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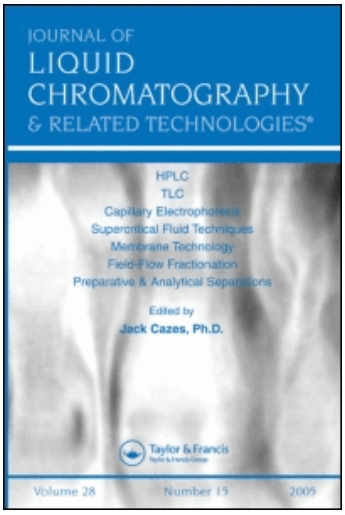

Journal of Liquid Chromatography & Related Technologies

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To cite this Article Lindner, Wolfgang F. and Hirschböck, Irmgard(1986) 'Chromatographic Resolution of Amino Acids Using Tartaric Acid Mono-N-octylamide as Mobile Phase Additive', Journal of Liquid Chromatography & Related Technologies, 9: 2, 551 — 571
To link to this Article: DOI: 10.1080/01483918608076653
URL: http://dx.doi.org/10.1080/01483918608076653

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JOURNAL OF LIQUID CHROMATOGRAPHY, 9(2&3), 551-571 (1986)

CHROMATOGRAPHIC RESOLUTION OF AMINO ACIDS USING TARTARIC ACID MONO-N-OCTYLAMIDE AS MOBILE PHASE ADDITIVE

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ABSTRACT

Chromatographic resolution of enantiomeric amino acids by a chiral ligand exchange system is presented using a novel chiral mobile phase additive, (R,R)-tartaric acid mono-n-octylamide (TAMOA) which sufficiently complexes copper II or nickel II ions. The chiral system is generated by dynamically coating a reversed phase packing, and shows selectivity for many amino acids. Under certain conditions also norepinephrine can be partially resolved. Retention and enantioseparation mechanisms in conjunction with the mobile phase composition and the ratio of TAMOA to the metal ion are discussed.

INTRODUCTION

Analytical techniques resolving α-amino acids or enantiomeric derivatives thereof are becoming of increasing importance for biochemical, chemical and geochemical studies. Besides the well developed enantioselective GC methods for α-amino acid derivatives (1) to determine their D,L-ratios in various samples (e.g. protein hydrolizate) also enantioselective systems in liquid chromatography have been developed. Especially the separation of optical isomers using ligand exchange liquid chromatography (CLEC) have become most popular since its first introduction by Davankov and coworkers (in 1968) (2), who subsequently stimulated this field by numerous original contributions (3).

At the beginning of CLEC the chiral ligands chelated by divalent metal ions, preferable copper II ions, were grafted on the support (originally polystyrene

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0148-3919/86/0902-0551$3.50/0

followed by silica gel) of the stationary phase by covalent bondings. Later,1979, Lindner,Karger and coworkers (4) introduced an alternative approach of chiral ligand exchange chromatography by adding chiral metal complexing agents to the eluent thus creating a dynamically and adsorptively coated chiral stationary phase using conventional achiral column packings, like RP phases.

In the same year also Hare and Gil-Av published a CLEC method based on the additions of a L-proline Cu II complex to the mobile phase, using a cation exchanger as non-chiral stationary phase (8). Subsequently this CLEC technique using reversed phase systems which are easy to handle, relatively stable and reproducable, became very popular. Many enantioselective CLEC systems, generated by the mobile phase additive mode, have been published yet and are summarized in Table I.

It is interesting to note, that in most cases α-amino acids and derivatives thereof are used as chiral chelating selector (SE), and that copper II ions seem to be preferred for the resolution of razemic molecules (SA) to α-amino acids or dansyl amino acids (Dns-D,L-AA). Other transition metal ions, like Zn II, Cd II, Ni II, Hg II, usually chelate much weaker than Cu II with the chiral selector (SE) and SA molecules. The formation of five-membered chelate rings of α-amino acids is also known to be preferred rather than six-membered rings, as it would be necessary by chelating ß-amino acids. Thus, ß-amino acids are hardly to resolve by CLEC. Basic considerations on this phenomenon have been made, especially on covalently bonded chiral ligand exchange phases, by Davankov (3) using models of ideally formed mixed chelate complexes. However, this explanation fits considerably well to the chromatographic results.
Theories on chiral separation mechanisms, stereoselective binding phenomena and chelation in the CLEC mobile phase additive mode seem to be more complicated, since a variety of additional equilibria in the chromatographic system have to be considered.

Reminding, CLEC deals with complexation mechanisms, thus the (chiral) selector (SE) and the (chiral) selectand (SA) have to have a certain molecular structure to fit into this scheme. Moreover, variations, e.g. coordination and geometry, of the ligand structure and the following parameters should be taken into consideration in the scope of CLEC:

(a) the nature of the ligand atoms (N, O, S, P)
(b) the functionality of the ligand atom (e.g.N in amine, amide or nitrile)
(c) the size of the chelate rings to be performed (4, 5, 6 or 7-membered rings)
(d) the number of chelate rings which can be accomplished by the ligand molecules (e.g. bi- or tridentate ligand)
(e) the steric effect determined by the size and position of the optically active substituents on the chelating ligand molecules (SE and SA)
(f) the nature of the central metal ion to be chelated, its oxidation stage as well as its coordination number (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{3+} etc.)

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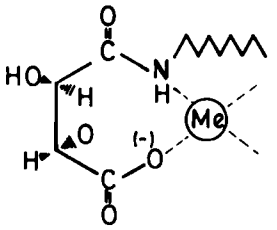

Figure 1
Structure of TAMOA-Me ion chelate complex.

(g) the temperature, the solvents in which the chelation process should take place, pH and the puffer system (summarized as mobile phase conditions).

Based on the above mentioned facts and in continuation of our work on chiral mobile phase additives, in the present study a new chelating additive, (R,R)--tartaric acid mono-n-octylamide (TAMOA) is presented for the enantioseparation of common and uncommon amino acids.

Structure features of racemic SA molecules are briefly elucidated in respect of their resolvability by TAMOA. In connection with it also the possible contributions of the diverse mobile phase components in the overall ligand exchange process are discussed, and a possible model of the (enantioselective) ligand exchange mechanisms in the mobile phase additive mode is drawn.

EXPERIMENTAL

Instrumentation : The chromatographic system was built up by the following components : HPLC Pump, Model 410 (Kontron) , injector, Model 7120 (Rheodyne) , equipped with a 20 µl loop ; detector, UV 254 nm, Model LC 15 (Perkin-Elmer) ; columns : analytical, 250x4.6 mm I.D., packed with Spherisorb ODS, 5 µm; pre-column as saturation column installed before the injector 100x4.6 mm I.D., packed with LiChrosorb RP 18 25-40 µm. The precolumn and the analytical column are placed in a Haake waterbath and kept at constant temperature (32°C or 50°C).

Reagents : All reagents used (methanol, acetonitrile, methylene chloride, diethylether, acetic acid, ammonia conc.,copper(II)-, nickel(II)-acetate, KOH, n-octylamine and (R,R)-O,O-diacetyl tartaric acid anhydride (DATAAN) were from Merck-Darmstadt. The water used was bidestilled in our laboratory. All amino acids were obtained from Sigma-München.

Preparation of (R,R)-+-tartaric acid mono-n-octylamide (TAMOA) :

(a) (R,R)-O,O-diacetyl tartaric acid mono-n-octylamide (I) :

33 g (0.15 M) DATAAN is dissolved in dichloromethane at 0°C, an equimolar

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TABLE I Chiral Mobile Phase Additives Performing Enantioselective Ligand Exchange Chromatography

Chiral Selector (SE)	Adsorbent (stationary phase)	Metal Ion	Selectand (SA) (racemic)	References
L-2-iospropyl-4-n-octyl--diethylene triamine (C_3-C_8-diene)	RP 8	Zn^{2+}	Dns-AA	4,5
L-prolyl-n-octylamide (L-Pro-C_8-amide)	RP 8	Zn^{2+},Cd^{2+}, Ni^{2+},Cu^{2+},Hg^{2+}	Dns-AA dipeptides nirvanol, ethosuccinimide	5,6
L-prolyl-n-dodecyl=amide	RP 8	Ni^{2+}	Dns-AA	7
L-Pro (e.g. D-Pro)	cation exchanger	Cu^{2+}	AA	8
L-Pro	RP 8	Cu^{2+}	AA	9, 10, 11
L-Pro	RP 8	Cu^{2+}	Dns-AA	12
L-Pro	silica gel	Cu^{2+}	thyroid-hormones	13
L-Arg	RP 8	Cu^{2+}	AA	11
L-His	RP 8	Cu^{2+}	AA	11, 14
L-His-O-CH_3	C_{18}	Cu^{2+}	AA	15, 16
n-alkyl-L-hydroxy=proline (n-C_7-;n-C_{10}-;n-C_{16}-)	RP 18	Cu^{2+}	AA	17
L-Phe	RP 18	Cu^{2+}	α-methyldopa, Trp 5-hydroxy-Trp	13
L-Phe	RP 18	Cu^{2+}	9-(3,4-dihydroxy=butyl) guanine	18
L-Phe	RP 18	Cu^{2+}	m- and p-hydroxy=mandelic acid	19
L-Asp-mono-alkylamide	RP 18	Cu^{2+}	AA	20, 21, 22
L-Asp-L-Phe-O-CH_3	RP 18	Cu^{2+},Zn^{2+}	DOPA,Trp,Tyr,Phe Val,Leu,Ileu,Nleu	23, 24
N,N-di-n-propyl-L-Ala	RP 18-TLC	Cu^{2+}	Dns-AA	25
N,N-di-n-propyl-L-Ala	RP 18	Cu^{2+}	Dns-AA α-methyl-α-AA	26, 27, 28
N,N-dialkyl-L-AA	RP 18	Cu^{2+}	AA	30

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TABLE I continued

N-(TOS)-L-Phe N-(TOS)-D-Phe	RP 18	Cu^{2+}	AA	31, 32
(2'S,4R,2'RS)-N-(2'-hydroxy--dodecyl)-4-hydroxyproline	RP 18	Cu^{2+}	4-thiazolidine=carboxylic acid	33
N,N,N',N'-tetramethyl-(R)--propanediamine-1,2	RP 18	Cu^{2+}	DOPA-Phe,Trp, mandelic acid	34

amount of n-octylamine (19.4 g) is added dropwise. Afterward the solution is stirred for 12 hours at room tamperature. After evaporating the dichloromethane the residue is dissolved in aqueous ammonia, filtered and thereafter the solution is acidified with HCl conc. A white product (I) precipitates, which is filtered off and washed with ice water. After drying, the resulted amide is suspended in 100 ml ether at room temperature for 1 hour, filtered off from the solution and dried again. Yield : 28 g (I).

(b) TAMOA :

28 g (72 mM) (I) is dissolved in 200 ml methanolic KOH (10%). The hydrolysis of the acetyl groups is rapid and after 30 minutes the precipitated potassium salt of TAMOA can be filtered off. The residue is dissolved in some water at elevated temperature, this solution is acidified with HCl conc. to a pH of about 2.0 . The resulted precipitate (TAMOA) is filtered off, washed with ice water and dried. It can be used without further purification.

Yield : 7 g ; Fp 152°C. The product is identified by IR and NMR spectroscopy. The specific rotation of TAMOA is $[\alpha]_{546}^{20} = +52^{o}$ (c = 3.9 /MeOH).

Preparation of mobile phase and coated stationary phase :

The desired molar amount of TAMOA , metal salt and acetic acid were dissolved in about 800 ml water. This solution was then adjusted with ammonia conc. to the appropriate pH value (e.g. 5.5). The solution was finally filled up to 1000 ml ; the pH checked and, if necessary, readjusted. From these stock solutions also the dilutions with the organic modifiers were made, whereby the pH in these mixtures was also readjusted. The chromatographic system was brought into equilibrium by pumping through the column, at thermostated conditions, about 40 to 50 column volumes of mobile phase which was discarded. Afterward, the mobile phase was recycled. For changing the metal ion in the system the columns were washed out with 200 ml mobile phase containing H_2O-MeOH-AcOH/50-50-1 . After running through pure water, the columns were ready for a new coating.

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RESULTS AND DISCUSSION

Separation of D,L-α-amino acids

We have previously reported the separation of dansyl derivatives of α-amino acids using the C_3-C_8-dien Zn(II) system and L-Pro-C_8 amide Ni(II) system (see Table I), whereby values of relative retention (α-values) up to 2.5 for the corresponding pair of optical antipodes could be achieved. However, the resolution of free α-amino acids was not sufficient. The present chiral metal chelate (TAMOA-Cu(II)-)system allows the separation of all common α-amino acids as listed in Table II. Resolution is found for aliphatic, basic and acidic α-amino acids. High chiral recognition is effected by the TAMOA--Cu(II) additive with α-values mostly sufficient for baseline separation. This is also due to the good column performance of the RP column which is maintained. Fig.2 shows typical enantioseparations of some α-amino acids.

As can also be noticed from Table II, the retention of the various amino acids is quite different and can be unpractically long for some of them at a given set of conditions. To get such compounds eluted in a reasonable k' region, the mobile phase has to be adjusted, either by changing the pH or by adding organic modifiers to the aqueous mobile phase, whereby at the same time the absolute concentrations of TAMOA, copper acetate and the buffer salts could also change. However, this shows the great variability of the mobile phase composition to be adjusted adequately in CLEC.

α-amino acids with a secondary amine function, like proline, allo-hydroxy= proline, dehydroproline or N-methyl-valine and N-methyl-leucine, can be also separated with high selectivity factors. The cyclic amino acids ,however, have a reversed elution order compared to the open ones.

(R,R)-TAMOA contains two chiral OH-groups and it has an α-hydroxy acid and an α-hydroxy amide structure (see Fig.1) which is quite different from the α-amino acids or derivatives thereof which are commonly used as chiral se= lectors in CLEC. This is noticeable since it is known, that α-hydroxy acids chelate much weaker than α-amino acids. However, TAMOA chelates relatively strong with transition metal ions (e.g.Cu(II), Ni(II)) and it should form a seven-membered ring between the carboxyl and the amide function. TAMOA could also be seen as an aliphatic α- or ß-hydroxy acid or acid amide (see Fig.1) which are known to chelate transition metal ions weakly.

It is not clear, whether the two OH-groups participate by forming a multi= dendate chelate complex or they do not. The postulated seven-membered ring structure is not proven yet, since the IR and NMR spectroscopic measurements were sufficiently interpretable due to broad signals observed. We are also lacking crystallographic data due to the fact, that we could not grow sufficiently good crystalls. However, the two C-atoms substituted by the OH-groups contain the stereochemical information by spatially controlled attrac= tion or repulsion to the chiral selector molecules (SE) to become resolved.

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TABLE II Separation of Free Amino Acids Using the (R,R)-TAMOA-Copper Complex as Mobile Phase Additive

Racemic Compound (SA)	k'_D	k'_L	$\alpha = \frac{k'_{long}}{k'_{short}}$ retained	Mobile Phase
Ala	0.7	1.2	1.7	a
Asp	4.3	3.6	1.2	a
Asn	5.3	6.1	1.15	a
Glu	4.9	3.6	1.36	a
Gln	6.1	9.1	1.5	a
$CySO_3H$	0.8	0.5	1.6	a
Val	7.2	13.1	1.8	a
Leu	>25	>25		a
	1.3	1.8	1.38	b
I-Leu	1.1	1.9	1.8	b
N-Val	0.5	0.9	1.8	b
N-Leu	1.45	2.6	1.8	b
Pro	2.7	1.2	1.7	a
OH-Pro(allo)	3.1	1.1	2.8	a
3,4-dehydro-Pro	5.5	1.8	3.9	a
pipecolic acid	0.8	1.05	1.3	a
α-amino butyric acid	2.0	4.0	2.0	a
ß-amino butyric acid	0.4	0.5	1.2	a
Ser	1.8	2.4	1.3	a
Thr	2.6	3.4	1.3	a
allo Thr	2.0	4.6	2.3	a
Homoser	1.7	2.2	1.3	a
Met	10.9	27.0	2.5	a
CySH	0.8	1.2	1.5	a
Ornithin	4.8	6.0	1.3	a

(continued)

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TABLE II continued

Citrullin	2.1	4.3	2.1	a
α-methyl ornithin	5.0	6.8	1.4	a
δ-difluoromethyl-ornothin	7.5	8.3	1.1	a
N-methyl-Val	1.5	5.6	3.7	a
Arg	7.4	16.9	2.3	a
His	>25	>25		a
	3.9	3.3	1.2	c
Phe	>25	>25		a
	7.4	8.4	1.1	d
Trp	>25	>25		a
	15	19.5	1.3	d
6-Me-Trp	17.2	21.2	1.2	d
7-Me-Trp	17.4	22.2	1.3	d
Phegly	1.6	2.0	1.4	d

Conditions : Column 250x4.6 mm I.D., ODS Spherisorb 5 µm, Temperature 50°C ; mobile phase composition : a) 0.1 M NH_4Ac + 0.4 mM TAMOA + 0.4 mM $Cu(Ac)_2$ (pH adjusted with NH_4OH to 6.5) ;
b) 85 mM NH_4Ac + 0.34 mM TAMOA + 0.34 mM $Cu(Ac)_2$ (pH adjusted to 7.0) - - acetonitrile / 850-150 ; Temperature 50°C ;
c) 0.1 M NH_4Ac + 0.15 mM TAMOA + 0.15 mM $Cu(Ac)_2$ (pH adjusted to 5.5) , Temperature 50°C ;
d) 60 mM NH_4Ac + 0.9 mM TAMOA + 0.9 mM $Ni(Ac)_2$ (pH adjusted to 7.5) - - methanol / 600-400 ; Temperature 35°C .

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Besides the enantioseparation of α-amino acids, TAMOA-Cu(II) supplies also some chiral information on the resolution of ß-amino butyric acid resulting in an α-value of 1.2 (see Table I). The resolution is certainly less pronounced compared to α-amino butyric acid (α = 2.0), but it is an indication, that CLEC is not restricted to SA molecules forming only five-membered chelate rings, like α-amino acids, α-hydroxy acids, ethanolamines etc.

Along this line we studied the effect of additional groups or substituents of the SA molecules, besides the most dominant chelating carboxyl and amino group, in terms of its participation on the chelate complex and in respect of stereoselectivity. As model substances served the structural isomers of tyrosine,o-,m- and p-Tyr (Fig.3). As can be seen from Fig.3, o-Tyr could act as a tridentate ligand forming a five-membered and a seven-membered chelate ring with the central metal ion (in this case Ni(II), whereby the binding increment of the OH-group is not quantified yet. In m-Tyr and even more in p-Tyr, the phenolic OH-group becomes less possible to participate on the chelate complex

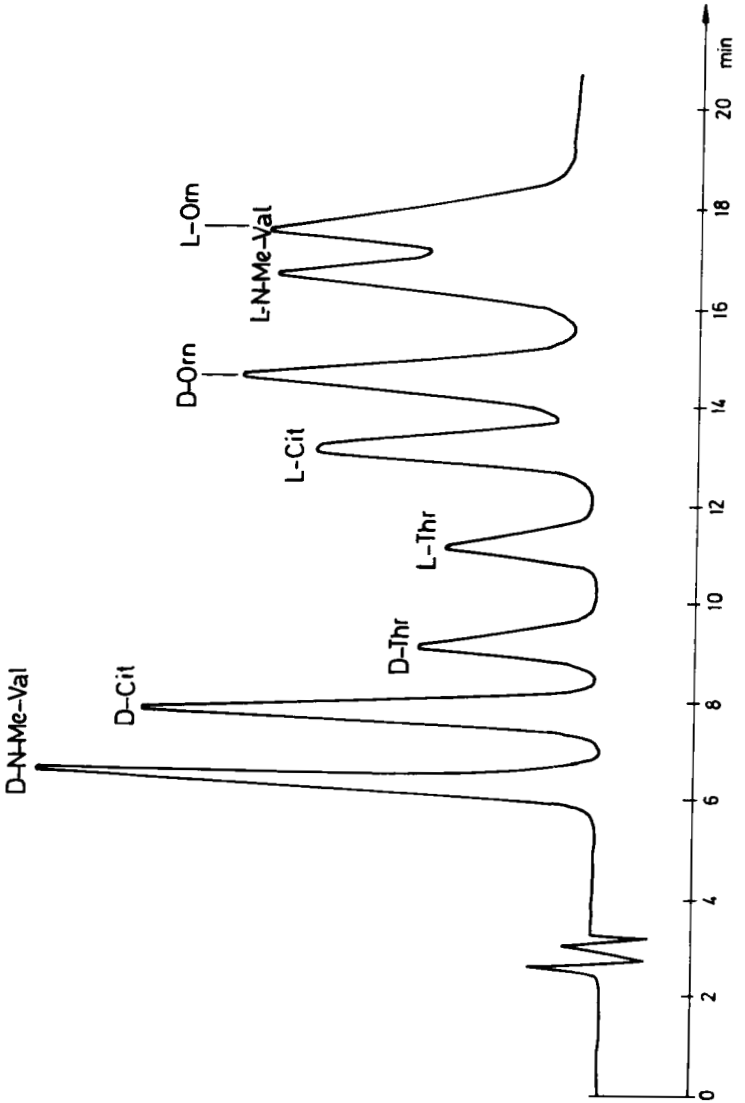

Figure 2

Chromatographic separation of D,L-threonine (Thr), D,L-citrulline (Cit), D,L-ornithine (Orn) and D,L-n-methyl valine (N-Me-Val) using the TAMOA-Cu(II) mobile phase additive. Conditions see Table II, mobile phase (a).

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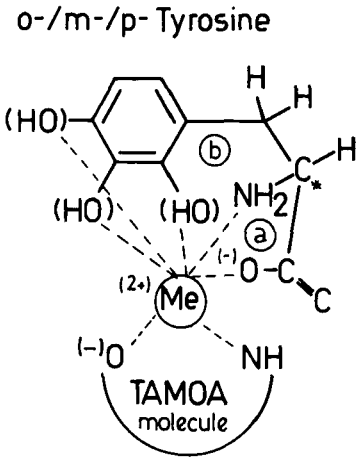

	k'_D	k'_L	$\alpha(\frac{k'_L}{k'_D})$
o-Tyr	13.9	14.7	1.06
m-Tyr	6.4	7.2	1.13
p-Tyr	2.8	3.3	1.17
Phegly	5.1	6.7	1.31
Phe	>30	> 30	-

Figure 3

Enantioseparation of tyrosine with (R,R)-TAMOA-Ni(II) chelate complex. Conditions : Column 250x4.6 mm I.D., Spherisorb ODS 5 µm, T = 32°C ; mobile phase : 60 mM NH_4Ac + 0.9 mM TAMOA + 0.9 mM $Ni(Ac)_2$ (pH adjusted to 7.5 with NH_4OH) - methanol / 600-400.

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due to their unfavoured position. As a result, the total retention time of the three isomers is significantly different. The retention time of o-Tyr is roughly two times higher than of m-Tyr and four times higher than of p-Tyr. Since in uncomplexed stage the three isomers should have nearly the same lipophilicity, the retention time of the chelated molecules should directly be connected with the capability to complex metal ions via a multidentate attachment resulting in differently stable complexes. However, a "strong complex" must not necessarily mean to observe also high enantioselectivity. This is only dependent on the stereochemical information of the chiral selector (SE) and the conformation of the chiral groups or substituents on the selectand (SA) molecule, both in its chelated form, and the resulted sterical dependent attraction or repulsion of the SA and SE molecules to each other.

In the present example, when a bidentate ligand becomes a tridentate one by incorporating an additional binding group, the conformation of the SA chelate changes, and thus also the enantioselectivity of the total mixed SE-SA complex can change. As a result, o-Tyr is resolved by an α-value of 1.06 which increases to 1.13 and 1.17 for m-Tyr and p-Tyr. The trend of retention time and enantioselectivity (α-values) is countercurrent.

Another speculative explanation for this phenomenon could also be a competitive two-dentated ring chelation of the metal ion, either by ring

(a) or by ring (b) (see Fig.3) which could have an equilibrium to each other. Thus, each ring creates its own conformational stereoselectivity, which also must not result in the same elution order and/or stability constants of the diastereomeric mixed complexes. The stereoselectivity created by the ring-formation (a) or (b) could theoretically be as different , that a reversal of the elution order could proceed. If this would happen, and the tendency of ring formation (a) and (b) is relatively equal, a total loss of enantioselectivity would be the result. Even if the direction of the stereoselectivity of ring (a) and (b) remains the same, but with a different magnitude, it does not necessarily implicate, that resulted chromatographic α-values must be additive and increase, since the bidentate ring formation (a) and (b) compete to each other. As a consequence of this consideration, the existance of more than two relatively equal binding (chelating) groups in chiral ligand exchange chromatography can cause severe phenomena in terms of the order of chiral resolution and its magnitude. It should be noticed, that the elution order of proline and other cyclic α-amino acids (L before D-isomer) is reversed in comparison to open chained α-amino acids besides histidine. Also the α-amino acids Asp, Glu, $CySO_3H$ and His which can form relatively stable tridentate or competitive bidentate chelate rings between the two carboxyl and the amine functions, show reversed elution orders ; observations could fit into the above made statement.

The experiment shown in Fig.3 contains also the values of phenylglycine (Phegly) and phenylalanine (Phe). The relative retention of Phegly is about the same to m-Tyr, whereas the k' values of D,L-Phe, lacking the OH-group of Tyr, are much higher than 30. Subsequently, the complex formation of Phe with Ni(II) and TAMOA is much stronger than of Phegly, since the increase of retention can notonly be attributed to the CH_2 increment , as the only difference between Phe and Phegly ; the retention must also deal with a more stable complex formation of Phe rather than of Phegly.

Besides the resolution of racemic amino acids we examined briefly the enantioselectivity of TAMOA towards racemic compounds with ethanolamine structure as shown in Fig.4, using the same set of conditions, as chosen for the resolution of Tyr isomers. As a result, the ethanolamine, ST 1059, can partially be resolved, whereas the related compounds, methoxamine and octopamine, remain unresolved. It seems obviously, that the methoxy group participates to some extent at the chelate formation, even more pronounced than the aliphatic OH-group. However, if the SE molecule contains an additional chiral center, as in methoxamine, thus resulting in the fact, that four stereoisomers can not be separated any more, probably due to insufficient chromatographic sufficiency of the chiral system. One can observe only a broad and unresolved peak. Comparing octopamine with p-tyrosine, it is clearly seen, that for chelation just the aliphatic OH and the amine function would be accessible, but the

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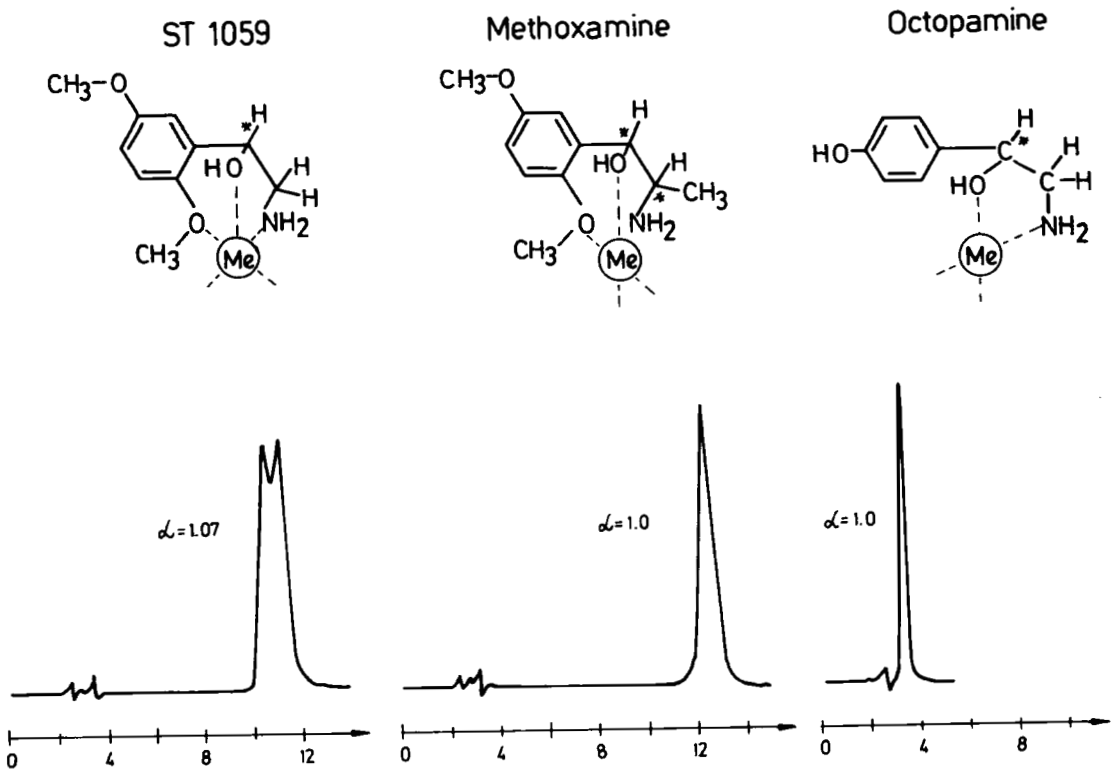

Figure 4

Enantioseparation of recemic ethanolamines with (R,R)-TAMOA-Ni(II) chelate complex.
Conditions see Fig.3.

OH-group chelates too weak, so octopamine is almost unretained and unresolved under these conditions.

As a consequence from the finding, that the phenolic OH-group in m-tyrosine participates to some extent at the chelate complex, the conclusion becomes obviously to try also the resolution of catecholamines as another example for racemic ethanolamines. As seen in Fig.5, partial resolution can be observed for norepinephrine, which must deal with a preferential chelate ring formation between the phenolic OH and the amino groups, thus building an eight-membered ring. Epinephrine and other catecholamines could not be resolved under these conditions.

CLEC phenomena, a speculative reflection

Discussing chiral ligand exchange chromatography phenomena, especially in mobile phase additive mode, effects caused by mobile phase components, like buffer salts, hydroxide ion concentration, water, organic modifiers and ammonia, which are always present, as well as the amount of the metal ion salt and the chelating agent and their ratio to each other should be considered.

Firstly we examined on an example the influence of the ratio of the chiral selector (TAMOA) to metal ion (Ni^{2+}) in terms of stereoselectivity (α-values), keeping other chromatographic parameters constant. The results are summarized in Table III. As can be seen, in a relatively low concentration range of TAMOA in the mobile phase, the greatest value of enantioselectivity was observed when the molar ratio of TAMOA to Ni(II) was 1:1. At this set of chromatographic conditions (0.9 mM TAMOA/ 1 mobile phase), no or little enantioseparation could be noticed if the ratio TAMOA to Ni(II)-acetate was 1:2 or 2:1. This result is not that astonishing, if one considers, that ligand exchange and chelation are not only caused by the SE and SA molecules, but also by the additional mobile phase components, especially ammonia and hydroxyl ions. In fact, in the CLEC, especially in the mobile phase additive mode, many equilibria of formed mixed chelate complexes are possible. Lam (16) has described the simplest form of stereoselective mixed chelation by the formation of ternary complexes consisting of a metal ion and two nonidentical ligands, such as SE and SA.

Me + 2 SE $\rightleftharpoons$ SE-Me-SE

Me + 2 SA $\rightleftharpoons$ SA-Me-SA

Me + SE SA $\rightleftharpoons$ SE-Me-SA

SE-Me-SE + SA-Me-SA $\rightleftharpoons$ 2 SE-Me-SA

The stability of the ternary complex is described by

$$\log k = 2 \log [\text{SE-Me-SA}] - \log [\text{SE-Me-SE}] - \log [\text{SA-Me-SA}]$$

A preferential formation of the ternary mixed complex is characterized by the sign and the value of log k and also by the concentration of SE, Me and

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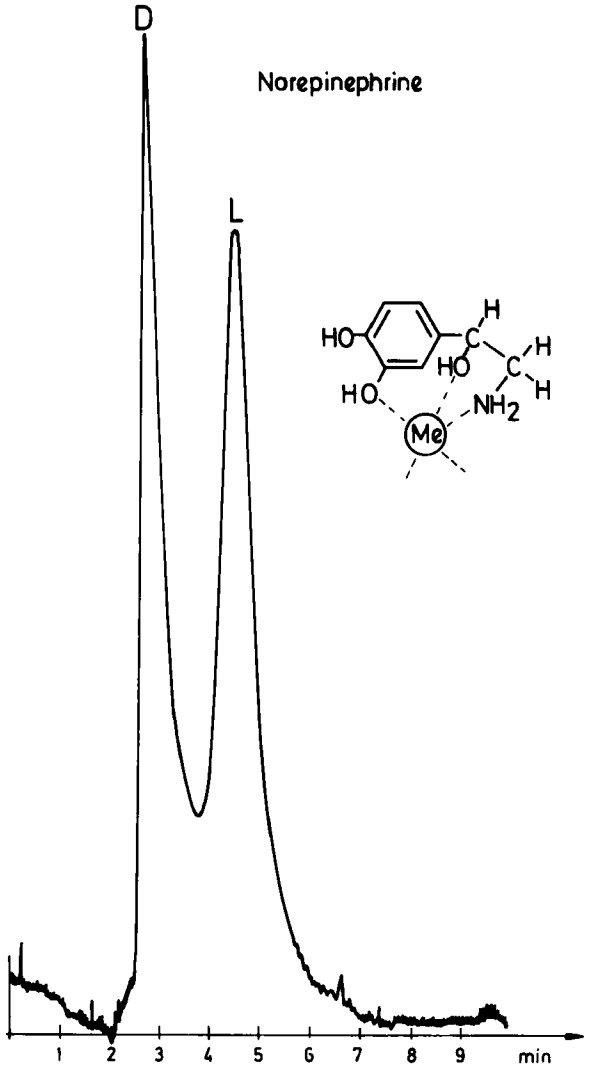

Figure 5

Enantioseparation of racemic norepinephrine with (R,R)-TAMOA-Cu(II) chelate complex. Conditions : Column like in Fig.3 , T = 50°C ; mobile phase : 0.1M NH_4Ac + 2 mM TAMOA + 2 mM $Cu(Ac)_2$ (pH adjusted to 6.5 with NH_4OH).

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TABLE III Enantioselectivity of TAMOA (SE) in Dependence of the SE-Metal Ion Ratio

(R,R)-(+)-TAMOA (mM) (x)	Ni(II) (mM) (y)	o-Tyr			m-Tyr			p-Tyr			PheGly			ST 1059		
		k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_L	k'_D	α	k'_1	k'_d	α
0.9	1.8	17.2		1.0	9.5		1.0	4.7		1.0	8.5	7.3	1.1	10.7		1.0
0.9	0.9	14.7	13.9	1.06	7.2	6.4	1.13	3.3	2.8	1.17	6.7	5.1	1.3	7.7	8.8	1.07
0.9	0.45	11.3		1.0	6.8		1.0	3.2		1.0	4.8	5.7	1.2	6.2		1.0
1.8	0.9	31.0		1.0	14.3		1.0	7.8	7.1	1.09	10.1	12.1	1.2	11.8	11.2	1.05

Conditions : Mobile Phase : 60 mM NH_4Ac + (x) mM(R,R)-TAMOA + (y) mM $Ni(Ac)_2$ (pH adjusted with NH_4OH) - MeOH/600-400
Column : 200 x 4.6 mm I.D., ODS 5 μm ; T = 32°C

SA. However, the ligand exchange will not only be performed by exchanging from the SE-Me-SE complex one partner by a (D)- or (L)-SA molecule forming the diastereomer complexes SE-Me-(D)-SA and SE-Me-(L)-SA ; it is also likely, that an intermediate complex summarized as (I), indicated in Fig.6, has to be formulated as enantioselective chelate complex with relatively easy exchangeable ligand molecules. By this extension of the traditional concept it has to be dealt with not precisely defined SE-Me-I complex as well as with the complexes SE-Me-SE, SA-Me-I and I-Me-I which all should be present to some extent in the chromatographic system. Consequently it will be fairly sensitive to get retention of SA via complexation with metal ions and mobile phase components, and enantioselectivity via stereospecific chelation with a chiral SE ligand. Thus, it becomes obvious, that such systems have to be balanced by e.g. pH and/or a source of metal ion to get moderately stable mixed complexes to force the enantioselective ligand exchange mechanisms. The stereoselectivity is determined by the three dimensional conformation of the diastereomer SE-Me-SA complex, of the spatial interaction (attraction or repulsion) of the chiral substituents within the mixed chelate complex.

It was also found, that the concentration of the components in the mobile phase and especially of the chiral selector (e.g. TAMOA) which is partially adsorbed on the stationary phase, will contribute significantly to the retention characteristics. At increasing concentration of TAMOA (its limitation is determined by the solvability of the TAMOA-Me-ion complex in the mobile phase), it becomes feasible, that even at a 2:1 ratio of TAMOA:Me-ion enantioselectivity is created , but slightly less pronounced than at a 1:1 ratio. Also the retention times increase remarkably, what is expected; but this says only, that more sites are excessible to create retention for the SA molecules.

As mentioned previously, retention is performed by the number of chelate complexes adsorbed on the stationary phase (SE-Me-SE and SE-Me-I, repsectively) if one ligand can be exchanged by SA molecules. If one makes the reverse experiment, doubling the metal ion concentration in respect to the amount of SE (the ratio of TAMOA to Ni(II) is 1:2), surprisingly also a remarkable increase on retention compared to the 2:1 ratio of TAMOA:Ni(II) is observed. Consequently, not only the amount of SE-Me-SE and SE-Me-I is responsible to create retention of SA via chelation, it is also the amount of the undefined complex I-Me-I capable to undergo ligand exchange mechanism thus forming I-Me-SA complexes which contribute also an increment to the overall retention, but not to enantioselectivity. The stability constants of the I-Me-I complex will be much lower than, for instance, for SE-Me-SA, however, the concentration of I is much higher than the one of SE, so this model could still be valid. Another aspect which is shown by this experiment, is the fact, that for a sufficient creation of enantioselectivity the number of

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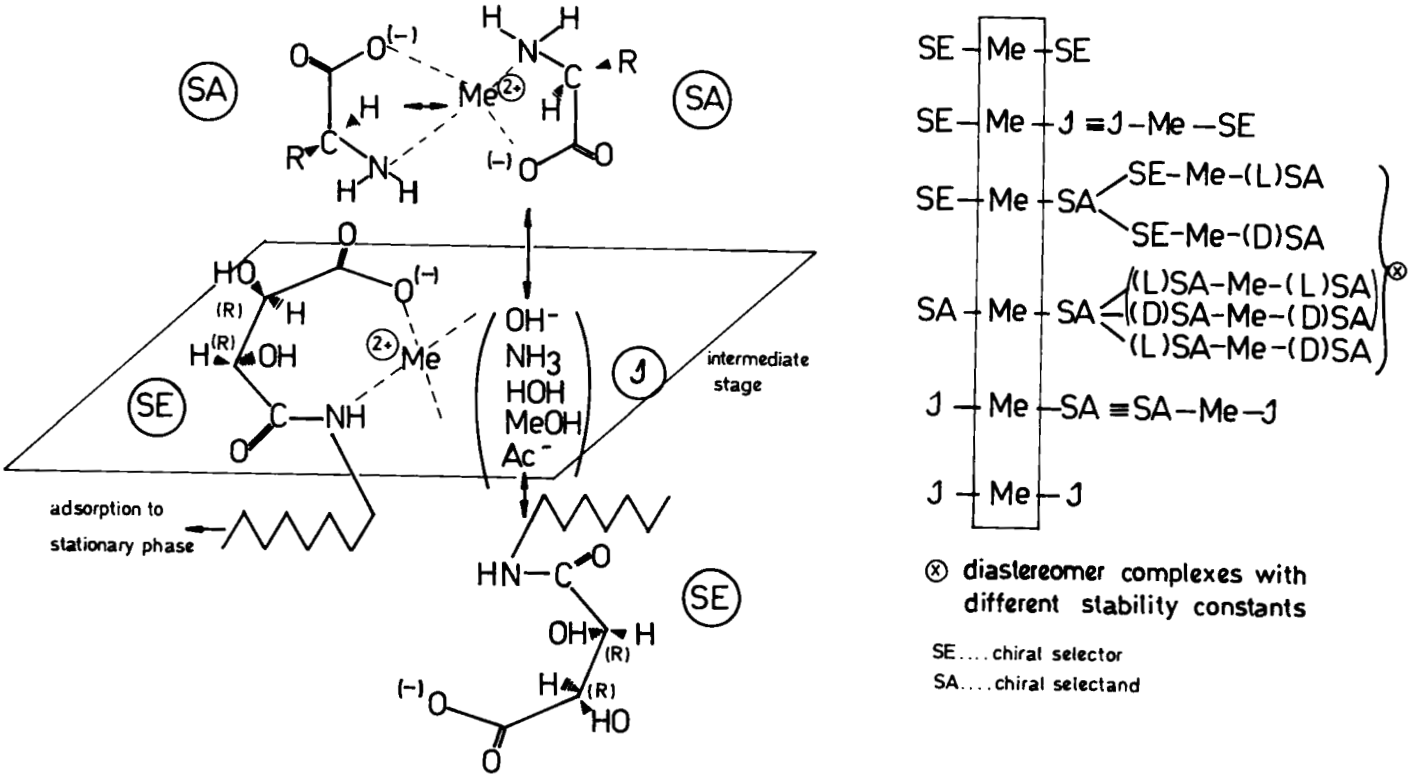

Figure 6

Model of ligand exchange mechanism in the mobile phase additive mode.

enantioselectively accessible SE-Me-SE and/or SE-Me-I molecules seems to be relatively low. The postulation of an 1:1 complex of SE:metal ion seems to be also in accordance to the results of Feibush et al (35) who designed a so= called "diluted" bonded stationary phase for CLEC, thus avoiding the dimonant formation of the 2:1 complexes (SE-Me-SE). The results of the authors were superior in terms of enantioselectivity and column efficiency, which could be attributed to the more rapid exchange of intermediate ligands than to the SE ligand.

In the following additional aspects of enantioseparation assuming ligand exchange mechanisms involving the chelation of metal ions should be discussed. If metal ions are present, such molecules will always elute as chelate com= plexes containing a central metal ion. Thus, the eluted and resolved peaks in the mobile phase additive mode of CLEC should be either the diastereo= isomer, SE-Me-(L)-SA and SE-Me-(D)-SA complexes, or the corresponding I-Me-(L)-SA and I-Me-(D)-SA complex, if one considers an exchange of SE and I . In the same way, SE or I could subsequently be exchanged by a SA molecule preferably with the same configuration (L or D) as the partner SA molecule, since the particular enantiomer is already concentrated in the respective peak fraction.

As a result, a complex, e.g. (L)-SA-Me-(L)-SA, would be formed representing the molecules of the (L)-peak. It is hard to distinguish between these prin= ciple ways. However, if the chiral selector SE is covalently bonded to the backbone, one will also observe enantioseparation by comparable ligand ex= change mechanisms, whereby the eluted fraction of the (L)-peak can only con= tain the (L)-SA-Me-(L)-SA or the I-Me-(L)-SA complex molecules.

The SA and SE molecules are in principle exchangeable and probably re= latively equal to each other in terms of lipophilicity. If so, SA could also serve as a chiral selector for another SA molecule forming SA-Me-SA complexes. Assuming a chromatographic system containing no chiral SE molecule present in the mobile phase or grafted onto the backbone , and injecting a nonracemic mixture of chiral SA molecules (unequal (D)-(L)-ratio), the accessible isomer (e.g. the (L)-form) could serve as achiral selector. In this case the following mixed chelate complexes could be performed :

(L)-SA-Me-(L)-SA (1)

(L)-SA-Me-(D)-SA (2)

(D)-SA-Me-(D)-SA (3)

The complexes (1) and (3) behave enantiomerically to each other, but dia= stereomerically to complex (2), what means, that (2) must be different from (1) and (3) in terms of complex stability constants and e.g. lipophilicity. Subsequently a resolution of (L)-SA and (D)-SA should be possible, espe= cially when the amount of (D)-SA is much smaller than of (L)-SA and there= fore the amount of the (D)-SA-Me-(D)-SA (complex (3) becomes small to negligible compared to the complex (1) and (2). This concept should theo=

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retically work, in consistence with the chiral mobile phase additive (SE) concept discussed previously.

Gil-Av and coworkers have made similar considerations (36) explaining the resolution of a nonracemic mixture without the usage of any other chiral source to resolve the enantiomers.

The concept of separating optical isomers via the formation of diastereomer molecule complexes with an optically active recourse (SE) in the mobile phase is a general one. The intermolecular binding forces to perform molecule associates are diverse, the formation of mixed chelate complexes is only one way to obtain the goal. Other chromatographic techniques following this line are the "solvation" of chiral molecules , e.g. salts of alkanolamines, with optically pure chiral solvents (e.g. tartaric acid esters), thus forming diastereomeric molecule associates (L)-SA-(R)-SE_x and (D)-SA-(R)-SE_x, as it was recently experimentically demonstrated by Prelog and coworkers (37).

The chiral mobile phase additive technique in non-aqueous systems, as shown by Hara et al (38), using (R,R)-tartaric acid amines as chiral selector (SE) to form diastereomeric molecule complexes, forced by hydrogen bonding and dipol-dipol interactions, follows also the general principle as well as the enantioselective ion pair chromatography , introduced by Pettersson and Schill (39).

All the different molecule complexes briefly discussed before should be described by equilibria and their constants which can be quite different. To which side the molecule association or dissociation is forced, is controlled by the concentration of each partner. Also the equilibria are superimposed, consequently, it is quite complicated to characterize them separately. The postulated molecule complexes in the additive mode of CLEC (see Fig.6) illustrates this clearly. Besides this, also kinetics of the various exchange processes should be discussed, since they are very important factors in terms of peak performance and especially noticeable in CLEC systems. E.g. increasing the temperature generally speeds up the exchange process resulting in better peak performance , however, often a decrease in enantioselectivity has to be taken in account. In particular Davankov has taken attention to this important aspect in CLEC (3).

CONCLUSION

Using (R,R)-TAMOA-Cu (II) as chiral mobile phase additive to create enantioselective ligand exchange systems in reversed phase LC allows the resolution of many free α-amino acids inclusively N-methylated ones. Its seven-membered chelate structure is unique, however, it will only create enantioselectivity for selectand molecules also capable to chelate a metal ion (e.g. Cu^{2+} or Ni^{2+}). Based on the diverse metal chelate complexes postulated, it becomes obvious, that the many variables (e.g. type of mobile phase

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components and its concentration) will influence the overall retention characteristics and also the enantioselectivity is dominated, however, by the molecular structure of SE and SA and the size and position of the chiral substituents and their spatial interaction.

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