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REVERSED-PHASE SEPARATION OF OPTICAL ISOMERS OF Dns-AMINO ACIDS AND PEPTIDES USING CHIRAL METAL CHELATE ADDITIVES*

W. LINDNER**, J. N. LePAGE***, G. DAVIES, D. E. SEITZ and B. L. KARGER§

Institute of Chemical Analysis and Department of Chemistry, Northeastern University, Boston, Mass. 02115 (U.S.A.)

SUMMARY

This paper is a continuation of the study of metal chelate additives to the mobile phase in reversed-phase liquid chromatography for the achievement of selective separations. In particular, through the use of the chiral chelate, L-2-isopropyl-4-octyl-diethylenetriamine–Zn(II), all common amino acids, with the exception of proline, as well as a number of other amino acids have been resolved into their optical isomers as their Dns derivatives. Remarkably high relative retentions are achieved, permitting rapid chiral separation of individual amino acids. Based on a study of pH and ionic strength, it is suggested that a selective interaction in basic media (pH 9) involves the electrostatic bidentate attachment of the Dns solute to the metal chelate. Attachment occurs through the carboxylate anion and the anion of the sulfonamide group whose proton is lost in the presence of the metal acid center. Other studies examined the role of metal ion on separation. In particular, when Ni(II), Cd(II), and Cu(II) were employed, differences in retention and selectivity were found. Interestingly, when Hg(II) was substituted for Zn(II), the elution order for D,L-Dns-amino acids was inverted, thus providing a simple means for checking chiral separations. Resolution of optical isomers of chiral dipeptides using Ni(II) chelate is also shown. Finally, several applications of this approach are discussed. Since little or no racemization was found in the dansylation reaction or upon standing, rapid assessment of optical purity of free amino acids was possible.

INTRODUCTION

We have recently shown that the selectivity of anionic solutes can be significantly altered by the addition of metal chelates to the mobile phase in reversed-phase liquid chromatography[1,2]. In particular, 4-dodecyldiethylenetriamine in a 1:1

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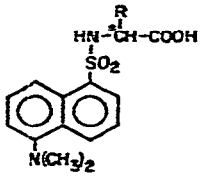
** Max Kade Post-Doctoral Fellow; present address: Institute of Pharmaceutical Chemistry, University of Graz, Graz, Austria.

*** Polaroid Corporation Graduate Fellow; present address: Organic Chemistry Division, W.R. Grace, Nashua, N.H., U.S.A.

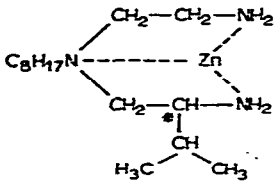
§ To whom correspondence should be addressed.

stoichiometric ratio with Zn(II) was found to provide functional group, steric and geometrical selectivity. More recently, using chiral metal chelate additives, we have reported the separation of optically active pairs of 5-dimethylaminonaphthalene-1-sulfonyl (Dns, dansyl) amino acids[3]:



where R determines the particular amino acid.

In this paper we will explore in detail a variety of factors which are of importance in the chiral recognition of the above substances, including pH, metal ion and the structure of the amino acid itself. We will show the resolution of all common protein amino acids, except proline, along with a number of other Dns-amino acids using L-2-isopropyl-4-octyl-diethylenetriamine–Zn(II) (C_3^*-C_8-dien–Zn(II)):



The results of this paper will illustrate the potential of this approach for the rapid trace analysis of amino acid and dipeptide enantiomers of synthetic and biological origin.

The use of metal ions for selective separation in liquid chromatography via ligand exchange chromatography has been utilized for a number of years as summarized in ref. 4. Optically active chelating exchangers have been bonded to a variety of stationary supports, and, after equilibration with an appropriate metal ion, have been successfully employed in the separation of optical isomers. Most notable in this area have been the studies of Davankov and Zolotarev[5], Lefebvre *et al.*[6], Foucault *et al.*[7] and Gübitz *et al.*[8]. D,L-Selectivity is found to be high; however, efficiency and peak symmetry are often poor and therefore at times unsuitable for analysis of complex mixtures.

A second approach to resolve optical isomers using metal systems is to add chiral agents to the mobile phase, as demonstrated by Nakazawa and Yoneda[9]. Recently, Hare and Gil-Av[10] have separated some underivatized D,L-amino acids on an ion-exchange column through the direct addition of the L- (or D-) proline–Cu(II) complex to the mobile phase.

It is worth noting that the resolution of enantiomers by liquid chromatography has recently been reviewed[11]. Gas chromatography has also been utilized for

separation of optical isomers, particularly of amino acids[12,13]. Typically, a non-volatile amino acid derivative is used as the stationary phase of a column. Free amino acids must undergo extensive derivatization before analysis.

EXPERIMENTAL

Equipment

Modular liquid chromatography systems were used in this work consisting of the various components in several combinations: Altex (Berkeley, Calif., U.S.A.) Model 100 and Waters Assoc. (Milford, Mass., U.S.A.) M6000A pumps; Rheodyne (Berkeley, Calif., U.S.A.) Model 7120 and Waters Assoc. U6K valve injectors; Laboratory Data Control (Riviera Beach, Fla., U.S.A.) Model 1206V ultraviolet absorbance detector; and Schoeffel (Westwood, N.J., U.S.A.) Model FS 970 spectrofluore monitor. For quantitative analysis a Hewlett-Packard (Palo Alto, Calif., U.S.A.) Model 3385A automation system was used. The columns were thermostated (typically at $30 \pm 0.1°$) with a Haake (Evanston, Ill., U.S.A.) Type NBE water circulator in combination with a Neslab (Durham, N.H., U.S.A.) cold finger. The pH of aqueous solutions was determined by a Beckman (Irvine, Calif., U.S.A.) Model 3500 digital pH meter.

Bonded phase packings and columns

Chemically bonded *n*-octyl (C_8) phases were synthesized in our laboratory as described previously[14]. Hypersil 5-μm particles (Shandon Southern, Sewickey, N.J., U.S.A.) were slurried overnight in 6 *N* HNO_3 and subsequently washed with distilled–deionized water to neutral pH (litmus paper). After bonding with *n*-octyldimethylchlorosilane, the unreacted accessible silanol groups were "capped" with trimethylsilane. Chemical analysis revealed bonded phase coverage of 3.4 μmole/m^2. Column tubing consisted of Analabs (Noth Haven, Conn., U.S.A.) "Anakro I.D." precision-bore stainless-steel tubing (4.6 mm I.D.) with drilled-out Swagelok end fittings and Whatman (Clifton, N.J., U.S.A.) 2-μm stainless-steel frits. The bonded phase particles were packed in the column by conventional slurry techniques. For most of the studies 15-cm lengths were used; however, in some cases 5-cm columns were employed. In order to increase column lifetime at the high pH values used in this work (*e.g.* pH 9), a 5-cm precolumn containing the bonded phase packing was placed before the injector.

Reagents and solvents

L-2-Ethyl-4-octyl-diethyltriamine (C_3^*-C_8-dien) was synthesized as described previously[3]. This synthesis gives low yields and produces a highly toxic intermediate (aziridine). Therefore, we have developed a new approach for L-2-isopropyl-4-octyl-diethylenetriamine (C_3^*-C_8-dien), the chelate that we have predominantly used in this study. Following Anderson *et al.*[15], we synthesized valyl-glycinamide using a mixed carbonic anhydride approach with isobutylchoroformate, *tert.*-butoxycarbonyl (BOC)-valine and N-octylglycinamide. After cleavage of the BOC group with trifluoroacetic acid, the peptide was reduced with BH_3 in tetrahydrofuran[16], yielding the dien in high optical purity. The starting material we have used for this work, octylamino-glycinamide, was synthesized by condensation of chloroacetamide and *n*-octylamine in ethanol under caustic conditions[17].

4-Dodecyldiethylenetriamine (C_{12}-dien) and dodecyltrimethylammonium bromide were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). The free amino acids, Dns-amino acids, 2,4-dinitrophenyl (DNP)-amino acids, BOC-amino acids, Dns chloride and pyridine-2-azo-*p*-dimethylaniline (PADA) were reagent grade and obtained either from Sigma (St. Louis, Mo., U.S.A.) or Pierce (Rockford, Ill., U.S.A.). *n*-Octyldimethylchlorosilane and trimethylchlorosilane were purchased from Silar (Scotia, N.Y., U.S.A.). The organic solvents were UV grade from Burdick and Jackson (Muskegon, Mich., U.S.A.). Water was obtained in purified form using a Milli Q system (Millipore Corp., Wayland, Mass., U.S.A.).

Mobile phase preparation

The chelating agent and metal salt were carefully weighed on an analytical balance and added to the appropriate amount of an aqueous ammonium acetate solution (pH $\approx$ 7.0). The pH was adjusted with either glacial acetic acid or aqueous NH_3 and the appropriate amount of organic modifier added. The mobile phase was then filtered and degassed with helium.

RESULTS AND DISCUSSION

Separation of D,L-*Dns-amino acids*

We have previously reported[3] the separation of a number of D,L-Dns-amino acids in reversed-phase liquid chromatography using the chiral chelate additive C_3^*-C_8-dien–Zn(II). Fig. 1 shows the separation of D,L-Dns-Thr and D,L-Dns-Ser using this metal chelate system. High chiral recognition is afforded by this mobile phase additive (*e.g.* α for D,L-Dns-Ser is 2.5), while at the same time good column performance is maintained. With such selectivities very short columns can be used to assess optical purity of amino acids (see later). Fig. 2 shows the separation of D,L-

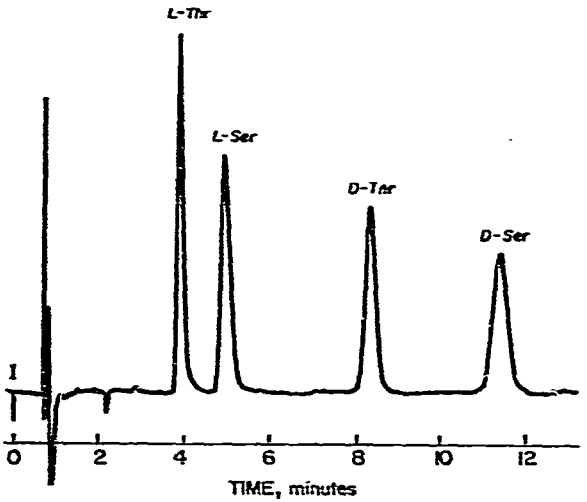


Fig. 1. Chiral separation of the Dns derivatives of D,L-threonine (Thr) and D,L-serine (Ser). Conditions: 0.8 m*M* C_3^*-C_8-dien–Zn(II), 0.19 *M* ammonium acetate, pH 9, acetonitrile–water (35:65, v/v), flow-rate 2.0 ml/min, 30°. Column: 15 cm × 4.6 mm, 5-μm Hypersil bonded with *n*-octyl groups.

Dns-Asn and D,L-Dns-Asp. Note in this case the reversal in elution order of the enantiomers of aspartic acid. As will be shown shortly, basic conditions (pH 9) turn out to be an important factor in the achievement of chiral recognition for the Dns-amino acids.

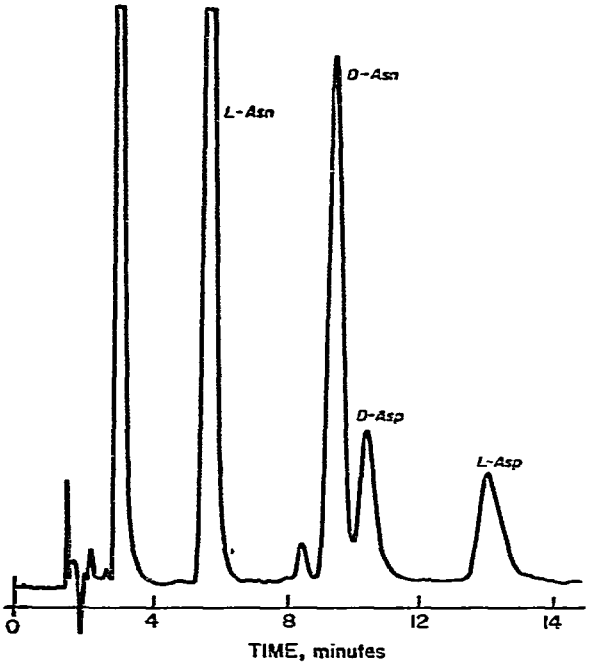


Fig. 2. Chiral separation of the Dns derivatives of D,L-asparagine (Asn) and D,L-aspartic acid (Asp). Conditions as in Fig. 1, except flow-rate 1.0 ml/min.

As described in the experimental section, using a precolumn consisting of the chemical bonded phase before the injection valve substantially reduces deterioration of the column due to the high pH conditions. This appears to be a result of equilibration of the mobile phase with silica before the eluent enters the analytical column[18]. Thus, the dissolution of the packing is significantly retarded. Use of high coverage monomeric and silanized bonded phases and NH_3/NH_4^+ as buffer (*vs.* metal hydroxides[19]) may also enhance column lifetime. In our experience the reversed-phase columns can last at least three months under the conditions shown in Figs. 1 and 2 (*i.e.* high pH) when a precolumn is employed.

Table I presents the retention and α values for all the common D,L-Dns-amino acids along with other amino acids. Separation is found for all pairs except D,L-Dns-Pro, the only amino acid listed with a secondary α-amino group. Relative retention values for separated pairs are found to vary greatly from as low as 1.10 (*e.g.* D,L-Dns-*allo*-Ileu) to as high as 2.2 (*e.g.* D,L-Dns-Thr) and 2.5 (D,L-Dns-Ser). In general, amino acids with additional polar groups (*e.g.* –OH, $-CONH_2$, $-SO_3^-$) appear to produce high α values. Further evidence of this specificity may be seen in the elution order of D,L-Dns-Asp where the D-isomer elutes before the L-isomer, unlike all the

TABLE I

CHIRAL SEPARATION OF Dns-AMINO ACIDS

Conditions: 0.8 m*M* C_3^*-C_6-dien–Zn(II), 0.19 *M* ammonium acetate, pH = 9, acetonitrile–water (35:65, v/v), 30°.

Dns-Amino acid	*k'*	*α(k'D/k'L)*	*Dns-Amino acid*	*k'*	*α(k'D/k'L)*
Gly	6.75	—	L-Ileu	5.0	1.20
			D-Ileu	6.0	
L-α-Ala	2.5	2.24			
D-α-Ala	5.6		L-*allo*-Ileu	4.9	1.10
			D-*allo*-Ileu	5.4	
L-α-NBu	2.5	1.40			
D-α-NBu	4.85		L-Phe	14.4	1.83
			D-Phe	26.3	
L-Nval	4.2	1.96			
D-Nval	8.2		L-Trp	18.0	1.80
			D-Trp	32.6	
L-Nleu	7.35	1.98			
D-Nleu	14.5		L-Ser	3.2	2.50
			D-Ser	7.8	
L-Val	3.0	1.50			
D-Val	4.5		L-Thr	2.5	2.20
			D-Thr	5.6	
L-Leu	6.2	2.07			
D-Leu	12.8		L-Pro	2.0	—
			D-Pro	2.0	
L-Asp	4.8	0.75			
D-Asp	3.6		L-Met	4.4	1.94
			D-Met	8.5	
L-Glu	0.7	2.10			
D-Glu	1.45		L-Met-sulfone	1.5	1.70
			D-Met-sulfone	2.6	
L-$CySO_3H$	4.15	1.63			
D-$CySO_3H$	6.8		L-Phenylglycine	8.9	1.65
			D-Phenylglycine	14.8	
L-Asn	2.25	1.95			
D-Asn	4.3		L-His*	69.3	1.45
			D-His	101.5	
L-Gln	0.8	1.15			
D-Gln	0.95		L-Tyr*	19.25	1.75
			D-Tyr	33.4	
L-Arg	0.6	1.67			
D-Arg	1.0		L-Lys*	37.1	1.50
			D-Lys	55.3	
			L-Ornithine*	33.25	1.65
			D-Ornithine	55.2	

* Di-Dns derivatives.

other Dns-amino acids studied to date. As a consequence of the generally large α values for all amino acid pairs, rapid separation of individual derivatized amino acids requiring only a relatively small number of theoretical plates is possible. On the other hand, as seen in the capacity ratios (k'), overlap of isomers of one amino acid with that of another is possible. Thus, when the optical purity of a number of amino acids is to be determined simultaneously, a coupled column approach in which the first column achieves a non-chiral separation of the Dns-amino acids may be necessary.

In a previous paper[2] we showed that the selectivity generated with metal chelate additives was significantly different from that observed in the ion-pair or reversed-phase mode. We have explored in more detail the causes of this special

selectivity and its role on chiral recognition. In particular, the study of the influence of pH on retention and relative retention with various structurally related Dns-amino acids has proven to be valuable in elucidating some aspects of the ion association process. The results of this study and its implications on separation are discussed in the next sections.

Influence of pH on retention and selectivity

Fig. 3 shows plots of log k' *vs.* pH for a number of Dns-amino acids as well as several monocarboxylic acids. In the cases of 2-phenylpropionic acid and phenylacetic acid, retention is seen to decrease with increasing pH. On the other hand, Dns-Gly, α-Ala, Nval and Nleu all have marked increases in retention with increasing pH. This enhanced retention with pH corresponds to an increase in α for the D,L-enantiomeric pairs in this pH region. We shall discuss the behavior of Dns-Pro, β-Ala and γ-aminobutynic acid (NBu) at a later point.

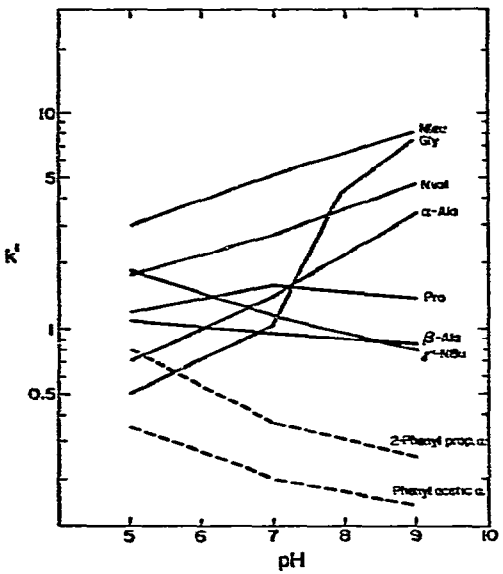


Fig. 3. Influence of pH on retention of L-Dns-amino acids with a metal chelate additive. Conditions: 0.65 m*M* C_3^*-C_8-dien–Zn(II), 0.17 *M* ammonium acetate, acetonitrile–water (35:65, v/v), 30°. Column as in Fig. 1.

The importance of basic pH in the selectivities observed using zinc-chelate additives can be further seen from the elution order of the homologous series of Dns-amino acids: Gly, α-Ala, α-NBu, Nval and Nleu. In this series, the amino acids each differ by one methylene unit and hence elution based on solvophobic retention would predict a monotonic increase in k' from Dns-Gly through Nleu[20,21]. This behavior is borne out in Fig. 4 which shows the retention of the series with typical ion-pair conditions using dodecyltrimethylammonium counterion at pH 5.3 and 9.0. Hydrophobic selectivity is observed in the retention of the negatively charged Dns-amino acid with the positively charged quaternary ammonium ion on the reversed-phase column.

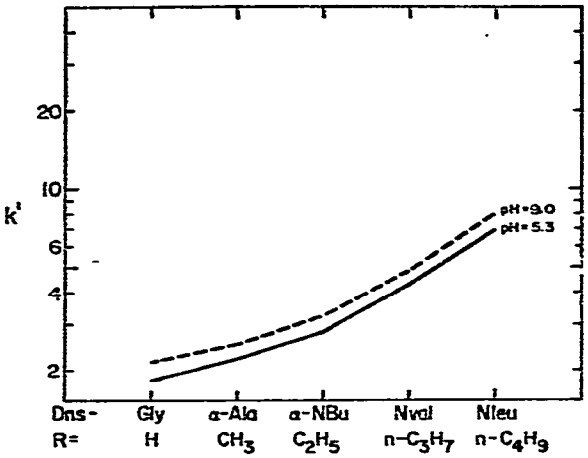


Fig. 4. Influence of pH on retention of L-Dns-amino acids with ion-pair chromatography. Conditions: 1 m*M* dodecyltrimethylammonium bromide, 0.13 *M* ammonium acetate, acetonitrile–water (35:65, v/v), 30°. Column as in Fig. 1.

Fig. 5 illustrates retention of this homologous series with C_2^+-C_8-dien–Zn(II) as counter ion at pH 5, 7 and 9. Separate studies revealed similar pH retention characteristics for C_3^+-C_8-dien–Zn(II). At pH 5 the hydrophobic retention order found with typical ion-pair additives is observed (Fig. 4). Increasing pH, in addition to yielding greater retention, also results in a change in the hydrophobic elution order. Indeed, at pH 9 a marked decrease in retention is found for the series Dns-Gly, α-Ala and α-NBu. The expected hydrophobic retention order is, however, observed for Dns-α-NBu, Nval and Nleu.

The retention trends for the Zn(II) chelate are also observed for Zn(II) addition to the mobile phase. As shown in Fig 6, retention and selectivity are found to change in the presence of Zn(II) as a function of pH; however, the effect is slightly less pronounced. The fact that the Dns-amino acids are retarded rather than accelerated on the reversed-phase column upon addition of Zn(II) at high pH (opposite to that observed in argentation chromatography[22]) may be attributed to increased hydrophobic character of the solute Zn(II) complex over the ionized solute due to charge neutralization. Alternatively, adsorption of the metal complex on silanol groups may in part account for the increased retention.

The change in the hydrophobic elution order in Figs. 5 and 6 for Dns-Gly, α-Ala and α-NBu reveals that the steric environment on the α-carbon influences significantly the complexation of the Dns-amino acids with the metal and metal chelate at high pH. This selective complexation effect must also be associated with chiral recognition, since α values for the D,L-pairs approach unity as the mobile phase pH is reduced from 9 to 7. Below pH 7, most optical isomers of Dns-amino acids are not separated.

Solute structural factors on complexation

The question now arises as to the structural features of the Dns-amino acids important for binding to the metal chelate at basic pH (*i.e.* pH $\approx$ 9). Given the strong

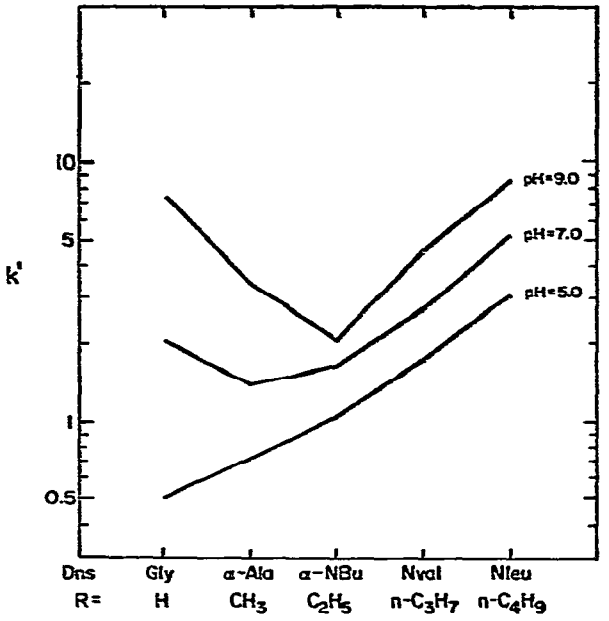


Fig. 5. Influence of pH on retention of L-Dns-amino acids with a metal chelate additive. Conditions: 0.65 m*M* C_2^*-C_8-dien–Zn(II), 0.17 *M* ammonium acetate, acetonitrile–water (35:65, v/v), 30°. Column as in Fig. 1.

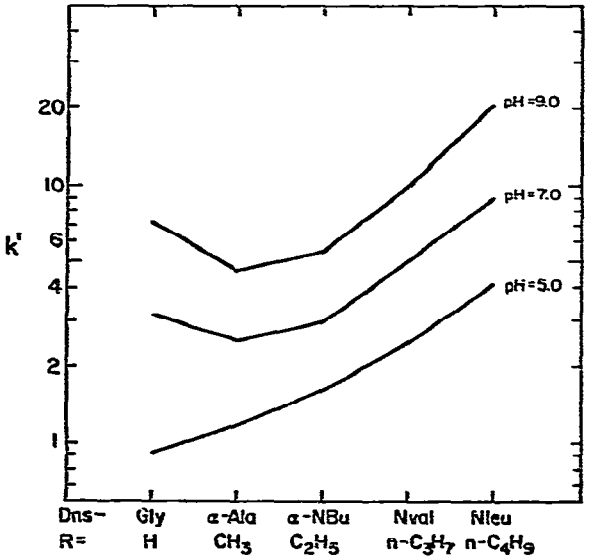


Fig. 6. Influence of pH on retention of L-Dns-amino acids with Zn(II) added to the mobile phase in reversed-phase liquid chromatography. Conditions: 1 m*M* $Zn(CH_3COO)_2$, 0.13 *M* ammonium acetate, acetonitrile–water (35:65, v/v), 30°. Column as in Fig. 1.

dependence of retention and selectivity (including chiral recognition) on pH and ionic species involved, it is clear that complexation via electrostatic interactions must play an important role. One obvious electrostatic site of attachment is the carboxylate anion of the Dns-amino acid. We will present evidence that suggests a second electrostatic interaction resulting from the ionization of the sulfonamide group.

This suggestion may at first seem surprising, given the weak acidity of the Dns-amino acid sulfonamide group[23]. Indeed, separate pH *vs.* retention studies of a number of Dns-amino acids on the bonded C_8 phase revealed that a pK_a value in excess of 10 in general exists for the sulfonamide group under the mixed aqueous-organic mobile phase conditions. However, it is known that the acidity of metals or metal chelates is such that proton loss can be induced from weakly acidic functional groups. Thus, in basic media (pH 9) the Dns-amino acids may act as dianionic species in the presence of dien–Zn(II) chelates.

In order to illustrate this electrostatic effect, we have measured k' as a function of ionic strength for a number of Dns-amino acids at pH 7 and pH 9, and the results are shown in Table II. Here, two ionic strengths have been used which differ by a factor of two. The ratio of k' for the two ionic strengths should theoretically be related to 2^n where n is the charge of the solute species. At pH 7 the Dns-amino acids follow the expected monoanionic behavior (k' ratio of 2) for Dns-Gly, α-Ala and Ser, as does the simpler carboxylic acid, 3-phenylbutyric acid. On the other hand, the diacids Dns-Asp and Glu have a k' ratio of 4, which is expected for dianionic species.

TABLE II

INFLUENCE OF IONIC STRENGTH ON RETENTION OF Dns-AMINO ACIDS AT pH 7 AND pH 9

Salt concentrations 0.065 *M* and 0.13 *M* ammonium acetate. Other conditions as in Fig. 1.

Dns-Amino acid	*k'[0.065 M NH_4Ac]/k'[0.13 M NH_4Ac]*	
	pH 7	*pH 9*
Gly	2.0	4.2
α-Ala	1.8	4.7
Asp	3.9	6.7
Glu	4.0	7.1
Ser	2.0	4.7
Pro	—	2.1
3-Phenylbutyric acid	≈2.0	≈2.0

At pH 9, the k' ratio remains at 2 for the monocarboxylic acid, but now the ratio increases to *ca.* 4 for Dns-Gly, α-Ala and Ser, indicating dianionic behavior. Bidentate attachment of the sulfonamide and carboxylate anions to the dien–Zn(II) chelate can explain this behavior. It is also to be noted that for the diacids the k' ratio increases to *ca.* 7 which is suggestive of a trianionic species. These high k' ratios also mean that care must be exercised in making up the mobile phase in order to maintain good retention reproducibility.

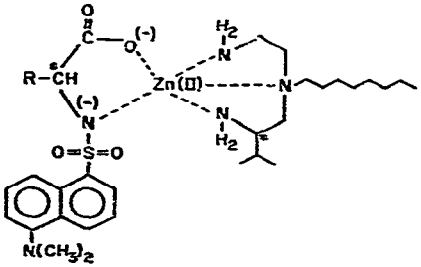
As further evidence for multiple ionization in the presence of the metal chelate, ionic strength effects were found to be pH independent when dodecyltri-

methylammonium bromide was substituted for the metal chelate. Thus, the Dns-monocarboxylic amino acids act as monoanions and the dicarboxylic amino acids as dianions when simple ion-pair chromatography is employed. In agreement with Fig. 4, no special selectivity is found in the association process when simple counter ions are employed.

It is seen in Table II that the k' ratio at pH 9 of Dns-Pro remains at 2. Since proline possesses a secondary amino group, there is no free hydrogen present when the Dns derivative is made. Thus, bidentate attachment via electrostatic interaction with the divalent metal chelate is not possible. Returning to Fig. 3, we observe that Dns-Pro does not bind as strongly to the metal chelate as when amino acids with primary amino groups are used. Here, retention of Dns-Pro does not vary greatly from pH 7 to 9 in contrast to such species as Dns-Gly. This weaker complexation may in part explain the lack of separation of D,L-Dns-Pro when a chiral chelate is employed (Table I).

We also note in Fig. 3 that an important component in bidentate attachment of the Dns-amino acid to the dien–Zn(II) chelate appears to be the relative position of the sulfonamide to the carboxylic acid. When the sulfonamide group is attached to the β-carbon (β-Ala) or the γ-carbon (γ-NBu), retention is nearly independent of changes in pH in this region. In the case of Dns-Gly, β-Ala and γ-NBu the sulfonamide group and the carboxylic acid are separated by 1, 2 and 3 methylene groups, respectively. If chelation proceeds through these groups, the corresponding chelate ring sizes would be 5, 6 and 7 for Dns-Gly, β-Ala and γ-NBu, respectively. It is known that metal chelates yielding 6- and 7-membered rings are less stable than those of the corresponding 5-membered ring complex[24]. However, it is interesting to note that 6-membered ring formation between the metal center and the sulfonamide and carboxylate anions may also influence retention to some extent. This is suggested by the fact that L-Dns-β-Ala (6-membered ring) has a k' value at pH 9 which is 25% larger than Dns-γ-NBu (7-membered ring).

The illustration below indicates a possible structure of the mixed complex dien–Zn (II) with a Dns-α-amino acid.



Zn(II) is generally believed to be tetracoordinated when complexed with dien[25]. However, as to be noted later, Zn(II) can rather easily expand its coordination number and pentacoordination, as shown above, is possible[26]. We shall refer back to the above structure in later discussions.

Returning to the aliphatic α-amino acids, the bulkiness of the group attached to the chiral center would be expected to influence the binding of the Dns-amino

acids to the chelate. The results in Fig. 5 can be understood in these terms with Dns-Gly binding strongest since R = H. This binding strength reduces with Dns-α-Ala (R = CH_3) and Dns-α-NBu (R = CH_2CH_3) beyond which retention increases due mainly to the hydrophobicity of the alkyl substituent. As seen in Table I, the retention of L-Dns-Nval (R = $CH_2CH_2CH_3$) is greater than that of L-Dns-Val (R = $CH(CH_3)$–CH_3). This result may be an indication of bulk effects close to the chiral center influencing the strength of chelate formation.

Consider next the acidic amino acids in Table I (Dns-Asp, Glu and $CySO_3H$). We note that the elution order of D,L-Dns-Asp (R = $-CH_2COOH$) is opposite to that of all other enantiomeric pairs. We have previously shown that at pH 7 Dns-Asp interacts quite strongly with the metal chelate[2]. However, D,L-Dns-Asp is not separated at pH 7 using C_3^*-C_8-dien–Zn(II), and thus it would appear that the sulfonamide anion interaction with the metal chelate at basic pH is an important component in chiral recognition. Besides the 5-membered chelate ring shown in structure I, it is also possible for Dns-Asp to form a 6-membered chelate ring between the metal center, the sulfonamide group and the second carboxylate group. We have already suggested that such a 6-membered chelate may influence retention (*cf.* Dns-β-Ala and Dns-γ-NBu). It may be possible that tridentate attachment of Dns-Asp to the metal center with the formation of a 5- and 6-membered chelate ring is a factor responsible for the reversal in elution order of this enantiomeric pair relative to other Dns-amino acids. It is interesting to note that others also have found a reversed elution order for underivatized Asp relative to most other amino acids[5].

In the case of Dns-Glu (R = $-CH_2CH_2COOH$) the normal elution order (*i.e.* D > L) is observed in Table I. Moreover, the α value is similar to that found for the corresponding aliphatic amino acids, and finally k' is relatively small for both isomers (*cf.* Dns-Asp). These results suggest that the 5 membered chelate ring system shown in structure I may be a predominant interaction for chiral recognition at basic pH. Note that for the other carboxylate group to interact with the metal center, a 7-membered chelate ring involving the sulfonamide group would be required. Such a ring would be expected to be relatively weak. In the case of Dns-$CySO_3H$ (R = CH_2SO_3H), it would appear that the 5-membered chelate ring system (structure I) is also important. We further note the relatively large α values for certain polar D,L-Dns amino acids (*e.g.* Ser, R = $-CH_2OH$; Thr, R = $-CH(OH)CH_3$; Asn, R = $-CH_2CONH_2$) may be due to the selective interaction of the hydroxyl and amide groups with the metal chelate. Obviously, more work is necessary to elucidate further these selective interactions.

We have previously argued that outer-sphere complexation may be an important binding mechanism between solute and metal chelate at pH 7. This model was based in part on the ability of high concentrations of acetonitrile (~25%, v/v) to displace the chelate dye, PADA, from the inner coordination of Zn(II) in the dien–Zn (II) metal chelate at pH 7 (1). We repeated this experiment at pH 9, but based upon the yellow color of the solution, PADA did not appear to complex with the metal chelate. Either ammonia, or more likely hydroxide, prevented PADA complexation at this basic pH, and we were thus unable to draw conclusions with respect to the PADA experiment.

Nevertheless, the fact that chiral recognition increases from near unity at pH 7 to the values shown in Table I at pH 9 and the ionic strength experiments

(Table II) lead one to the reasonable conclusion that inner sphere complexation of the type shown in structure I is occurring at pH 9. With the dien chelating agent, Zn(II) is believed to form a distorted tetrahedral configuration[25]; however, as already noted, Zn(II) is also characterized as a metal which can relatively easily expand its coordination number to 5 or even at times to 6 (ref. 26). Rates of exchange are more rapid when solute binding involves the last coordination sites[27], and this may in part explain the relatively good efficiency at pH 9 (Figs. 1 and 2) in spite of the fact of potential multidentate binding of the solute to the metal chelate. Further studies should elucidate in more detail binding differences between enantiomeric pairs.

Other parameters influencing retention and selectivity with dien–Zn(II)

In addition to examining the role of pH and ionic strength on retention and selectivity of Dns-amino acids, the influence of metal chelate concentration in the mobile phase and temperature of the column was studied. Retention was found to increase nearly linearly with chelate concentration in the region 0.1 m*M* to 5 m*M* C_3^*-C_8-dien–Zn(II). Thus the complexation can be assumed to be a reversible process. Interestingly, although selectivity between the individual L-Dns-amino acids was found to vary, enantiomeric selectivity did not change to a significant extent in this metal chelate concentration region. Due to this behavior and the reasonable retention obtained at *ca.* 1 m*M*, most experiments were conducted at roughly this C_3^*-C_8-dien–metal(II) concentration.

Changes in temperature were found to have a dramatic influence on retention in the region of 30 to 70°. ΔH values were typically found to be *ca.* −7 kcal/mole (*cf.* −3.5 kcal/mole for anisole) which corresponds to a twofold change in k' for a 20° change in temperature. This strong temperature dependence is not surprising considering the complexation processes occurring in the column. Enantiomeric selectivity as calculated using $(\alpha - 1)$ values was typically found to decrease by a factor of two in raising the temperature from 30 to 70°. For the dien–Zn(II) additive, efficiency was not significantly increased at higher temperatures due to the already high efficiency obtained at 30°. For the dien–Zn(II) system, we have therefore chosen to work at 30° in order to take advantage of the higher selectivity. Increasing temperature with other dien–metal additives was found to have a dramatic effect on efficiency as discussed in the next section.

Role of metal ion

A main component of the complexation process occurring with the Dns-amino acids and the dien–Zn(II) chelate is presumed to be an induced proton loss of the sulfonamide group in the presence of the metal chelate additive. From a purely electrostatic standpoint, it follows that a major driving force in the complexation process would then arise from the acidity or charge to radius ratio of the metal ion[2]. Acidity is only one of many metal ion properties that will influence the structural and functional requirements of the solute necessary for specific complexation and chiral recognition. Other metal ions were therefore examined as to their influence on the retention and chiral separation of the Dns-amino acids as a function of pH. In particular, Cd(II), Ni(II), Hg(II) and Cu(II) were selected due to their large dien–metal(II) complexation constants[24].

It is interesting to examine first the elution of Dns-Gly *vs.* α-Ala for the various metal ions as a function of the pH of the mobile phase using C_2^*-C_8-dien–Zn(II). We may presume that if, at a given pH, k' of Dns-Gly $< k'$ of Dns-α-Ala, that hydrophobic retention predominates. On the other hand, from what has been discussed previously, if k' of Dns-Gly $> k'$ of Dns-α-Ala, then specific complexation of the derivatized amino acids may be presumed. For purposes of comparison, we determined the pH at which the k' of the two Dns-amino acids was equal, given the same mobile phase composition. The order of increasing pH was found to be Cu(II) ≈ Ni(II) < Zn(II) < Cd(II). This order follows the decreasing charge to radius ratio or decreasing metal ion acidity. Based on this metal ion order and the fact that the elution order of Dns-Gly and Dns-α-Ala is pH dependent, it would appear that the other metals in the series follow a similar behavior to that of Zn(II). Thus, electrostatic bidentate attachment of the carboxylate anion and the sulfonamide anion arising from the induced proton loss from the acidic metal center would seem to play a role in the complexation of these metals. Therefore, formation of the 5-membered chelate ring, as outlined in structure I, may be presumed to occur. However, because of other properties of the metal ions such as coordination number and structure we may expect differences in retention and selectivity beyond the pH effect.

Table III summarizes a number of separation and detection characteristics of the Dns-amino acids with the various metals. For purposes of comparison, we have included an aliphatic amino acid, Dns-α-Ala, and a polar amino acid, Dns-Thr. We first note that at pH 9 some chiral recognition was achieved in all cases; however, for Cu(II) this was rather low. Second, although Cd(II) and Ni(II) resolved polar Dns-amino acids, poor chiral recognition was found for the aliphatic Dns-amino acids. This behavior may be inherent for these metal ions or alternatively in the case of Cd(II), a higher pH may need to be employed, as this metal ion is less acidic than

TABLE III

INFLUENCE OF METAL ON CHROMATOGRAPHIC CHIRAL SEPARATION

0.8 m*M* C_3^*-C_8-dien–metal(II), pH 9, 30°, flow-rate 2.0 ml/min, acetonitrile–water (35:65, v/v). Column: 15 cm × 4.6 mm, 5-μm Hypersil C_8.

Metal ion	*Dns-amino acid*	$\alpha = k'_D/k'_L$	*UV**	*Fluorescence (%)***
Ni(II)	α-Ala	1.00	+	≈70–90
	Thr	0.42		
Cu(II)	α-Ala	≈1.00	—	≈70–90
	Thr	≈1.00		
Zn(II)	α-Ala	1.75	+	≈100–300
	Thr	2.22		
Cd(II)	α-Ala	1.00	+	≈100–200
	Thr	1.30		
Hg(II)	α-Ala	0.77	limited	≈60–100
	Thr	0.85		

* 254 nm; + = low background absorbance, — = very high background, limited = high background.

** Relative to 100% value with no additives; C_{12}-dien chelate used.

Zn(II). Third, Hg(II) was found to generate chiral selectivity for both polar and non-polar Dns-amino acids. Interestingly, a change in enantiomeric elution order was observed for Ni(II) and Hg(II), relative to Cd(II) and Zn(II).

Efficiency was found to vary significantly with the metal ion studied. As previously reported[2], Zn(II) and Cd(II) yielded good efficiency and peak symmetry. At 30°, Ni(II), produced relatively low efficiency; for Hg(II), a marked improvement in efficiency and band symmetry occurred at 50°. This behavior can be clearly seen in Fig. 7 which shows the separation of D,L-Dns-Ser and D,L-Dns-Thr. Poor resolution is obtained at 30°, whereas almost baseline separation is achieved at 50° in the case of D,L-Dns-Thr.

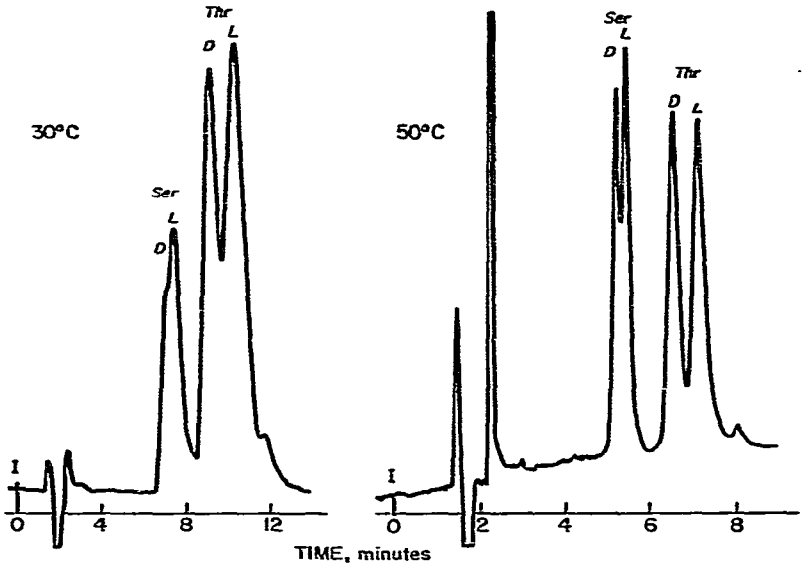


Fig. 7. Influence of temperature on the separation of D,L-Dns-Ser and -Thr using dien–Hg(II). Conditions: 0.8 m*M* C_3^*-C_8-dien–Hg(II), 0.19 *M* ammonium acetate, pH 9.0, acetonitrile–water (40:60, v/v), flow-rate 2.0 ml/min. Column as in Fig. 1. Temperature as indicated. Fluorescence detection used.

In terms of detection, both Cd(II) and Zn(II) can be successfully used at 254 nm, whereas Cu(II) cannot be employed and Hg(II) will find only a limited use due to a high background. With respect to fluorescence, both Zn(II) and Cd(II) can provide signal enhancement, possibly as a result of the complexation of the Dns-amino acid to the metal chelate. On the other hand, Ni(II), Cu(II) and Hg(II) chelates produce decreased fluorescence signals. Thus, when fluorescence is used for detection, Zn(II) has a decided advantage (along with Cd(II)).

L-C_3^*-C_8-dien–Hg(II) may provide a useful complement to the corresponding Zn(II) chelate in that Dns-amino acids invert their elution order with Hg(II). Good separation of the aliphatic and polar Dns-amino acids occurs with Hg(II) as with Zn(II), and thus Hg(II) may be used to check chiral separation on Zn(II). Obviously, another approach to this end is to employ the D-C_3^*-C_8-dien–Zn(II) chelate.

Hg(II) also offers special selectivities in certain cases. For example, Fig. 8 shows the separation of the four Dns-Ileu isomers at 50°, using the chiral dien chelate. This separation could not be achieved with Zn(II) or Ni(II) under conditions studied.

L-C_3^*-C_8-dien–Ni(II) could only be used to separate a few Dns-amino acids, in particular those possessing additional polar or ionic groups such as Dns-Asp, Asn, $CySO_3H$, Ser and Thr. In these cases an elution order opposite to that found for Zn(II) was obtained. In general, poor column efficiencies which could not be rectified by raising the temperature were found. However, in special cases, such as D,L-Dns-Cys (Fig. 9), good selectivity and reasonable efficiency were achieved.

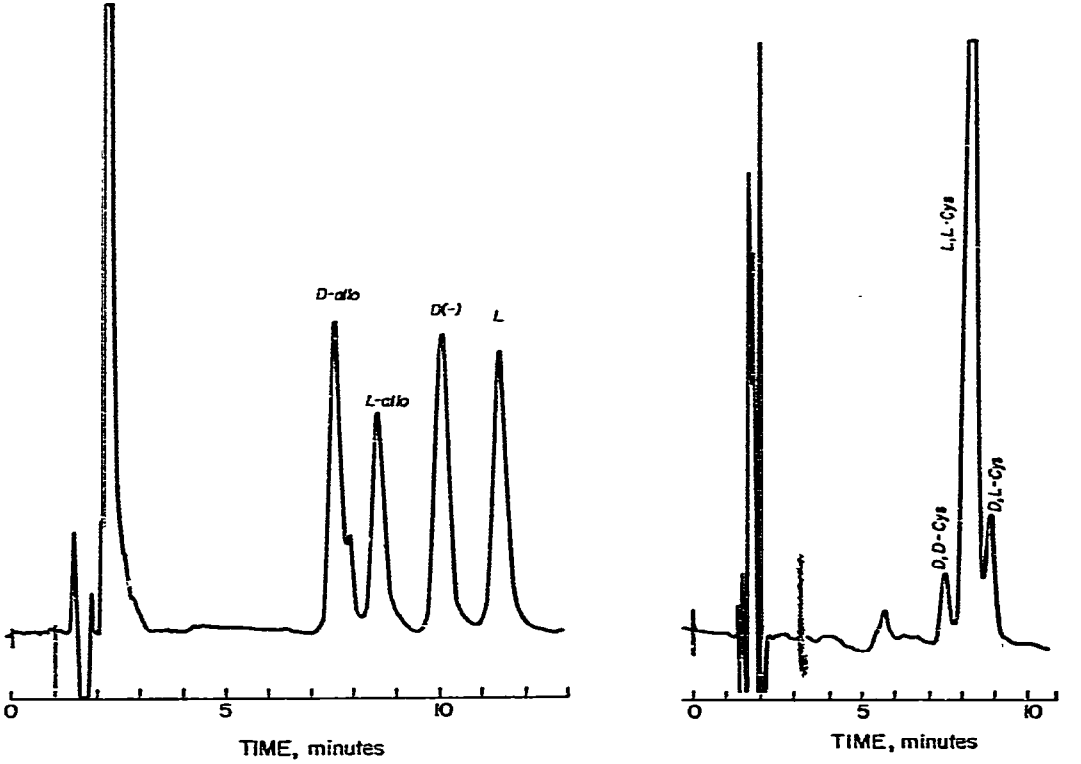


Fig. 8. Separation of the four isomers of Dns-Ileu. Conditions as in Fig. 7 except pH 9.2, flow-rate 1.0 ml/min and temperature 50°.

Fig. 9. Separation of D,L-Dns-Cys. Conditions: 0.8 m*M* C_3^*-C_8-dien–Ni(II), 0.19 *M* ammonium acetate, pH 9.0, tetrahydrofuran–water (35:65, v/v), flow-rate 1.0 ml/min, 30°. Column as in Fig. 1.

The selectivity found with polar Dns-amino acids using the chiral dien–Ni(II) led us to examine D,L-Dns-dipeptides, for which excellent selectivity and good column performance were obtained. This is shown in Fig. 10 in the separation of Dns-glycyl-D,L-amino acid dipeptides. Table IV presents retention and relative retention data for a number of the above dipeptide types along with several D,L-Dns-amino acid-Gly-dipeptides, using L-C_3^*-C_8-dien–Zn(II) and Ni(II). In all cases, Ni(II) achieved higher retention and relative retention values, with an inversion in elution

order. The use of the corresponding Cd(II) and Hg(II) chelates also yielded enantiomeric selectivity for most of the Dns-dipeptides, with chiral recognition intermediate between Zn(II) and Ni(II). Thus, while the Zn(II) chelate exhibited the highest separation of all the metals for the Dns-amino acids, it produced the lowest selectivity in the case of the Dns-dipeptides. Clearly, the coordination number and structure of the metal play a significant role in the results.

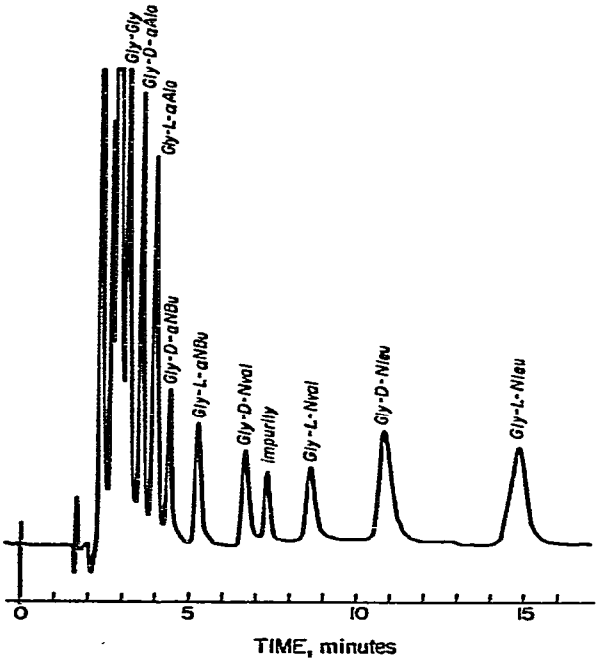


Fig. 10. Separation of Dns-glycyl-D,L-amino acid dipeptides. Conditions: 0.8 m*M* C_3^*-C_8-dien–Ni(II), 0.19 *M* ammonium acetate, pH 9.0, acetonitrile–water (35:65, v/v), flow-rate 1 ml/min, 30°. Column as in Fig. 1.

Finally, it is interesting to note that in the case of dien–Zn(II), Dns-Gly-D,L-α-Ala for both isomers had greater retention than D,L-Dns-α-Ala-Gly, and likewise, Dns-Gly-D,L-Leu for both isomers was longer retained than D,L-Dns-Leu-Gly. This result may be further evidence of the importance of the 5-membered chelate rings possibly forming between the sulfonamide, amide and carboxylate groups. Since Gly is smaller than either α-Ala or Leu, the dansyl group will tend to bind tighter when Gly is the amino acid that is derivatized. In summary, the results in Tables III and IV reveal that metal ion variations can be powerful tools for control of retention and selectivity.

Applications

The high α values of the D,L-Dns-amino acids with C_3^*-C_8-dien–Zn(II) will permit the convenient determination of D,L ratios of 1 to 5000 for most enantiomeric pairs. Thus, this approach can be used as a sensitive and rapid screening procedure for the assessment of optical purity of free amino acids. One need only mix an excess

of Dns chloride in acetonitrile with a freshly buffered sodium bicarbonate solution (pH 9.5) of the amino acid. After roughly 5 min at room temperature the reaction solution can be directly injected onto the reversed-phase column containing the chiral metal chelate additive. Fig. 11 shows an example in the analysis of D,L-Dns-α-Ala on a 5-cm column in less than 1 min with good removal of the dansylation byproducts. In this analysis it is not necessary for the dansylation reaction to go to completion, as one is simply interested in the ratio of the two isomers (*i.e.* the optical purity of a particular isomer). Other studies show that the optica purity of BOC-amino acids can also be rapidly and conveniently determined by this procedure following cleavage of the BOC group. It should also be pointed out that through the use of short columns rapid changes in mobile phase conditions and reequilibration can be achieved ($\approx$ 10 min). Thus, the metal chelate or metal ion can be easily changed either to invert elution order or to achieve a separation not possible with a particular additive.

In order for the above procedure to be valid, it is necessary that racemization in the dansylation reaction be negligible. We have studied the extent of racemization in the formation of the Dns derivative and its subsequent stability for a number of optically pure free L-amino acids. Since basic conditions are known to lead to racemization of free amino acids, after the amino acid was dissolved in the buffer solution (pH 9.5), it was immediately mixed with the Dns chloride reagent solution. The reaction was then allowed to proceed for 12 h at room temperature. The derivatized amino acids were then separated using the chiral dien–Zn(II) additive. No detectable amount of the D-isomer (<0.02%) could be observed for the following L-amino acid enantiomers: Dns-α-Ala, Val, Leu, Ileu, Thr, Asn, Phe, Met, α-NBu, Glu, and Asp. In the case of Dns-Ser, 0.4% of the D-isomer was found. It is not clear whether this was the result of racemization during reaction or whether the starting material was optically pure. (Ser is known to be susceptible to racemization[28].) This result needs further checking. In any event, little or no racemization occurs during or after dansylation, and thus the approach illustrated in Fig. 11 is valid for rapid assessment of optical purity of starting materials.

That racemization does not appear to occur to any significant extent can be important for several other reasons. First, one need not be concerned with racemization during solute travel through the reversed-phase column, so that what is injected will be faithfully recorded at the detector. Second, this approach may be used for racemization of peptide end group amino acid analysis[29]. In this case dansylation of the N-terminal group is first allowed to take place. Hydrolysis of the peptide bonds is next performed, followed by separation and detection of the dansyl amino acid. Recent work has shown that no racemization occurs during N-terminal amino acid analysis by this approach.

Up to this point we have discussed potential applications for which only the D,L ratio of the amino acids need be determined. Obviously, quantitation of amino acids is also of general interest. As has been pointed out in the past[30–32], difficulties have been found in the quantitative conversion of some free amino acids to their Dns derivatives, as a consequence of a side reaction of excess Dns chloride with the carboxylic group, leading to degradation of the Dns-amino acids. We have recently been able to achieve quantitative reaction of all Dns-amino acids by some changes in reaction conditions. Thus, a quantitative amino acid analysis by precolumn derivati-

TABLE IV

CHIRAL SEPARATION OF DIPEPTIDES

0.8 m*M* C_3^*-C_8-dien–metal(II), 0.17 *M* ammonium acetate, pH 9, 30°, acetonitrile–water (35:65, v/v).

Solutes (Dns derivatives)	*Zn(II)*		*Ni(II)*	
	k'	*α*	*k'*	*α*
Gly-L-α-Ala	1.2	0.92	1.35	1.15
Gly-D-α-Ala	1.3		1.15	
Gly-L-α-NBu	1.6	0.94	2.35	1.3
Gly-D-α-NBu	1.7		1.8	
Gly-L-Nval	2.5	0.94	6.35	1.4
Gly-D-Nval	2.65		3.15	
Gly-L-Nleu	4.1	0.94	8.1	1.4
Gly-D-Nleu	4.35		5.7	
Gly-L-Val	2.5	0.96	3.7	1.55
Gly-D-Val	2.6		2.35	
Gly-L-Leu	3.95	0.94	8.45	1.5
Gly-D-Leu	4.2		5.7	
Gly-L-Met	2.2	0.96	4.05	1.35
Gly-D-Met	2.3		3.0	
Gly-L-Thr	1.1	0.92	1.3	1.3
Gly-D-Thr	1.2		1.0	
L-Ala-Gly	0.8	0.94	—	—
D-Ala-Gly	0.85			
L-Leu-Gly	2.0	0.95	5.2	1.25
D-Leu-Gly	2.1		6.15	

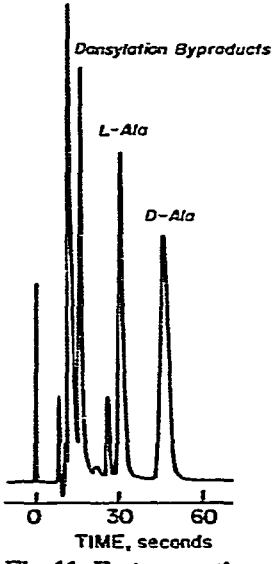


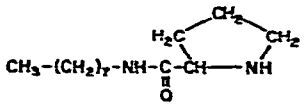
Fig. 11. Fast separation of D,L-Dns-α-Ala with metal chelate additive. Conditions as in Fig. 1 except 0.5 m*M* C_3^*-C_8-dien-Zn(II). Column: 5 cm × 4.6 mm, 5-μm Hypersil C_8.

zation with Dns chloride can be realized. A second column for enantiomeric separation could then be coupled to the first column in order to obtain the quantitation of all amino acid enantiomers. Using UV detection, picomole analysis is possible and with fluorescence, subpicomole analysis should be achievable. This work will be reported elsewhere.

CONCLUSIONS

The use of chiral metal chelate additives to the mobile phase in reversed-phase liquid chromatography has been shown to be a powerful tool for the separation of D,L-Dns-amino acids and dipeptides. By manipulation of pH, ionic strength, metal ion and temperature, a good control on separation can be achieved. This work can be extended in many directions.

We have recently begun to explore other metal chelate additives. One particularly interesting chiral additive is L-proline-*n*-octylamide (L-Pro-C_8 amide):



which with a stoichiometric amount of Ni(II) is able to separate some free amino acids as well as Dns-amino acids, as shown in Fig. 12. Note that the elution order of

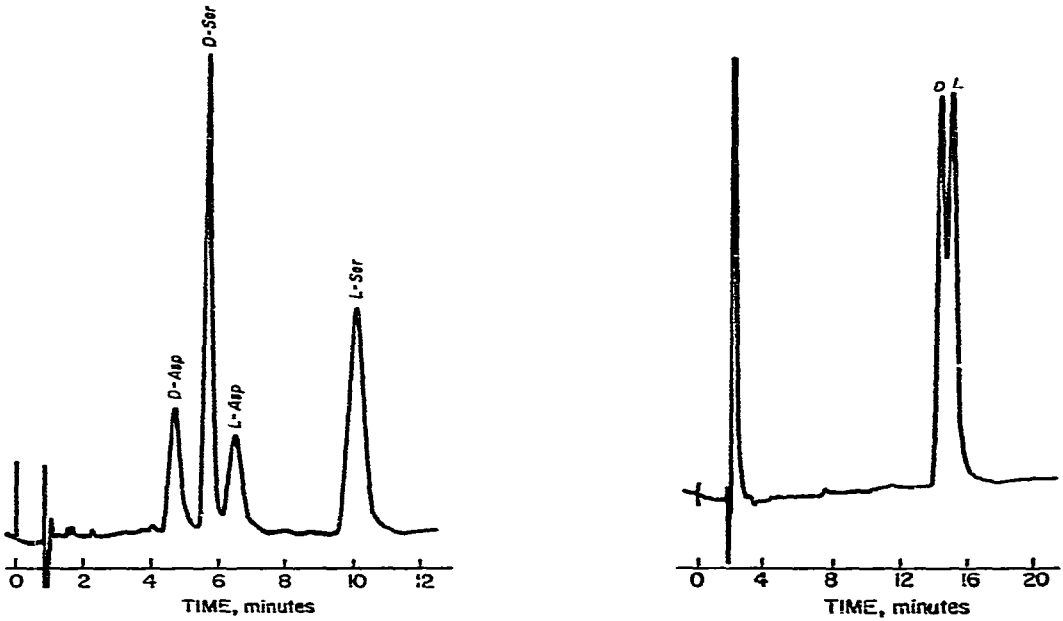


Fig. 12. Separation of D,L-Dns-amino acids with L-Pro-C_8-amide Ni(II). 0.13 *M* ammonium acetate, pH 9.0, methanol–water (35:65, v/v), flow-rate 2.0 ml/min, 30°. Column as in Fig. 1.

Fig. 13. Separation of D,L-Dns-Pro using L-Pro-C_8-amide Ni(II). Conditions and column as in Fig. 12 except flow-rate 1.0 ml/min.

Dns-Asp is the same as that of the other amino acids, such as Dns-Ser. Not all Dns-amino acids to-date have been separated by this metal chelate; however, Dns-Pro is resolved into its enantiomers (see Fig. 13). Thus, various metal chelate additives may be used to achieve particular separations. Work is continuing on the study of metal chelate additives for the resolution of optical isomers.

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