsilon°$'s) and dielectric constants,[9] matching the viscosity of the TEABC reduced diffusion broadening when compared to that of less viscous solvents such as acetonitrile, Figure 2.

Retention ratios were not linear for short development distances, Figure 3. To obtain good resolution and reproducible R_f's, the solvent front should travel at least 10 cm. Lack of linearity has been attributed to demixing[10] of a small amount of triethylamine which forms a concentration gradient in the first 2–4 cm of development. Faint blue gradients have been observed when dry plates are sprayed with ninhydrin. Spraying stock solutions of distilled triethylamine spotted on silica TLC plates has produced the same blue color.

The selectivity and retention properties of the solvent system change after about 4 days of continuous use, or after thirty to forty 5 × 10 cm plates have been developed. Preparative separations also deplete the solvent system. Selectivity does not appear to change but retention increases as successive plates are run. Fresh solvent system should be used for each

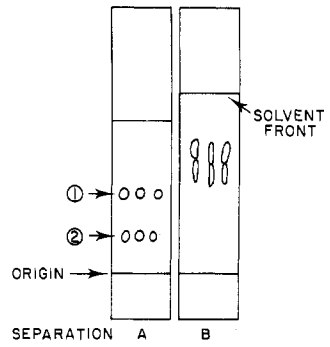

Figure 2. Effect of solvent viscosity on the separation of a diazotization reaction mixture of glycylhistidylglycine and diazotized arsanilic acid. The solvent in separation A was isopropyl alcohol/TEABC (IS/TEA), 50:50. Solvent B was CH_3CN/TEABC, 50:50. Compound 1 is arsanilazophenol; compound 2 is arsanilazoglycylhistidylglycine. Plates were spotted with 1-μL aliquots of 1 mM solutions.

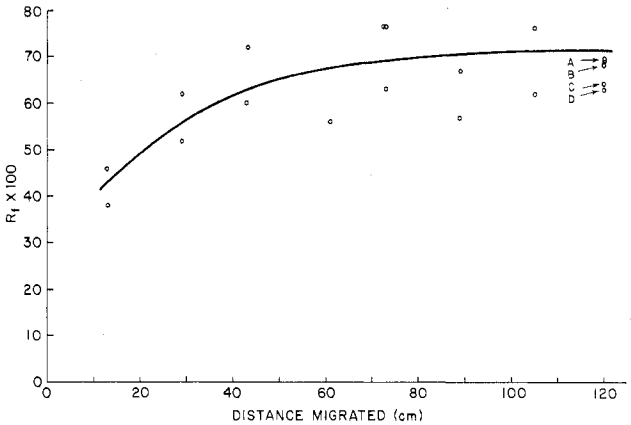

Figure 3. Variation of R_f with distance migrated: separation of (A) Δ-*s-cis*-[Co(EDDA)(L-Asp)], (B) Λ-*s-cis*-[Co(EDDA)(L-Asp)], (C) Λ-*u-cis*-[Co(EDDA)(L-Asp)], and (D) Δ-*u-cis*-[Co(EDDA)(L-Asp)], with the 50:50 IS/TEA solvent system. Note that the four components are not resolved before 120-cm development. Plates were spotted with 1–2-μL aliquots of 10 mM solutions of the complexes.

preparative run (two 20 × 20 × 1.5 mm preparative plates constitute a single run) or whenever it is important to obtain reproducible R_f's.

Retention was controlled by varying the ratio of isopropyl alcohol to TEABC. Anionic, neutral, and mono- and dipositive compounds were usually well separated from one another (anionic materials were retained least with the exception of azo dyes) when a 50/50 mixture (v/v, isopropyl alcohol/TEABC) was used. Tripositive compounds required 30/70 or 20/80 mixtures or multiple developments in a 50/50 mixture to obtain any elution. The more polar mixtures exhibited poorer resolution than the 50/50 mixture.

Thin-Layer Separations. Co(III) Complexes. There are numerous references in the literature pertaining to the separation of geometrical isomers of coordination complexes on thin layers.[2,6,11] In some cases resolution was incomplete and in others the solvent system reported worked only for a single compound.

The system described here has proved highly versatile and effective for the purification of a *wide* variety of compounds including the separation of diastereomers as well as isomers possessing more subtle differences than simple cis/trans isomerism. Modification of the solvent system's retention characteristics was straightforward, allowing the purification of a variety of different metal complexes and ligands, Tables I and II. Moreover analytical LC can be used in conjunction with the TLC techniques for rapid quantitative screening of reaction mixtures.[12]

Table I. Isomer Separations[a]

sepn no.	compd	10^2R_f	comments	ref
1	Δ-*s-cis*-[Co(EDDA)(L-Asp)]	68	partial separation in a single run	13
	Λ-*s-cis*-[Co(EDDA)(L-Asp)]	67		
	Λ-*u-cis-fac*-[Co(EDDA)(L-Asp)]	59	complete separation in single run	
	Δ-*u-cis-fac*-[Co(EDDA)(L-Asp)]	56		
	Δ-*u-cis-mer*-[Co(EDDA)(L-Asp)]	47		
2	*s-cis*-[Co(EDDA)(CDP)]	72	partial separation in single run	5b
	u-cis isomers	75, 77		
3	*s-cis*-[Co(dien)(L-Asp)]ClO_4	11		14
	u_1- and u_2-*cis*-[Co(dien)(L-Asp)]ClO_4	18		
4	*s-cis*-[Co(dien)(*erythro*-(2*S*)-3-MeAsp)]ClO_4	11		3
	μ_1- and μ_2-*cis*-[Co(dien)(*erythro*-(2*S*)-3-MeAsp)]ClO_4	18		
5	Λ-[$Co(en)_2$(L-Asp)]Cl	32	30/70 IS-TEA (see text)	15
	Δ-[$Co(en)_2$(L-Asp)]Cl	41		
	Λ-[$Co(en)_2$(L-Ala)]Cl_2	17		15
	Δ-[$Co(en)_2$(L-Ala)]Cl_2	17		
	Λ-[$Co(en)_2$(L-Hse)]Cl_2	13		15
	Δ-[$Co(en)_2$(L-Hse)]Cl_2	13		
	Λ-[$Co(en)_2$(L-Tyr)]$^{2+}$	11		15
	Δ-[$Co(en)_2$(L-Tyr)]$^{2+}$	15	Δ isomer contaminated with some Λ material	
6	*trans*-[$Co(NH_3)_4(NIC)_2]^+$	90	trace constituent of reaction mixture, isolated preparatively	16
7	[$Co(en)_2(CO_3)$]Cl	50		
	K[$Co(en)(CO_3)_2$]	80		
8	*s-cis*-[Co(EDDA)(en)]Cl	35	complete separation of all four compounds in a mixture	
	u-cis-[Co(EDDA)(en)]Cl	15		
	s-cis-[Co(EDDA)(pn)]Cl	46		
	u-cis-[Co(EDDA)(pn)]Cl	26		
9	[Co(EDDA)(arsanilazo-*N*-acetyltyrosine)]	67		
	[Co(EDDA)(arsanilazo-*N*-acetylhistidine)]	77	corresponds to C-2 and C-4 diazotization of the imidazole ring and subsequent formation of two discrete complexes	18

[a] Unless otherwise indicated separations were analytical and performed with 50/50 IS/TEA (see text).

Table II. Azophenol Separations[a]

compd	10^2R_f	comments
3-(4-arsanilazo)-2-naphthol[18]	90	analytical separation resolves all components
2-(4-arsanilazo)phenol	77	
2-(4-sulfanilazo)phenol	66	
2,6-bis(4-arsanilazo)phenol	57	
2-(4-arsanilazo)-1-hydroxybenzene-4-arsonic acid	47	
2,6-(bis(4-arsanilazo))-1-hydroxybenzene-4-arsonic acid	41	
arsanilazo-*N*-acetyltyrosine[18]	78	prepared by preparative TLC
bis(arsanilazo)-*N*-acetyltyrosine	59	
arsanilazo-*N*-acetylhistidine	89	
bis(arsanilazo)-*N*-acetylhistidine	79	
arsanilazoglycylhistidylglycine	80	
glycylhistidylglycine	68	
N-acetyltyrosine	95	
N-acetyl-*O*-acetyltyrosine	100	
N-acetylhistidine	87	

[a] Unless otherwise indicated separations were analytical and performed with 50/50 IS/TEA (see text).

In some cases very closely related isomers have been separated on TLC where, formerly, prolonged ion exchange was required in conjunction with tedious extractions to remove the eluting salt from the separated isomers. In some cases separations were observed for the first time. For example, Δ- and Λ-*s-cis*-[Co(EDDA)(L-Asp)] could not be separated by anion-exchange chromatography,[13] nor could the *u-cis-mer*-[Co(EDDA)(CDP)] (CDP = 2-(4-carboxyphenylazo)-4,5-dimethylphenol) isomer be completely separated from a mixture of *s-cis* and *u-cis-fac* isomers.[5b] The current method resolves the isomers in these mixtures to varying degrees (Table I, separations 1 and 2, respectively). Three isomers of [Co(dien)(L-Asp)]ClO_4, u_1, u_2, and *s-cis*, required 35 days to separate by conventional ion-exchange chromatography.[14] The three isomers of a similar complex, [Co(dien)(*erythro*-(2*S*)-3-MeAsp)]ClO_4, required 30 days for resolution.[3] The corresponding TLC separation resolves the *s-cis* from the *u-cis* isomers but fails to separate the *u-cis* isomers from one another (Table I, separations 3 and 4, respectively).

The synthesis and characterization of the isomers of $[Co(en)_2(L\text{-}Tyr)]^+$ was abandoned because of adsorption difficulties during ion-exchange purification procedures.[15] This adsorption has been attributed to the presence of aromatic groups. The two diastereomers of the complex are resolved sufficiently on a single preparative TLC run to generate the characteristic CD spectrum of the pure Δ isomer. The Λ isomer appears to be contaminated with some additional Δ material (Table I, separation 5). Analytical TLC of the reaction mixture showed 17 other metal-containing components. Separation data for several other $Co(en)_2$(amino acid)$^{2+}$ diastereomers are presented in Table I, separation 5. It appears that a "dangling" ionizable group improves separation for this type of complex by providing enough interaction with the stationary phase.

Purifications have been obtained for compounds from reaction mixtures which either could not have been purified by other means or required a series of steps which greatly reduced yields. The complex *trans*-$[Co(NH_3)_4(NIC)_2]^+$ (NIC = nicotinamide), a minor constituent of the reaction mixture, could only be isolated in trace quantities after a series of tedious chromatographic steps and crystallizations;[16] however, the preparative TLC method allowed separation of the desired product from the reaction mixture in one step. Interestingly the reaction solvent, ethylene glycol, did not interfere with sample application or development (Table I, separation 6).

The isolation procedure[17] for $K[Co(en)(CO_3)_2]$, an important synthetic intermediate, requires many steps, during which hydrolysis of the desired product can occur. Employing the TLC method described here, we can separate the complex from the crude reaction mixture in a single step, greatly reducing the time required for preparation of this material (Table I, separation 7).

The separation of simple geometrical isomers by TLC is not particularly novel, but in the case of [Co(EDDA)(en)]Cl and [Co(EDDA)(pn)]Cl not only were the *u-cis* and *s*-cis isomers of each compound separated but also both sets of isomers were well resolved from one another when a mixture of all four species was chromatographed. The corresponding isomers differ only by a methyl group (Table I, separation 8).

Cobalt complexes of diazotized *N*-acetylhistidine and *N*-acetyltyrosine and related azo dyes have been used as spectrophotometric models for Co^{III}(EDDA)(azoprotein) complexes.[5,18] Formation of these complexes has been followed by spotting the reaction mixture at intervals on plates and then developing. In this way optimal conditions conducive to the formation of these species have been found (Table I, separation 9).

Azo Dye Derivatives. The spectrophotometric characterization of diazotized tyrosine and histidine containing peptides and proteins has been reported.[19,20] Certain inconsistencies appeared when reproduction of some of these modifications were attempted. We have shown[18] that these problems stemmed from the impure model compounds that were originally synthesized.[19] The impurity of these compounds was established by employing this solvent system to separate components of amino acid diazotization reaction mixtures both analytically and preparatively, Table II.

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Registry No. Isopropyl alcohol, 67-63-0; TEABC, 15715-58-9.

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Characterization of $[Ni(en)_x]^{2+}$ (x = 1, 2, 3; en = Ethylenediamine) on the Surface of Montmorillonites

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The exchange of en complexes of Ni^{2+} on the bidimensional surface of montmorillonite-type minerals gives a mixture of $[Ni(en)_3]^{2+}$, $[Ni(en)_2]^{2+}$, and enH^+ on the surface. These complexes were characterized on the surface by IR and electronic spectroscopy. The relative concentrations depend on the exchange conditions, but the bis complex is preferred. The mono complex disproportionates in the presence of these clay minerals to $[Ni(en)_2]^{2+}$ and $[Ni(H_2O)_6]^{2+}$. The bis complex on the surface is diamagnetic with a characteristic absorption band at 21 500 cm^{-1}. As a consequence the surface is a very weak axial ligand, if a ligand at all. The tris complex decomposes in vacuo below 473 K to the diamagnetic $[Ni(en)_2]^{2+}$ form, which itself is destroyed in vacuo above 473 K. The protonated en molecules decompose below 473 K.

Introduction

The stability of metal uncharged ligand complexes on solid surfaces can be quite different from that in solution.[1,2] Both enhanced stability and destabilization have been found.[2] Thus, the overall formation constant β_2 of $[Cu(en)_2]^{2+}$ on the bidimensional surface of montmorillonite-type clay minerals is $10^{23.1}$ and $10^{21.35}$ on a resin. These numbers are to be compared with the value of 10^{20} in aqueous solution.[1] In the three-dimensional cage network of synthetic faujasites, $[Cu(en)_2]^{2+}$ is destabilized.[2,3] The origin of these effects is not well-known at present. However, it was found that $[Cu(en)_2]^{2+}$ on a Camp Berteau montmorillonite acquired an extra crystal field stabilization energy (CFSE) of 16–23 kJ mol^{-1} with respect to its CFSE in aqueous solution.[4] This was not accompanied by a physically significant change in the Cu–N bonding characteristics except for a slight increase of the covalent

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