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EFFECTS OF STRUCTURE OF PEPTIDE STATIONARY PHASES ON GAS-CHROMATOGRAPHIC SEPARATIONS OF AMINO-ACID ENANTIOMERS

by

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Brief

Asymmetry at the amide end of a dipeptide derivative has been shown to be more important than at the ester end. Structural effects have been examined using a tripeptide and substituents of different types.

Abstract

The gas-chromatographic behavior is described for a series of systematically-substituted, optically-active, peptide derivatives used as stationary phases. Results with pairs of phases containing one racemic center indicated that the major part of the separation occurred at the amide end of the dipeptide. Nevertheless, α values were sensitive to changes in the structure of the side group at the ester end. A steady decrease in α occurred as the bulkiness of the peptide side-groups decreased. A tripeptide derivative produced α values nearly as large as the corresponding dipeptide and showed a small, but significant, increase in chromatographic temperature-stability. However, underivatized solid peptides used as stationary phases did not show enantiomer separations. An increase in the bulkiness of the solute ester group produced an increase in α on all phases studied. An increase in bulkiness at the solute α carbon atom produced a consistent, but not straightforward, effect.

Introduction

N-TFA(trifluoroacetyl) - L-valyl-L-valine cyclohexyl ester (vv) has been shown to be an excellent stationary phase for the separation of the enantiomers of a wide variety of amino acid derivatives (1,2,3). Recently, a second dipeptide phase, N-TFA-L-phenylalanine-L-leucine cyclohexyl ester, was synthesized which also gave good enantiomer separations and could be used at higher operating temperatures (4). The particular ease of separability associated with the dipeptide phase was attributed to the possibility of formation of three hydrogen bonds in a "diastereomeric" bridged association complex between the solute and solvent.

While there have been detailed studies of solute behavior on the above stationary phases, there has been no study of systematic changes in the peptide stationary phase itself. The main purpose of this study was, therefore, to investigate those factors which were most important in leading to an enantiomer separation with peptide phases so that, hopefully, new and better stationary phases, suitable for specific applications, could be prepared. First, a study was done to determine which end of the stationary phase was more significant in bringing about the separation and whether two asymmetric centers were necessary for resolution. Then, the effects of systematic changes in the side chains of the peptide were determined. Specifically, we were interested in determining if the two amino-acid portions of the peptide acted independently of one another and the significance of a change on the amide end relative to a similar change in the ester portion. Also, since N-PFP (pentafluoropropionyl) solutes have been shown to be more stable and, in some cases, to give larger α values than the corresponding

N-TFA derivatives, it was of interest to compare the chromatographic behavior of the N-PFP and N-TFA stationary phases with respect to N-PFP and N-TFA solutes (5,6).

Previous investigators have worked only with dipeptide stationary phases. Since it was thought that higher-polymer peptides might have better chromatographic temperature-stability, a tripeptide was derivatized for comparison with the corresponding dipeptide derivative. Also, since enantiomers have recently been separated on an optically active solid adsorbent, insoluble, solid, underivatized peptides, stable to above 200 ^{o}C, were investigated (7).

Experimental

Reagents

The N-t-BOC (tertiary-butyloxycarbonyl) derivatives of L-valine, L-leucine and glycine, N-CBZ (carbobenzoxy) derivatives of DL-valine, L-valine, L-leucine, DL-alanine ethylester hydrochloride, DL-alanine methylester hydrochloride, and DL-valine methylester hydrochloride were purchased from Mann Research Laboratories, New York, N.Y. The glycine, DL-alanine and trifluoroacetic acid were obtained from Matheson, Coleman and Bell, Norwood, Ohio. The poly-L-phenylalanine, and N-TFA-L-valyl-L-valine cyclohexyl ester were from Miles Laboratories, Elkhart, Indiana. The 30% HBr in glacial acetic acid and dicyclohexylcarbodiimide were from Eastman Organic Chemicals, Rochester, N.Y. The L-Leucyl-L-leucyl-L-leucine was obtained from Schwarz BioResearch, Orangeburg, N.Y. and the L-valyl-L-leucine from Nutritional Biochemicals Corp., Cleveland, Ohio. The trifluoroacetic anydride was from Aldrich Chemical Co., Milwaukee, Wisconsin, and the pentafluoropropionyl anhydride from K and K Laboratories, Plainview, N.Y. All chemicals were used without further purification.

The individual peptide stationary phases were prepared in one of two ways. Either the free peptide was esterified and trifluoroacetylated or an amino acid ester was coupled to a t-BOC or CBZ-protected amino acid followed by trifluoroacetylation.

The N-PFP-L-valyl-L-leucine cyclohexyl ester (p-vl), N-TFA-L-valyl-L-leucine cyclohexyl ester (vl) and N-TFA-L-leucyl-L-leucyl-L-leucine cyclohexyl ester (lll) phases were prepared by the direct esterification procedure which consisted of the following. One gram of the peptide was mixed with 100 ml of reagent grade cyclohexanol. The mixture was heated to 100 ^{o}C and dry HCl bubbled in for 60 minutes to produce a clear solution. The reaction mixture was then maintained at 100 ^{o}C for 10 hours with occasional addition of HCl. At the end of this period, the cyclohexanol was removed by vacuum distillation to produce a clear gum. In the vl case, the peptide ester hydrochloride was precipitated by addition of dry ether to produce a white solid. (However, the lll derivative could not be precipitated in that way. So the next step was carried out on the entire solution). The peptide ester was dissolved in 50 ml of CH_2Cl_2, cooled in an ice bath, and reacted with a five-fold excess of anhydride for 1 hour. The solvent, excess anhydride and by-products were removed with a rotary evaporator producing an oil which was taken up in approximately 5 ml of dry ether.

The product was then purified by preparative gas chromatography using SE-30 as a stationary phase. In general, the reaction mixtures contained 3-5 impurity peaks. The product was always the last compound to emerge and was well separated from the other components (peptide derivative peak k≈4). Final product yield was 50-75%.

The N-TFA-L-leucyl-L-valine cyclohexyl ester (lv), N-TFA-L-leucyl-L-leucine cyclohexyl ester (ll), N-TFA-glycyl-L-valine cyclohexyl ester (gv), N-TFA-L-valyl-glycine cyclohexyl ester (vg), N-TFA-DL-valyl-L-valine cyclohexyl ester (DL-L) and N-TFA-L-valyl-DL-valine cyclohexyl ester (L-DL) phases were prepared by a coupling procedure similar to that described by Koenig et al. with the following minor modifications (4). The amino acid ester reaction mixtures were heated to 100 °C, rather than leaving them at room temperature, so as to achieve solution. The crude amino acid ester products were precipitated with $CHCl_3$ prior to recrystallization from CH_2Cl_2 and ether.

In the DL-L case, the CBZ derivative of DL-valine was used rather than the t-BOC derivative. The CBZ protecting group was removed by treatment with 25 ml of 30% HBr in glacial acetic acid at 35 °C for 30 minutes. The free peptide ester was then precipitated by addition of 500 ml of dry ether. The rest of the procedure was the same.

The peptide trifluoroacetylation procedure for all phases was the same as described above. Unlike the procedure of Koenig et al. (4), we were unable to find a suitable recrystallization method for these phases. Therefore, the phases were purified by preparative-scale gas chromatography in the same manner as with the direct esterification products.

The identity and chemical purity of the stationary phases were carefully considered. The preparative-scale gas chromatographic purification procedure afforded a high degree of purity not possible by most other purification methods. However, as a check, s

a microanalysis was run on the lv phase. The results showed that it was the desired product and was indeed in very high purity. The identities of all products were checked by IR and mass spectrometry using the Miles vv phase as a reference. There was no question that the phases were the desired compounds.

Optical purity was also of prime concern. It is generally accepted that no racemization takes place during esterification under acid conditions (8). However, as a check, the N-TFA derivatives of the L- and DL-valine cyclohexyl esters were put through a vv column, and they produced a single peak and two peaks in a ratio of 1:1, respectively. The stationary phases prepared by the direct peptide esterification method were assumed to be optically pure since the starting peptides were optically pure and the process did not involve any steps which would have led to racemization. Also, the coupling process, performed under the given conditions should not have produced racemization (9). Therefore, all stationary phases were assumed to be pure ones of the desired optical composition.

The amino-acid derivatives used as solutes in the study were prepared from commercial or laboratory-prepared ester hydrochlorides by a previously described method (7). The identity and chemical purities of the amino-acid derivatives were confirmed by NMR and mass spectrometry.

Apparatus

For all work with capillary columns, an Aerograph 660 gas chromatograph, modified to minimize the dead volume in the system and equipped with a Hamilton inlet splitter, was used. The chromatograph was operated at a detector temperature of 180 °C,

an injection temperature of 200 °C, and at different column temperatures. During studies of the effect of temperature change, fifteen minutes were allowed for equilibration after each new temperature had been reached, unless otherwise stated. A flow rate of approximately 2ml/min of nitrogen, dried using 5A molecular sieve traps at ambient temperatures, was usually used. An Esterline-Angus Speed Servo recorder recorded the chromatograms.

Open tubular, Pyrex columns were used throughout the study of enantiomer separations. The 40m by 0.25 mm columns were cleaned, drawn, and conditioned as previously described (7). They were coated using 20% w/v solutions in dry ether (10) except the poly-L-phenylalanine and solid tripeptide columns which were prepared from saturated solutions of these compounds (≈3%w/v) in dichloracetic acid and water, respectively.

For preparative-scale purification of the stationary phases, an Aerograph 202 gas chromatograph equipped with a thermoconductivity detector was used. The stainless steel columns were 3.1 m by 4 mm packed with 5% SE-30 on DMCS-treated, acid-washed, 80/100 mesh Chromosorb G. The column was maintained at 285 °C, the injection port at 310 °C, and the detector at 310 °C with a helium flow rate of approximately 40 ml/min. The sample concentrations were adjusted so that a 50 ul injection produced 2-3 mg of product upon collection.

Capacity ratio, $\underline{k}$, separation factor, α, and resolution, $\underline{R}$, were calculated in the same way as before (7). The $\underline{k}$ and α values were reproducible within $\pm$ 0.003. Values for $\underline{R}$ could be reproduced within 0.1 unit.

Results

Effect of Individual Asymmetric Centers

To determine the relative contribution to the overall enantiomer separation of each of the two centers in the dipeptide, two pairs of compounds, L-DL/DL-L N-TFA-valyl-valine cyclohexyl ester and vg/gv, were synthesized. It was assumed that, in the L-DL and DL-L cases, the L and D fractions of the racemic center would contribute equal and opposite interactions and, thus, cancel out the enantiomer separating ability of that center. Because glycine is not optically-active, the vg and gv dipeptides made with that amino acid contained only one asymmetric center.

As shown in Table I, the L-DL phase gave excellent enantiomer separations with α values for the isopropyl alanine derivative which were only approximately 0.1 unit less than the vv phase. Surprisingly, the DL-L compound gave no detectable enantiomer separations. These results suggested that the amide end of the stationary phase made a far greater contribution to the separation than the ester end.

Unfortunately, the gv and vg stationary phases had quite different physical properties so that a completely comparable study could not be made. Although the gv phase behaved similarly to the other dipeptide phases (mp. $\approx$90 $^{\circ}$C), the vg phase was a high-melting white solid (m.p. =155 $^{\circ}$C) which made it unsuitable as a stationary phase. Like the DL-L valyl-valine which produced no observable separation of the enantiomers of the isopropyl alanine derivative, the gv produced only a very small separation. The k values at 100 $^{\circ}$C were 10.46 and 10.57 respectively, which represented an α

value of approximately 1.01. Again, these results suggested that the differential interaction at the ester end of the stationary phase produced only a small enantiomer separation.

Effects of Systematic Changes in the Amino-Acid Composition of Peptide Stationary Phases

To determine the effects on the α values of enantiomers of the side groups on a dipeptide, comparisons were made of isobutyl (leucyl) and isopropyl (valyl) derivatives. In all cases, ln k vs $1/T$ plots were essentially straight lines between 90 ^{o}C and 110 ^{o}C so Table II shows only the results at the temperature extremes. When valine was replaced by leucine in a series of dipeptide stationary phases, α for the isopropyl and ethyl alanine derivatives was always less by nearly the same amount, regardless of whether the substitution was at the amide end or ester end. However, the ethyl valine derivative was an exception on both mixed phases (vl, lv), the α value for lv being greater than that for vv.

Apparently, the larger α values associated with the valine-containing phases were the result of greater steric interaction with the isopropyl group, where the branching is closer to the asymmetric carbon, than with the isobutyl group of leucine. However, the results with the ethyl valine solute indicated that this was not a rigid rule and depended on the specific solute involved. In any case, the nearly equal effects on α of substituents at the amide and ester ends of the dipeptide indicated that important secondary steric contributions were made by the ester end of the dipeptide, even though the primary "bonding" interaction occurred at the amide end.

The stationary phases showed similar abilities to separate the individual amino-acid derivatives (the compounds themselves, not their enantiomers). For example, using the data in Table II to calculate an α_1 value from the k_1 value for each of two compounds, one finds that the α_1 values for the ethyl valine derivative relative to the isopropyl alanine derivative ranged between 1.51 and 1.57 at 90 °C. This behavior was not unexpected considering that the only difference between a leucine moiety and a valine moiety was an additional CH_2 group in the former.

Surprisingly, in light of the virtually identical α_1 values, Table III shows that the various peptide phases differed greatly in their relative abilities to resolve different pairs of enantiomers. In other words, the relative widths of the peaks were greatly different even though the ratio of k_1 (and k_2) values were nearly the same. To minimize the effect of column instability, the pairs of compounds shown in Table III were chromatographed immediately after one another in as short a time-span as possible. Because the resolving abilities of the columns were changing slowly with time and the sample sizes were not exactly the same, the reported values were precise only within $\pm$ 0.1. However, some of the observed differences were well outside this range of experimental error. Apparently, the band broadening for the otherwise symmetrical peaks depended strongly on the individual solute and differed from phase to phase. This latter aspect should be considered in any future attempts to tailor individual phases for special applications.

Dipeptide Phases vs. Larger Polymers

Table II also presents a comparison of α values on a tripeptide derivative (lll) and its corresponding dipeptide derivative (ll). This particular pair was chosen because the starting materials were readily available. All solutes gave slightly smaller α values on the tripeptide phase than on the corresponding dipeptide phase. However, the absolute differences were quite small and, for many practical applications, the tripeptide could be substituted for the dipeptide.

The fact that the tripeptide produced a separation indicated that the ester and amide ends of the molecule did not need to be in close proximity so as to cause a differential steric interaction. Apparently, the mechanism does not necessarily involve a joint interaction with both ends of the phase since the distance between the amide and ester portions was too great to be spanned by the solutes, assuming a linear tripeptide conformation.

One reason for preparing the tripeptide phase was to investigate its chromatographic temperature-stability relative to the corresponding dipeptide. It was thought that, since it was a larger molecule with a higher boiling point (as evidenced by a longer retention time on the SE-30 column), the tripeptide phase might be more suitable for use at high temperatures than the more volatile dipeptide phase. This was indeed the case. The rate of loss of resolution was much less at 120 ^{o}C for the tripeptide than the dipeptide phase. Operation at 120 ^{o}C for 60 minutes produced losses in $\underline{R}$ of 0.3 and 0.1 units for the dipeptide and tripeptide respectively. In general, the tripeptide phase, at approximately 10 C^{o} higher

temperature than the corresponding dipeptide, had the same rate of loss of resolution. Attempts to use the tripeptide phase at 130 °C showed that the rate of degradation was so great that the column was essentially useless after 30 minutes.

Another interesting point was that each of the three tripeptide columns used always produced k values which were approximately 30% smaller than the dipeptide phases, even though the coating solutions were the same weight/volume percent. Since there was no way of accurately measuring the amount of stationary phase in the column, smaller k values may have been the result of a smaller quantity of stationary phase retained in the column or of a smaller effective fraction of active sites.

Since enantiomer separations have been done by adsorption on a solid stationary phase (7), attempts were made to use solid poly-L-phenylalanine (molecular weight range 3,000-5,000) and the solid underivatized L-leucyl-L-leucyl-L-leucine tripeptide. These materials were solids which decomposed without melting at temperatures in excess of 200 °C. Columns coated with these compounds were quite difficult to prepare since both were nearly insoluble in most common solvents.

As adsorbents, both of the solid peptides produced very small k values and no detectable enantiomer separations. Representative k values at 70 °C for the isopropyl alanine derivative were 1.74 and 0.29 for poly-L-phenylalanine and for the tripeptide, respectively. The fact that the k values were small and the peaks were sharp indicated that there was little or no exposed glass. Hence, adsorption on the solid underivatized peptide appears to hold little promise.

N-PFP vs. N-TFA Stationary Phases

N-PFP derivatives produced larger α values than the corresponding N-TFA derivative on a solid ureide stationary phase (7). Parr et al. reported a larger α value for the enantiomers of some N-PFP-DL derivatives than for the corresponding N-TFA derivative (5,11). Also, in a study of catecholamine metabolites, N-PFP derivatives were shown to be more stable than N-TFA derivatives (6). Therefore, it was of interest to compare the chromatographic properties of an N-PFP phase with its corresponding N-TFA derivative. We were particularly interested in determining if the N-PFP phase produced larger α values with N-PFP solutes.

Table IV shows that for the p-vl and vl phases used for this study, the N-PFP solute gave only slightly smaller α values but much smaller k values and R values than the corresponding N-TFA derivative on both phases. The same was true at all temperatures. The ratio of α values on both stationary phases was quite temperature sensitive, those on the N-PFP changing somewhat more. For example, at 90 °C, the α values of N-PFP and N-TFA DL-alanine ethyl ester were virtually equal (1.087 vs 1.089) while at 110 °C the former were somewhat smaller (1.063 vs 1.069). The same was true for the corresponding α values for the N-TFA stationary phase.

In summary, N-PFP stationary phases can be readily synthesized and did produce reasonably good enantiomer separations. However, no advantages over the N-TFA derivatives were apparent.

Solute Effects

A complete evaluation of solute behavior was beyond the scope of this study. However, as shown in Table II, an increase in bulkiness of the solute ester group produced larger α values on all the stationary phases studied. Nevertheless, an increase in bulkiness at the α carbon atom produced a consistent but poorly-understood effect. For instance, the ethyl alanine derivative (methyl side group) produced smaller k values, larger α values, and better resolution on all phases at all temperatures than the ethyl valine derivative (isopropyl side group). It is clear from these results, as well as from those of Nakaparksin et al. (3), that more work is needed before the effects of individual solutes will be completely understood.

Discussion

A variety of optically-active, dipeptide (and higher polymeric) stationary phases can be obtained in high chemical and optical purity in the laboratory. Good yields are possible and purification is simple by preparative-scale gas chromatography. Hence, it should be possible to tailor the individual phase to the experimenter's needs.

This study indicated that the best separations of enantiomers of amino acid derivatives, using peptide stationary phases, would be achieved using a dipeptide stationary phase possessing an N-TFA group, bulky side groups, and a similarly bulky ester group, with a column operated at the lowest feasible temperature. A

stationary phase containing more sterically hindered side groups, such as t-butyl, might or might not produce better separations than existing phases, depending upon the effect of added crowding. However, the results with poly-L-phenylalanine indicated that an adsorption mechanism or an underivatized polypeptide will not work and suggested that there was a practical limit on the length of the peptide chain.

The derivatives of the volatile solutes should also possess a bulky ester group (such as t-butyl or isopropyl) and an N-TFA group. Somewhat greater chromatographic temperature-stability can be achieved, with a small sacrifice in α, by going to derivatized tri- or higher peptides.

Since the amide end of a dipeptide appeared to make the primary contribution to the separation, and since only one end of the peptide molecule had to be derivatized, it should be possible to produce a high-temperature stationary phase by derivatizing the amide end and attaching the carboxylic acid group to a stable, non-volatile optically-inactive substrate. However, since the substrate would not contribute to the enantiomer separation, care would have to be taken to maintain a high concentration of active sites.

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Table I. k_1, α, and R Values for Enantiomers of N-TFA-DL-Alanine Isopropyl Ester as a Function of Temperature on Dipeptide Stationary Phases Containing only One Asymmetric Center

Stationary Phase	90 °C			110 °C		
	k_1	α	R	k_1	α	R
N-TFA-L-Valyl-DL-Valine Cyclohexyl Ester	9.56	1.102	1.39	3.89	1.073	0.83
N-TFA-DL-Valyl-L-Valine Cyclohexyl Ester	3.53	a	a	2.16	a	a

[a] No enantiomer separation was observed.

Table II. k, α, and R Values for Enantiomers of a Series of Amino Acid Derivatives on a Series of Peptide Stationary Phases as a Function of Temperature

Stationary Phase	Amino Acid Derivative: N-TFA-DL-Alanine Isopropyl Ester, 90 °C k_1	α	R	110 °C k_1	α	R	N-TFA-DL-Valine Ethyl Ester, 90 °C k_1	α	R	110 °C k_1	α	R	N-TFA-DL-Alanine Ethyl Ester, 90 °C k_1	α	R	110 °C k_1	α	R	Methane
N-TFA-L-Valyl-L-Valine Cyclohexyl Ester	9.27	1.126	2.40	4.18	1.112	1.23	14.57	1.080	1.51	6.18	1.064	0.80	8.30	1.107	1.79	3.70	1.079	1.00	111
N-TFA-L-Valyl-L-Leucine Cyclohexyl Ester	8.71	1.118	2.75	3.65	1.095	1.17	13.85	1.061	1.44	5.33	1.043	0.60	7.83	1.095	2.07	3.24	1.075	1.00	114
N-TFA-L-Leucyl-L-Valine Cyclohexyl Ester	13.04	1.114	2.52	5.95	1.090	1.23	19.59	1.087	2.14	8.55	1.066	0.76	11.37	1.095	2.31	5.35	1.075	1.05	110
N-TFA-L-Leucyl-L-Leucine Cyclohexyl Ester	9.88	1.103	1.91	4.21	1.076	1.01	14.77	1.062	1.22	6.00	1.049	0.70	8.70	1.081	1.40	3.77	1.064	0.85	114
N-TFA-L-Leucyl-L-Leucyl-L-Leucine Cyclohexyl Ester	6.67	1.096	2.15	2.59	1.072	1.11	10.07	1.061	1.60	3.81	1.045	1.00	5.73	1.076	1.75	2.32	1.058	1.09	110

Table III. Ratios of Resolutions of Enantiomers of Amino Acid Derivatives as a Function of Temperature for a Series of Peptide Stationary Phases

Stationary Phase	90 °C		110 °C	
	R_1^a/R_2	R_1/R_3	R_1/R_2	R_1/R_3
N-TFA-L-valyl-L-valine cyclohexyl ester	1.4	1.2	1.5	1.2
N-TFA-L-valyl-L-leucine cyclohexyl ester	1.9	1.3	1.9	1.2
N-TFA-L-leucyl-L-valine cyclohexyl ester	1.2	1.1	1.6	1.2
N-TFA-L-leucyl-L-leucine cyclohexyl ester	1.5	1.3	1.4	1.2
N-TFA-L-leucyl-L-leucyl-L-leucine cyclohexyl ester	1.3	1.2	1.1	1.0
N-PFP-L-valyl-L-leucine cyclohexyl ester	1.6	1.2	1.6	1.0

[a] R_1 = Resolution of enantiomers of N-TFA-DL-alanine isopropyl ester.

R_2 = Resolution of enantiomers of N-TFA-DL-valine ethyl ester.

R_3 = Resolution of enantiomers of N-TFA-DL-alanine ethyl ester.