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PRACTICAL CONSIDERATIONS IN THE CHIRAL SEPARATION OF Dns-AMINO ACIDS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY USING METAL CHELATE ADDITIVES*

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SUMMARY

Practical aspects of the analysis of amino acids are addressed in this paper. Conditions for the resolution of enantiomers of all the common amino acids as their Dns derivatives using the chiral mobile phase additive: L-prolyl–*n*-octylamide–Ni(II) are reported. Variable D,L selectivity is observed, while maintaining good column efficiency. L-prolyl–*n*-dodecylamide–Ni(II) is found to distribute on the surface of a *n*-alkyl-bonded stationary phase and may act as a chiral immobilized phase. The separation of all the common Dns-amino acids within ≈35 min using reversed-phase conditions and a linear gradient is presented. Approaches to enantiomeric composition analysis of amino acids in a mixture are discussed, and a coupled column strategy linking the two separations reported above is proposed. In the combined system amino acid analysis is performed in the first part, with switching of individual amino acids of interest to the second part of the system for D,L-enantiomeric composition determination.

INTRODUCTION

The resolution of enantiomers by liquid chromatography (LC) is currently of great interest[1–4]. Much of the effort has focussed on the use of ligand-exchange chromatography for the resolution of amino acids. Of particular interest have been the studies of Davankov and Kurganov[4,5] in which cross-linked organic resins were modified by appropriate chiral ligands and Cu(II) was employed as the complexing metal. A similar approach has also been used by other workers for the ligand-exchange separation of enantiomers[6–8]. Recently, phases in which the chiral ligand has been bonded to small particle silica gel have been described[9–13]. Chiral bonded phases without the use of metal ions have also been successfully employed[14,15].

    An alternative approach for achieving chiral resolution involves the addition of the chiral metal chelate to the mobile phase. The latter approach, in the reversed-phase mode, was used by this laboratory for the resolution of Dns-amino acids (Dns-AAs)[16,17]. Free amino acids have been resolved on an ion-exchange resin[18] and in the reversed-phase mode[19-22]. The separation of optically active Co(III) complexes using tartaric acid salts added to the mobile phase was also reported[23].

    Using either the chiral bonded phase or the chiral additive approach, high chiral recognition is achieved for a number of D,L-amino acids (free or derivatized) with optically active metal chelate systems. Both approaches would appear to have a role to play in chiral separations. In general, the bonded-phase method would find greater value in preparative separations, since the mobile phase tends to be less complicated than with the additive approach. The bonded-phase systems are often characterized by relatively poor efficiency (however, see ref. 5) and may be less flexible in terms of manipulating conditions (and chiral resolving agents) for optimizing separations. The additive method would appear to be more associated (but not exclusively) with analytical-scale separations, as a consequence of greater flexibility with respect to the selection of chiral resolving agents and generally better efficiency.

    A new approach intermediate between the above two methods involves immobilizing a surface active metal chelate to the bonded phase and having little or no chelate in the mobile phase. This method has briefly been studied in ion-pair chromatography[24,25]. In this paper we will show some initial results using this approach for the D,L separation of Dns-AAs.

    In our previous work a chiral triamine–Zn(II) system for the resolution of Dns-AA was employed. Good resolution of all common Dns-AAs except proline was achieved[17]. In this paper, we present results with a new chelate system L-prolyl–*n*-alkylamide–Ni(II). Besides offering differing selectivities from our previous results, we are now able to achieve the resolution of all common Dns-AAs. Moreover, the simple synthetic approach to this chelate permits the facile variation of the structure of the system (*e.g.*, alkyl chain, chiral center, etc.), for the manipulation of the chiral separation.

    In order to be able to use a given resolution method of amino acids with "real" samples, some practical matters need to be considered. Besides the obvious desire to have a system able to resolve all the common amino acids (or other amino acids according to needs) with good efficiency and peak symmetry, one wants a system with sufficient flexibility for the D,L separation of several, up to all, amino acids in a given mixture in one run. Since achiral hydrophobic retention and chiral selectivity are variable from one amino acid to another, the potential of overlap of peaks from a given isomer of one amino acid to that of another is high. Moreover, LC is the method most often used as an achiral amino acid analyzer[26]. Therefore, we have developed a column switching procedure which permits the reversed-phase separation of a mixture of the common protein amino acids as the Dns derivatives on the first column, followed with appropriate switching, by the chiral resolution on the second column.

    In this paper we will present the principles and some practical details of this approach. The method involves an amino acid analyzer with a chiral separation module. By this procedure one can analyze the optical isomer composition of as many amino acids as desired.

EXPERIMENTAL

Equipment

Modular LC systems were used in this work. The pumps were Altex Model 100 (Berkeley, CA, U.S.A.) and Waters Assoc. M6000A (Milford, MA, U.S.A.); the injectors were Rheodyne Model 7120 and Model 7105 (Berkeley, CA, U.S.A.); and the switching valve used in the coupled column experiment was a Rheodyne Model 7000 six-port valve. The detectors were Laboratory Data Control Model 1205 V, 254 nm UV monitor (Riviera Beach, FL, U.S.A.) and Schoeffel Model FS 970 spectrofluoro monitor (Westwood, NJ, U.S.A.). The gradient system consisted of a Waters Assoc. Model 660 solvent programmer and two M6000A pumps. The columns were thermostated with a Haake water thermostat and circulator (Evanston, IL, U.S.A.). Chromatograms were recorded with a Hewlett-Packard Model 3385 A automation system (Avondale, PA, U.S.A.) or with a Linear Instruments Model 255 recorder (Irvine, CA, U.S.A.). pH values were determined using a Beckman Model 3500 digital pH meter (Irvine, CA, U.S.A.).

High-performance LC columns

Columns from various sources were used in this work. Some separations were performed with commercially available Supelco (Bellefonte, PA, U.S.A.) Supelcosil LC-18 or LC-8 150 × 4.6 mm I.D., (5 μm) columns. Other columns in various lengths were prepared in our laboratory using chemically bonded Hypersil 5-μm particles (Shandon Southern, Sewickey, NJ, U.S.A.) and conventional slurry packing techniques. In order to increase column lifetime at the high pH values used in this work, a 5-cm precolumn containing the bonded-phase packing was placed before the injector.

Reagents and solvents

Free and Dns-derivatized amino acids, CBZ-amino acids and Dns chloride (Dns-Cl) were obtained from either Sigma (St. Louis, MO, U.S.A.) or Pierce (Rockford, IL, U.S.A.). Other reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.). LC-grade solvents were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). $Ni(AcO)_2 \cdot 4H_2O$ was a Fisher certified reagent, Fisher Chemical (Fair Lawn, NJ, U.S.A.).

A stock solution of all the D,L free amino acids was prepared by dissolving the carefully weighed amino acids in 0.1 *N* hydrochloric acid to provide a concentration of 2.5 μmol/ml. These stock solutions were stored in the freezer. A given total amount of the amino acid mixture was taken by volume into a vial and evaporated to dryness. The dry sample was dansylated according to the procedure described below.

For the chiral separation studies, commercially available Dns-AAs were used when possible (Sigma and Pierce). Each of the enantiomers was injected individually to ascertain the correct assignment. In those cases where the Dns derivatives were not commercially available, and for the standard mixture of D,L-amino acids, a Dns-derivatization procedure which has been developed in this laboratory[27] was employed. A 1-ml volume of Dns-Cl solution [1.5 mg (5.56 μmol)/ml, in acetonitrile] was added to the free amino acid sample dissolved in a vial in 2 ml of 40 m*M* Li_2CO_3 buffer (pH 9.5 with hydrochloric acid), gently shaken for 2 min and then allowed to stand at room temperature in the dark. In case of the D,L standard mixture the reaction

was terminated after 35 min by adding 100 μl of a 2% methylamine hydrochloride solution in water.

Mobile phase preparation

Buffered mixtures of organic modifier and water were prepared by adding a given amount of glacial acetic acid to the appropriate amount of organic modifier, adding 90% of the water portion of the mixture, adjusting the pH to the desired value with aqueous ammonia, and then filling to the mark with an additional amount of water. The chiral additive and the metal salt were weighed on an analytical balance and added to the appropriate amount of the buffered mixture. Buffer concentrations were calculated based on the acetic acid added. Mobile phases were degassed with helium prior to use.

Preparation of L-prolyl–n-octylamide

The L-prolyl–n-octylamide was prepared in two steps. First, CBZ-L-proline (7.5 g, 30 mmoles) was dissolved in 100 ml of dry tetrahydrofuran (THF). The solution was cooled to $-20°C$ and N-methylmorpholine (3.3 ml, 30 mmoles) was added, followed by slow addition of isobutylchloroformate (4.35 ml, 33 mmoles). After several minutes, freshly distilled n-octylamine (5.28 ml, 32 mmoles) was added. The mixture was stirred for 15 min at $-20°C$ and allowed to warm to room temperature. The reaction mixture was filtered to remove the N-methylmorpholine hydrochloride salt and the filtrate stripped of THF. The resulting oil was taken up in ethyl acetate and extracted three times with a cold solution of 5% sodium hydroxide in water, three times with a cold 5% tartaric acid solution in water, and then once with water. The organic layer was dried over sodium sulfate and subsequently stripped of ethyl acetate. The resulting oil was dried under vacuum to produce a low melting white solid. The yield of CBZ-L-prolyl–n-octylamide was 95%. Nuclear magnetic resonance (NMR): (C^2HCl_3) δ 7.25 (s, 5H), 5.10 (s, 2H), 3.0–4.5 (m, 6H), 1.8–2.4 (m, 4H), 1.25 (s, 12H), 0.8–1.0 (m, 3H). Infrared (IR): 3300, 2930, 2860, 1740, 1700, 1660, 1550, 1440, 1300, 1125, 775 cm^{-1}.

The second step involved dissolving the CBZ-L-prolyl–n-octylamide (9.0 g) in ≈ 100 ml of methanol to which a small amount of Pd/C 10% was added and hydrogenating the mixture overnight at room temperature at ≈ 40 p.s.i. The catalyst was removed by filtration and the solvent evaporated. A clear oil was obtained with a yield of 88%. NMR: (C^2HCl_3) δ 2.8–4.0 (m, 6H), 1.6–2.2 (m, 4H), 1.3 (s, 12H), 0.8–1.0 (m, 3H). IR: 3320, 2930, 2860, 1660, 1525, 1465, 1420, 1370, 1250, 1100 cm^{-1}.

The optical purity of the n-propylalkylamide was determined using the standard $6 N$ HCL hydrolysis procedure with a subsequent Dns derivatization of the resulting proline and injection onto the chiral separation column. No D-proline was observed.

RESULTS AND DISCUSSION

Separation of D,L-Dns-AAs with L-Pro–C_x–amide–Ni(II)

In a previous paper[17] we briefly reported on the use of L-prolyl–n-octylamide–Ni(II) for the separation of D,L-Dns-AAs. We have explored further the use of this metal chelate and have obtained conditions to resolve all of the common amino acids

TABLE I

CHIRAL SEPARATION OF Dns-AMINO ACIDS

Conditions: 4 mM L-Pro–C$_8$–Amide–Ni(II), 0.088 M ammonium acetate, pH 9, methanol–water (60:40), 25°C. Columns: 60 × 4.6 mm or 150 × 4.6 mm, 5-μm C$_8$ Hypersil.

Dns-amino-acid	k'	$\alpha(k'_L/k'_D)$	Dns-amino acid	k'	$\alpha(k'_L/k'_D)$
D-Pro*	36.3	1.03	D-Nval	13.0	1.25
L-Pro*	37.3		L-Nval	16.2	
D-Met-sulfone*	25.7	1.75	D-Nleu	20.6	1.28
L-Met-sulfone*	45.1		L-Nleu	26.4	
D-Arg*	2.5	1.24	D-Met	12.3	1.48
L-Arg*	3.1		L-Met	18.2	
D-Asn*	12.0	1.23	D-αNBu	7.8	1.23
L-Asn*	14.7		L-αNBu	9.6	
D-Val*	50.3	1.05	D-Gln	3.1	1.32
L-Val*	52.9		L-Gln	4.1	
D-Ser	8.5	3.48	D-Ileu	15.56	1.08
L-Ser	29.6		L-Ileu	16.80	
D-Leu	19.3	1.22	D-Cit	3.1	1.19
L-Leu	23.5		L-Cit	3.7	
D-Ala	7.3	1.82	D-Phe**	4.6	2.24
L-Ala	13.3		L-Phe**	10.3	
D-Asp	9.6	0.69	D-Trp**	4.5	2.49
L-Asp	6.6		L-Trp**	11.2	
D-Thr	3.9	1.51	D-His**·***	10.4	1.75
L-Thr	5.9		L-His**·***	18.2	
D-Glu	19.8	1.21	D-Lys**·***	7.9	1.20
L-Glu	24.0		L-Lys**·***	9.5	
D-CySO₃H	18.0	0.92	D-Tyr**·***	18.7	1.71
L-CySO₃H	16.5		L-Tyr**·***	32.0	
			D-Orn**·***	7.8	1.20
			L-Orn**·***	9.4	

* Methanol–water (50:50), all other mobile phase conditions the same as above.
** Methanol–water (70:30), all other mobile phase conditions the same as above.
*** Di-Dns derivatives.

values for the D,L-Dns-AAs. A representative chromatogram is shown in Fig. 1. It is noted that as with the dien-Zn(II) system[17], this metal chelate affords in most cases high chiral recognition while also maintaining reasonable column efficiency. Under into their enantiomers. Table I presents the retention (k') and relative retention (α) the given set of conditions the α values range from 1.03 for D,L-Dns-Pro to 3.48 for D,L-Dns-Ser. The low value for D,L-Dns-Pro may result in part from the weak complexation of this amino acid to the metal chelate system[17] as well as from the significant hydrophobic retention under the column operating conditions. Through an understanding of the basic chromatographic retention and separation processes[28] selectivity can be optimized based on variation of pH, buffer concentration and
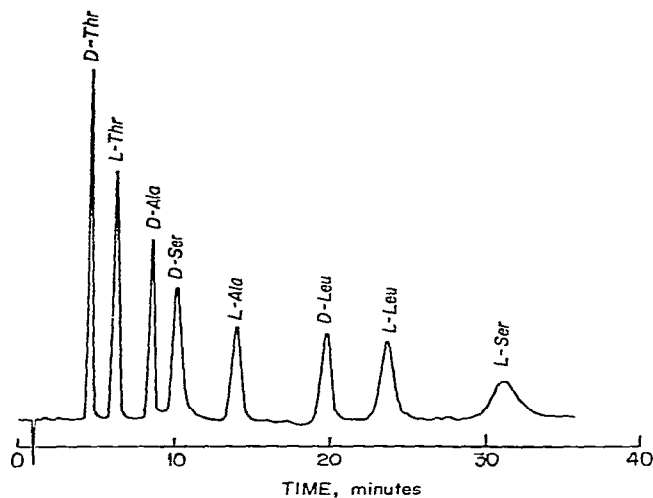
Fig. 1. Separation of D,L-Dns-AAs. Conditions: $4 \cdot 10^{-3}$ M L-prolyl–n-octylamide–Ni(II), $8.75 \cdot 10^{-2}$ M ammonium acetate, pH 9.0, methanol–water (60:40), flow-rate 2.0 ml/min., 25°C. Column: 15 cm × 4.6 mm, 5-μm C$_8$ Hypersil.

mobile phase composition and higher α values can be found in many cases, including for Pro.

It is interesting to compare the elution order in this system with that found for the dien-Zn(II) system[17]. In all cases in Table I, except for several acidic amino acids, the D isomer elutes before the L isomer, which is opposite to that found for the dien-Zn(II) system. However, substitution of Ni(II) for Zn(II) in the dien system did lead to an elution sequence of D before L. Whereas only D,L-Dns-Asp involved reversal of elution order for the dien-Zn(II) system, in Table I both D,L-Dns-Asp and D,L-Dns-CySO$_3$H are inverted*. Evidently, in the L-prolyl–n-octylamide–Ni(II) system the weaker ligand –CySO$_3^-$ is able to interact within the complex in a similar fashion as the stronger –CO$_2^-$ ligand on aspartic acid. Interestingly, in both chelate systems D,L-Dns-His elutes in the same order as the non-polar amino acids whereas reversal is found for His where it is a free amino acid[4,21]. Evidently, the Dns-derivatization of the imidazole nitrogen in His minimizes the binding of the imidazole ring to the metal chelate, thus preventing inversion in elution order.

Long-chain ion-pairing agents are known to adsorb to a significant extent on the bonded phase, leading to a dynamic ion-exchange mechanism of retention[24]. We have obtained data that indicates that this also takes place with the metal chelate system. L-Prolyl–n-dodecylamide–Ni(II) is distributed on the surface of a n-alkyl-bonded stationary phase and may act as a chiral immobilized phase. The equilibration of the metal chelate with the reversed-phase column based on the retention and relative retention variation of D,L-Dns-Ser, was examined and the results are shown in Fig. 2. An increase in k' for both Dns-AA enantiomers with the number of column volumes of mobile phase containing the metal chelate passing through the column is observed. It is also seen in Fig. 2 that α increases in a non-linear fashion as the chelate

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* In ref. 17, the elution order of D,L-Dns-Asp was incorrectly given as D before L.

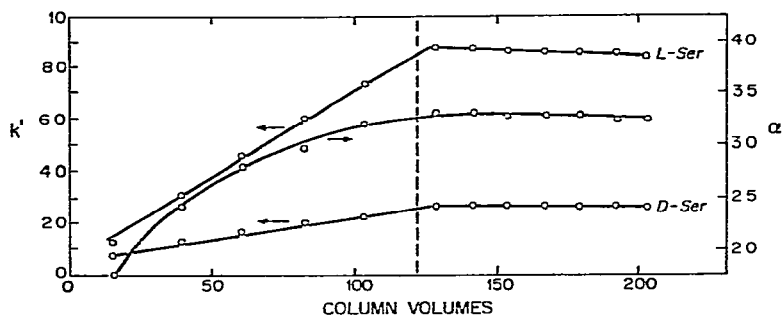Fig. 2. Change in k' and α of D,L-Dns-Ser as a function of addition of $3.6 \cdot 10^{-3}$ M L-prolyl-n-dode-cylamide–Ni(II) to the mobile phase. Conditions, same as Fig. 1, except column: 15 cm $\times$ 4.6 mm, 5-μm C_{18} Hypersil. At the dashed line, the mobile phase is switched to one containing no chiral chelate ($3.6 \cdot 10^{-3}$ M Ni(II) was present).

is loaded onto the support. This may be a direct result of the finite hydrophobic retention of D- and L-Dns-Ser under the mobile phase conditions without chelate present. As the chelate is loaded onto the support, the achiral hydrophobic retention process is decreased. When α levels off, the hydrophobic retention process contributes only to a negligible extent to the overall retention. Full mechanistic details will be published separately[28].

Assuming that retention is based mainly on ligand exchange with the adsorbed metal chelate in the system described above, it was interesting to examine what would happen if the chelate was removed from the mobile phase. The experimental findings are shown in Fig. 2. After passing approximately 125 column volumes of mobile phase, the chelate was removed, leaving all other conditions the same. Note that Ni(II) was maintained in the mobile phase at a concentration of $3.6 \cdot 10^{-3}$ M.

It can be seen that the column appears to be relatively stable up to at least 200 column volumes. The constancy further confirms the importance of the ligand-exchange step on the stationary phase. The results are a natural consequence of the slow rate of desorption of the metal chelate, as found in other ion-pair systems[24]. Indeed, by reducing the organic modifier content of the mobile phase, the chelate may remain immobilized for long periods of time. This immobilized layer approach has been briefly employed in ion-pair chromatography[24] and is an approach of potential utility in ligand-exchange systems[29]. Secondly, one can change chelates on the column at will for either alternative selectivity or for regenerating a stationary phase that has been chemically degraded. Thirdly, the metal may be released from the adsorbed chelate by manipulating the mobile phase, followed by reloading of another metal. More data on this approach will be presented at a later date.

Practical achievement of chiral separation of amino acids using coupled columns

Table I reveals that the Dns-AAs have a broad range of retention. Moreover, because of the large variation in α values, overlaps of chromatographic bands from an enantiomer of one amino acid with that from another amino acid will occur.

If one desires to have an analytical method which has the potential to resolve all D,L-amino acid pairs in one sample, two approaches appear possible. In one case a column could be constructed which has the capability to resolve over 50 components in one run. Based on current efficiencies possible with ligand-exchange chromato-

graphy, quite long columns would be required. This would mean relatively high-pressure operation and analysis times of some hours, even with gradient elution.

The second approach, which we have adopted, involves dividing the analysis into two separate steps. Here, the Dns-AAs are separated on a reversed-phase column in a conventional manner, and subsequently, individual peaks are samples for separation on a second column consisting of the chiral additive. A schematic diagram of this apparatus is shown in Fig. 3. This approach has the advantage of high flexibility allowing any number of amino acids to be analyzed for their D,L ratios on one sample, depending on the needs of the analysis. Moreover, separate steps for both amino acid and D,L ratio analysis means that the system can operate as a routine amino acid analyzer and a separate module for the chiral separation can be added as appropriate.
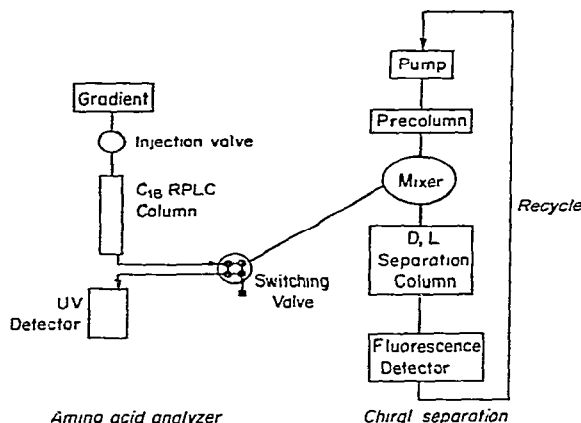

Fig. 3. Schematic diagram of an amino acid analyzer with a combined chiral separation module. Specific details are given in text.

It is interesting to note that the single-column approach is taken in the gas chromatographic (GC) analysis of D,L-amino acids using capillary columns[30]. While this method is valuable, amino acid analysis has generally been performed by LC, one reason being a lesser number of chemical steps prior to injection of the sample. This is especially true as one analyzes complex sample systems (e.g., broths). Moreover, as a consequence of the chemistry involved in the derivatization steps in GC, both Gln and Asn cannot be directly analyzed as their D,L pairs.

Let us now consider the various components of the coupled column arrangement shown in Fig. 3. On the left hand side, a standard LC gradient system can be set-up for the separation of the Dns-AAs using a chemically bonded reversed-phase column. If this amino acid analyzer is to be used successfully, and especially in the coupled column system, then several criteria must be met. First, the derivatization step must yield accurate quantitative results. Secondly, the chromatographic separation must be reproducible from run to run and over a period of time. In addition, column-to-column reproducibility should be good.

Consider first the quantitation steps. A separate paper will discuss this issue in full[27]. Here we will only summarize the main points. For compatibility with reversed-phase chromatography, we converted from the more standard derivatization conditions[31,32] using acetone, to one with two parts aqueous buffer to one part aceto-

nitrile. We have used pH 9.5 and Li_2CO_3 buffer. In order to standardize reaction conditions, we quench the reaction with a primary amine after a set time (35 min). This amine is selected such that it does not interfere in the chromatogram.

In this fashion, we have been able to achieve high yield-reproducible analysis of all the amino acids independent of the ratio of Dns-Cl to amino acid. Moreover, Trp, His, Lys, Tyr, and Orn are converted to the di-Dns derivatives under these conditions.

Consider next the separation of the Dns-AAs on the reversed-phase column. Others have published on this separation using both normal-phase and reversed-phase LC[33-36]. One obviously requires in the coupled-column approach a reproducible and reliable separation. Fig. 4 shows a separation in roughly 35 min of a mixture of all the common Dns-AAs including Dns-sulfonic acid, Dns-amide and Dns-methylamine (from the quencher). A linear gradient has been used to permit good run-to-run reproducibility and to allow easy transfer of the method from one LC system to another.
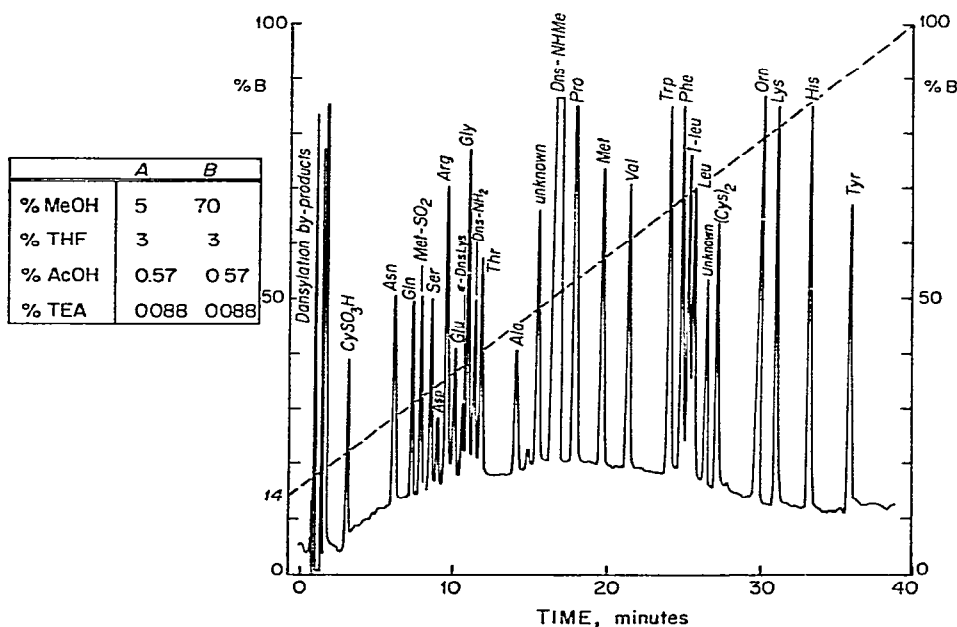

Fig. 4. Reversed-phase separation of Dns-AAs on a 5 μm C_{18} Hypersil column, 15 cm $\times$ 4.6 mm. Gradient conditions given in the figure. AcOH = acetic acid, TEA = triethylamine. See text for further details.

The linear gradient consisted of a variation in percentage of methanol in the mobile phase with a small amount of THF and an acetic acid buffer at pH 4.0 in both the A and B solvents. A trace of triethylamine (TEA) was also added to minimize the effect of any accessible silanol groups present on the bonded phase surface. For improved efficiency and selectivity the column was thermostatted at 50°C. In this separation a UV detector has been used for picomole analysis. With a fluorescence detector we have been able to reach the femtomole range.

Using the above system with a C_{18} bonded phase (Hypersil), the run-to-run retention reproducibility has been found to range from 0.1 to 0.5% relative standard deviation (R.S.D.) for individual Dns-AAs. We have also found that the reproducibility is 0.5% to 1.5% R.S.D. from mobile phase to mobile phase, if care is taken in making up the mobile phase. Using the same batch of silica, we have been able to achieve 0.5–2.6% R.S.D. for the retention of various Dns-AAs. These results undoubtedly arise from the high bonded phase coverage and trimethylsilyl end-capping treatment step. As expected, such species as Dns-Arg and Dns-NH$_2$ are most sensitive to small changes in mobile phase composition (e.g., concentration of acetic acid and TEA) or stationary phase (e.g., unreacted and accessible silanol groups). Based on the retention reproducibility and the quantitative aspects of the derivatization step, the system performs quite satisfactorily as an amino acid analyzer and can be potentially coupled to a second column for chiral separation.

We next turn to the coupling step itself. The first point to note is that on the chiral column we are interested only in the ratio of the amount of D- to L-Dns-AA, not in the total amount of amino acid present. Since both isomers elute at precisely the same time on the achiral column, it is thus only necessary to sample a portion of the chromatographic band eluting from the amino acid analyzer column. In particular, as shown in Fig. 5, it is possible to sample the band after the peak maximum so that the amino acid can be quantitated by peak height measurement on the first column. In addition, the system is set up at present for a 6 sec sampling time which is equivalent to a volume of 200 μl at a mobile phase flow-rate of 2 ml/min. This band volume does not significantly reduce column performance when it is delivered to the second column. Finally, use of fluorescence detection permitted good sensitivity in spite of the fact that only a small fraction of the band was transferred to the second column.
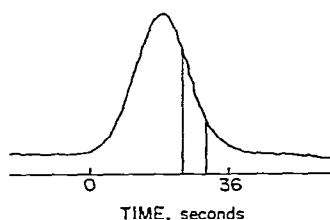

TIME, seconds

Fig. 5. Representation of the switching procedure of a chromatographic band eluting from the achiral column for transfer to the chiral separation column (see Fig. 4). Note sampling occurs after the peak maximum with a time window of ≈ 6 sec.

The achiral separation is achieved at pH 4.0 whereas high pH (≈ 9) is needed for the chiral separation. Moreover, under current operation the mobile phase for D,L separation contains L-prolyl–C$_8$–amide–Ni(II). Note also that the temperature of the chiral column is 25°C whereas that of the achiral column is 50°C. Therefore, an efficient and rapid means of changing solution conditions in the peak volume is required, in order to achieve high efficiency and separation in the chiral column. We have employed a specially designed mixer[37] for rapid premixing of the sample volume with the second mobile phase prior to injection on the column. Through the use of 200-μl sample volumes, only a small amount of acid had to be neutralized in the mixer

section. Note that in one sense this coupled column arrangement is similar to post-column reaction detection systems. Here we convert the sample to an appropriate set of conditions for further separation and detection after elution from the first column.

The column used for chiral separation in Fig. 3 is a C₈ Hypersil (5 µm) 6 cm × 4.6 mm I.D., prepared in our laboratory. Such a short column allows the D,L analysis of most of the Dns-AAs. For poorly resolved Dns-AA enantiomers, a longer column or alternative mobile phase conditions would be needed.

The mobile phase containing the chiral additive was used in the recycle mode to minimize loss of the chelate. Use of the recycled mobile phase showed no deterioration in chiral separation for prolonged periods of time (*e.g.*, over a month). The pH of the mobile phase, however, was adjusted daily with a concentrated ammonia solution, in order to overcome the slight changes in pH arising from the addition of acidic solutions from the first column.

Figs. 6 and 7 show chromatograms of chiral separations of several amino acids achieved with the coupled column approach. The time axis is from injection of the mixture on the first column, and the switching times may be recognized by the baseline disturbances. Good separation and peak shape are observed. Note that some of the separations shown in these two figures would be difficult to achieve on a single column. Thus, from Table I we can calculate that α for L-Dns-Gln and D-Dns-Thr is
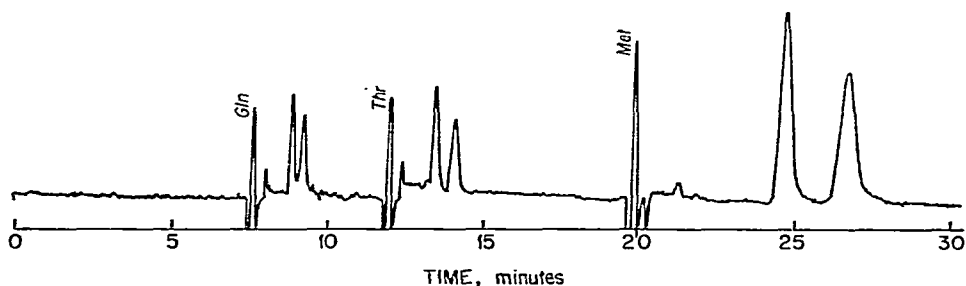

Fig. 6. Chiral separation of three D,L-Dns-AAs which have been first eluted from the achiral column and transferred into the chiral column (see Fig. 4). Time scale is from injection in the first column, with baseline interruptions marking the switching times. Conditions: achiral column see Fig. 3, chiral column: $4 \cdot 10^{-3}$ M L-prolyl–*n*-octylamide–$8 \cdot 10^{-3}$ M Ni(II), $8.75 \cdot 10^{-2}$ M ammonium acetate pH 9.2, methanol–water (60:40), flow-rate 2 ml/min. Column: 6 cm × 4.6 mm, 5-µm C₈ Hypersil.
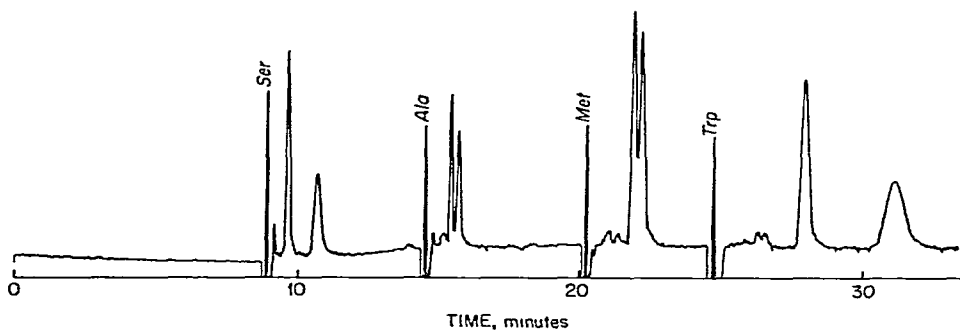

Fig. 7. Chiral separation of four D,L-Dns-amino acids which have been first eluted from the achiral column. Conditions as in Fig. 6 except $1.75 \cdot 10^{-1}$ M ammonium acetate, and flow-rate 3 ml/min.

only 1.05, for which ≈25,000 theoretical plates would be necessary for baseline separation. As another example, the a value for L-Dns-Ala and D-Dns-Met is only 1.08, and separation would again require a large number of plates. Even a worse situation can be faced, *e.g.*, with D-Dns-Cit and D-Dns-Gln which both have k' values of 3.1.

The mobile phase conditions in Fig. 6 differ from those in Fig. 7 in that the ammonium acetate concentration is doubled in the latter case. This larger concentration of buffer permits more rapid elution of long retained substances such as D,L-Dns-Trp. Obviously, a variety of mobile phase conditions such as concentration of chiral chelate, pH, temperature and organic modifier type and concentration can be manipulated to optimize separation. Also, one can conceive of using an immobilized layer of chiral additive on the bonded phase with little or no additive in the mobile phase.

The design shown in Fig. 3 permits sampling of individual amino acids in a complex mixture. This approach could be useful, for example, in the determination of racemization of aspartic acid for geochemical dating[38]. On the other hand, one may desire to have the capability to analyze all common amino acids as their D,L isomers in one sample. Here, one would develop chiral separation conditions to achieve resolution of groups of amino acids in one run on the chiral column. Grouping of individual amino acids into sample loops would be accomplished with a multiport valve. If necessary, several short chiral separation columns could be employed for simple use of different mobile phase conditions. With appropriate timing, all amino acids could be automatically analyzed as their D,L pairs in one run. These ideas are currently under study and evaluation in our laboratory.

In this paper we have addressed some of the practical aspects of the separation of D,L-amino acids using ligand-exchange chromatography. The prototype system shown in Fig. 3 offers a flexible means for analysis of a wide variety of mixtures for D,L composition of amino acids. The system can obviously be also employed for the resolution of substances other than amino acids as their D,L-pairs in complex mixtures containing a large number of components. The approach may also prove useful in the preparative scale fractionation of optical isomers in complex mixtures.

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