MOLECULAR WEIGHT INDUCED PEAK DOUBLING IN LIQUID CHROMATOGRAPHY

A Thesis

by

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Submitted to the Graduate College of

Texas A&M University

in partial fulfillment of the requirement

for the degree of

MASTER OF SCIENCE

May 1980

Major Subject: Chemistry

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ABSTRACT

Molecular Weight Induced Peak Doubling

in Liquid Chromatography (May 1980)

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Chairman of Advisory Committee: Dr. George I. Glover

Peak doubling was observed in the separation of certain blocked high-molecular weight amino acids and peptides on silica gel using high pressure liquid chromatography with an isopropanol-methylene chloride solvent system. The peak doubling appears to be a combination of molecular weight considerations and protic solvent interactions within the system, and can be prevented by the use of aprotic solvent systems such as acetonitrile-methylene chloride or toluene-ethyl acetate.

DEDICATION

This work is dedicated to Dr. Sherrel C. Smith, whose knowledge in the field of liquid chromatography is outweighed only by his willingness to share this knowledge with others. The work presented herein would not have been possible without the active interest and knowledgable, patient advice provided by Dr. Smith during the long hours of experimentation and interperatation required to prepare this work.

ACKNOWLEDGEMENT

I would like to thank Dr. George I. Glover who, as chairman of my committee, proved a constant source of advice and encouragement throughout the course of my research. I would also like to thank the members of my committee for the thoroughness with which they scrutinized my research, as well as the constructive advice they offered during the actual writing of the thesis.

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INTRODUCTION

During the course of synthetic work in this laboratory, severe band-broadening effects including peak doubling were observed during the preparative separation of diastereomers of *tert*-butyl 3-hydroxy-4-phthalimido-5-phenylpentanoate [THPPP] (Figure 1), as well as certain other high molecular weight amino acids and peptides, using high pressure liquid chromatography with an isopropanol-methylene chloride

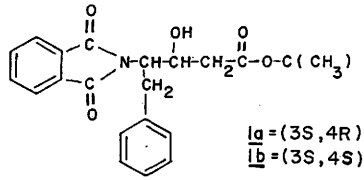
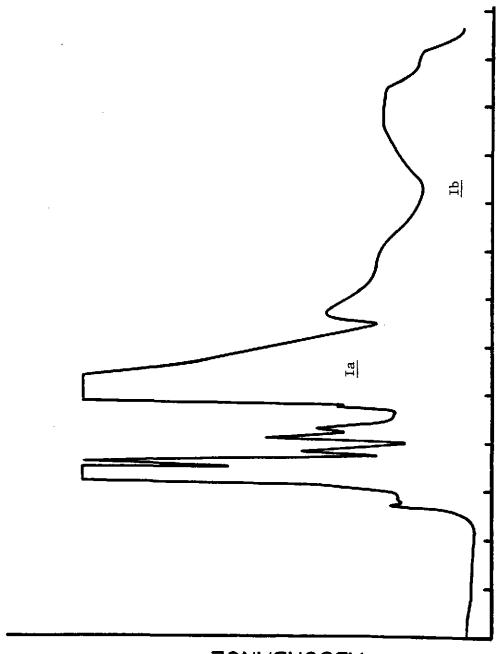


Figure 1. *tert*-Butyl 3-hydroxy-4-phthalimido-5-phenylpentanoate.

solvent system. It is apparent from the chromatogram (Figure 2) that purification of these diastereomeric amino acids under these conditions is very difficult. When analytical injections of purified Ia and Ib are made, Ia was eluted as a single, sharp peak (Figure 3) while Ib exhibited a frontal asymmetry even at low loading capacities (Figure 4) which, at higher loads progressed from a left-handed "shoulder" to a doubled peak.

This thesis follows the style of The Journal of Chromatographic Science.

Figure 2. The preparative separation of THPPP in an isopropanol-methylene chloride solvent system.


ABSORBANCE
Ia
Ib
1
2
3
4
5
6
TIME (hours)

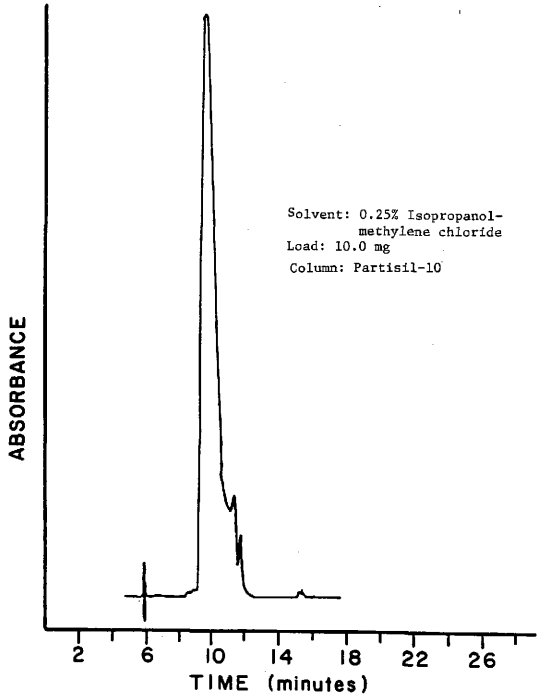
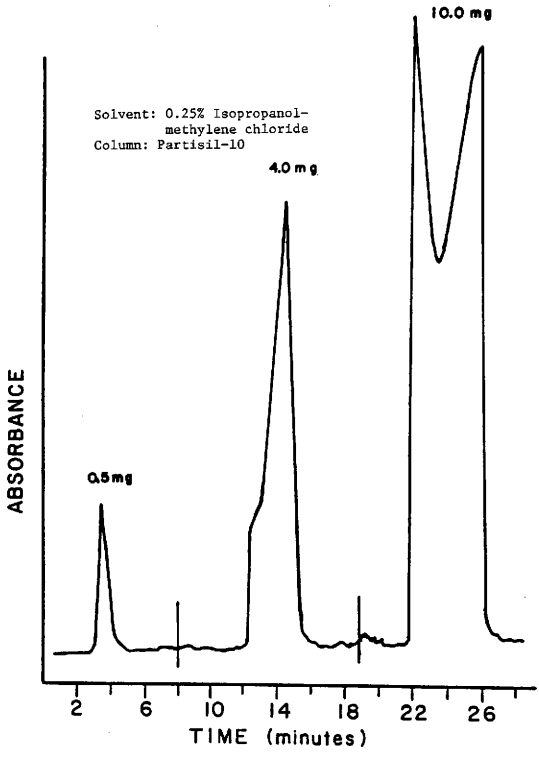


Figure 3. An analytical injection of THPPP(Ia) in an isopropanol-methylene chloride solvent system.

Figure 4. Analytical injections of THPPP(Ib) in an isopropanol-methylene chloride solvent system.


10.0 mg
Solvent: 0.25% Isopropanol-
methylene chloride
Column: Partisil-10
4.0 mg
0.5 mg
ABSORBANCE
2
6
10
14
18
22
26
TIME (minutes)

Before any attempt is made to understand the observed asymmetry effect, a basic understanding of silica gel adsorption chromatography is necessary.

The column packings used in this experiment are porous particles of silica gel. Silica gel is precipitated polysilicac acid $(H_2SiO_3)_n$; its structure is amorphous (non-crystalline) with Si-OH electron-acceptor groups lining the surface of the pores. (Figure 5).

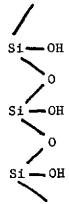


Figure 5. A generalized representation of the Si-OH lining of a silica gel pore.

Under normal conditions, solute molecules pass into these pores and are retained or removed, depending on the competitive strength of the mobile phase, under more or less equilibrium conditions.

Figure 6 shows the hypothetical separation of a three-component mixture using a silica-gel column [1].

As the solvent flows through the column, the sample molecules will interact with the Si-OH groups of the column packing as has already been mentioned. The relative strengths of these interactions produce differential rates of migration through the column allowing separation

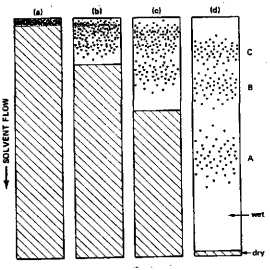


Figure 6. A hypothetical separation of a three-component mixture on a silica gel column.

to occur. As Figure 6 also shows, molecules will show a tendency to spread as they move down the column. This broadening of the solute distribution is not due to differences in equilibrium distribution, but rather to four basic processes (Figure 7) [1].

1. Eddy Diffusion
2. Mobile Phase Mass Transfer
3. Stagnent Mobile Phase Mass Transfer
4. Stationary Phase Mass Transfer

Eddy diffusion results from the differing flow paths the solute can take through the column packing. This results in some molecules taking a longer path through the column than others, resulting in more time being spent in the column by some molecules over others.

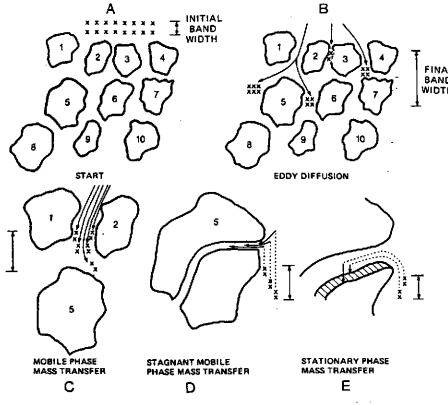


Figure 7. A schematic representation of the various contributions to band spreading within a chromatographic column.

Mobile phase mass transfer effects are the result of the laminar flow characteristics of the solvent as it travels through the flow path. Because of laminar flow, the flow rate of the solvent adjacent to the packing particle will be slower than at the center of the flow stream, resulting in a more rapid elution of some molecules than others.

Porous column packing particles contain some mobile phase within the pores that is stagnent or unmoving some solute molecules diffuse only a short distance into these stagnent pools while other molecules diffuse farther in. Those that diffuse father into the stagnent mobile phase spend less time in the moving mobile phase than do those which

diffuse only a short distance, producing stagnent mobile phase mass transfer effects.

Similar to this effect is stationary phase mass transfer in which solute molecules which become adsorbed deeper in the pores of the packing will spend more time in the packing particle than those molecules which are adsorbed close to the surface.

The contributions of all of these mass transfer effects to asymmetry result in the gaussian shape of a typical elution chromatogram such as Figure 8 [2].

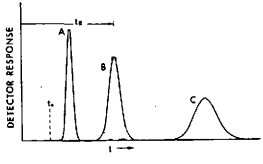


Figure 8. A generalized representation of a chromatogram resulting from the elution of a three-component mixture (see Figure 6, p. 8).

Deviations from the normal gaussian peaks, ideally observed in chromatography, can be the result of mechanical effects. Band tailing [3] can be classified into (see Figure 9):

(a) "Chemical" tailing occurs because of a mismatch between the sample and stationary or moving phases.

(b) "Solvent" tailing is the result of trying to separate a small band in the presence of a much larger band.

(c) "Poisson" tailing is caused by using poorly packed columns.

(d) "Exponential" tailing is the slight amount of asymmetry present in all systems which cannot be avoided.

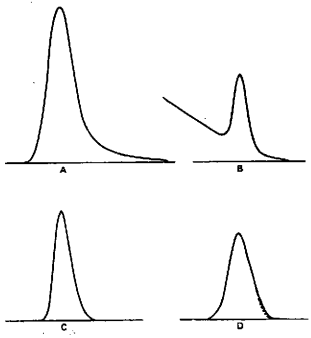


Figure 9. Band tailing effects in liquid chromatography. (See text for discussion).

None of these types of band-broadening effects could adequately account for the frontal asymmetry with which we were dealing since they all ultimately lead to right-hand tailing of the observed peak.

The possibility that overloading of the system could be responsible for the observed asymmetry was also discounted because this failed to account for the appearance of a frontal "shoulder" at relatively low load capacities.

Peak asymmetry has recently been studied by Geeraert and Verzele, [4] who determined that extra-column effect (injection, detection mode,

product purity, eluent velocity) did not result in a significant change in the peak shapes and concluded that an internal column process, manifesting the inhomogeneity of the column bed, was responsible for the observed asymmetry. Godbille and Devaux [5] attributed the asymmetry to sample impurities. In running colored compounds through our preparative columns (Merck EM-Size B), symmetrical bands were observed upon elution, indicating to us that inhomogeniety was not operating in this case. Likewise, prior purification of our sample compounds eliminated the possibility of sample impurities being involved in the peak doubling effects as was the case in the studies of Godbille and Devaux. Kirkland [6] observed that solvent systems modified with alcohols increased the possibility of a non-linear isotherm which is, in effect, what we were observing with an isopropanol-methylene chloride solvent system.

The confusion in peak identification and the decrease in resolution of the columns brought on by this effect made it necessary to determine the cause of the observed asymmetry in order that an alternative system could be found to successfully carry out the preparative and analytical separation of Ia from Ib.

We chose then to investigate the asymmetry phenomenon further using common amino acids and peptides with simple blocking groups having structure or functional group similarities to I.

EXPERIMENTAL PROCEDURE

Liquid Chromatography

Experiments were carried out on a chromatograph constructed from one Model 396-57 Milton-Roy pump, a modified injection valve as described by Smith, [7] and a Pharmacia Model 110 Ultraviolet detector. Pressure was indicated on a Bourden Gauge of 0-5000 lbs. Separations were made on 30 cm x 4.1 mm stainless-steel columns slurry-packed with Partisil-10, Chromasorb LC-6, and Vydac 101 TP. Components were joined by using 1/16 in O.D. stainless-steel tubing and Swagelok fittings. All fittings used downstream from injection point were zero dead-volume and tubing was 0.010 in I.D. A Hamilton 701N ten microliter syringe and a Hamilton 710N one hundred microliter syringe were used for injections. All separations were carried out at a flow-rate of 2 ml/minute at a pressure of 1000 p.s.i. Detection was at 254 nm.

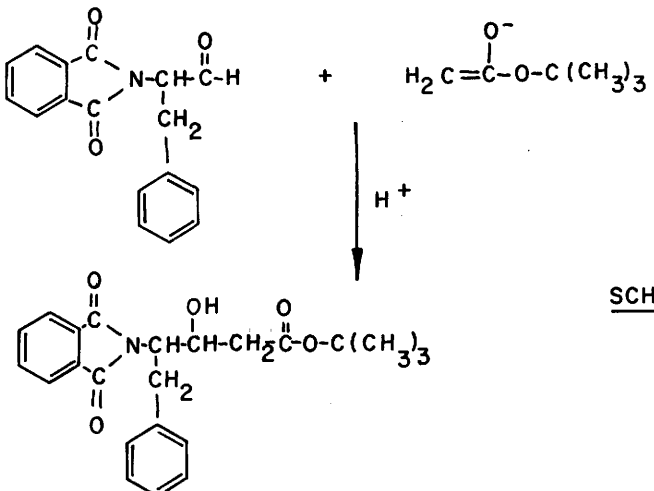
Sample Preparation

Synthesis of Ia and Ib. Diastereomers of I were prepared by the condensation of phthalylphenylalanal [8] with the zinc enolate of tert-butyl acetate [9] (Scheme 1).

N-Carbobenzyloxy (Cbz-) blocking groups were coupled to amino acids by the method described in Greenstein and Winitz [10].

Phthalyl blocking groups were coupled by the addition of phthalic anhydride to the amino acid in the presence of triethylamine [10].

Amino acids and peptides were esterified with methanol in the presence of acetyl chloride.



SCHEME I

Scheme 1. Synthesis of *tert*-butyl 3-hydroxy-4-phthalimido-5-phenylpentanoate(THPPP) I.

N-Cbz-alanyl-glycylglycine methyl ester, N-Cbz-L-alanyl-L-serine methyl ester, and N-Cbz-L-alanyl-L-valine methyl ester were obtained from Sigma Chemical Company.

All compounds were characterized by NMR and checked for purity by thin-layer chromatography.

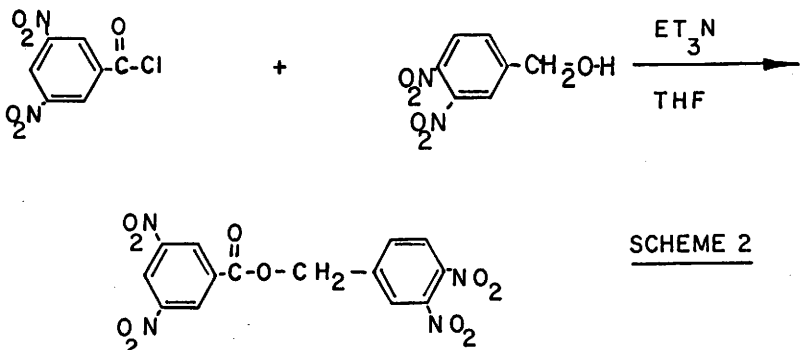
Synthesis of 3,5-dinitrobenzyl 3,4-dinitrobenzoate. The compound was synthesized by the esterification of 3,5-dinitrobenzoyl chloride with 3,4-dinitrobenzyl alcohol in the presence of triethylamine (Scheme 2). The alcohol (1.72 g, 8.68 mmol) was dissolved in 200 ml CH_2Cl_2 and stirred for 10 min. with 1.05 g (1.2 eq) triethylamine. The acid chloride (2.2 g, 9.54 mmol) was then added to the solution and stirred at 25°C for 3 h. Solution was extracted twice with 5% $NaHCO_3$ and twice with 1 M HCl. Organic layer was dried over Na_2SO_4 and rotovapped to a volume of 10 ml. To remove any residual alcohol, the concentrate was placed on a column containing 3 g of silica gel and eluted with 100 ml of CH_2Cl_2 which was rotovapped to dryness. The reaction yielded 3.0 g (88%) of the dinitrobenzyl ester. Melting point = 118.0°-119.0°C. Anal. ($C_8H_8N_4O_{10}$) C, H, N.

All compounds were diluted 1 g/10 ml (100 µg/µl) with Spectranalyzed methylene chloride for chromatography.

Solvent Preparation

Solvent strengths were determined so as to give a k' of 3-5, where [11]:

$$k' = \frac{t_r - t_o}{t_o}$$



SCHEME 2

Scheme 2. Synthesis of 3,5-dinitrobenzyl 3,4-dinitrobenzoate X.

$$k' = \frac{t_r - t_o}{t_o}$$

t_r = retention time (mins)

t_o = time for unretained molecules to move from one end of the column to the other.

t_o was determined for each column by injection of one microliter of benzene.

Solvents were prepared using volumetric flasks and serological pipettes and stored stoppered and sealed in Parafilm.

Column Preparation

Columns were slurry-packed as follows (Figure 10): About 2.1 g of packing material was added to about 20 ml of 1,3-dibromopropane (Aldrich). The mixture was sonicated for about 13 sec and the resulting slurry was added to the column with a pasteur pipette. A packing column filled with 1,3-dibromopropane and the remaining slurry was attached to the column to be packed and this, in turn, was connected to a helical coil of 1/4" in stainless-steel tubing filled with 70:30% v/v hexane:acetone and, with a flow rate of 20 ml/min, brought to 5000 p.s.i. This pressure was then released in the direction of the slurry-filled column and the solvent allowed to escape through the open-ended bottom of the column. This "ramming" process was then repeated three times and then the packing column was removed and a 7-micron stainless-steel frit was placed at the top of the column and finally closed off with a zero dead-volume fitting.

The column parameters were defined as follows:

Flow Rate = 2 ml/min

Reference Compound = 2-p-nitrophenethanol

($p\text{-}NO_2\text{-}C_6H_4\text{-}CH_2CH_2OH$) 1μ l injection

Solvent = 0.7% Isopropanol-methylene chloride

The number of theoretical plates (N) was calculated using the equation [11]:

$$N = 4\left(\frac{t_r}{0.607 \times \text{peak height}}\right)$$

The number of effective plates (N_{eff}) is given by [11]:

$$N_{eff} = N\left(\frac{k'}{1 + k'}\right)$$

N = number of theoretical plates
k'= k' for reference compound

The height of an equivalent theoretical plate (H or HETP) is determined by [12]:

$$H = L/N$$

L = length of column
N = number of theoretical plates

The reduced plate height (h) is given by the equation [11]:

$$h = H/d$$

H = height of an equivalent theoretical plate
d = diameter of packing particle

(See Appendix I for a description of what these parameters indicate).

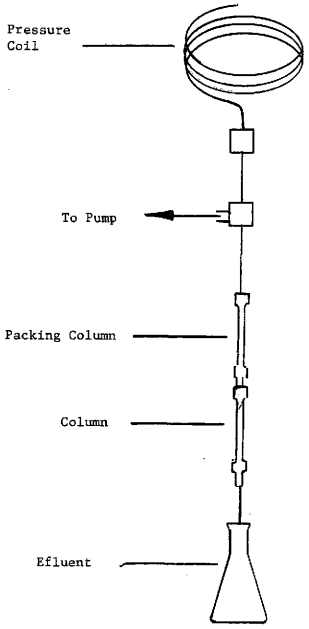


Figure 10. Apparatus for packing chromatographic columns

RESULTS AND DISCUSSION

Peak Doubling as a Function of Molecular Weight

Since the peak doubling was initially observed during the separation of a fairly large (M. W. = 397) and bulky compound, we decided to test whether the effect could be a function of the molecular weight and/or structure. The compounds used provided variations in individual properties in both molecular weight and side-chain composition (Figure 11).

Phthalyl-threonine methyl ester was chosen both for the presence of the phthalyl- blocking group (present in I) and for the secondary hydroxyl group on the threonine side chain (also present in I).

Cbz-threonine methyl ester contains the secondard hydroxyl function without the phthalyl- blocking group.

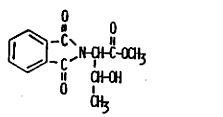
Cbz-valine methyl ester was chosen to examine the effect of a relatively low molecular weight amino acid with a bulky side chain on the peak symmetry.

Cbz-alanyl-serine methyl ester is a dipeptide of larger molecular weight with a hydroxyl function present in the side chain.

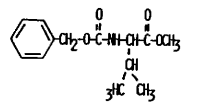
Cbz-valyl-valine methyl ester introduces a much larger molecular weight dipeptide with two bulky side chains and ny hydroxyl function.

Cbz-valyl-phenylalanine methyl ester has not only a large molecular weight and a bulky side chain, but also contains the phenyl-alanyl functionality present in I.

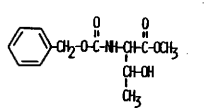
Cbz-alanyl-glycyl-glycine methyl ester was chosen to determine what effect a tripeptide with no functionalities would have on the peak



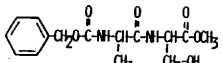
Phthalyl-Threonine
Methyl Ester



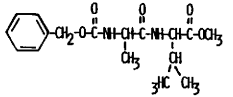
Cbz-Valine
Methyl Ester



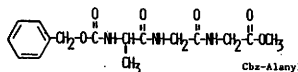
Cbz-Threonine
Methyl Ester



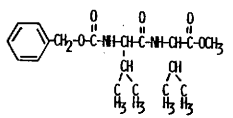
Cbz-Alanyl Serine
Methyl Ester



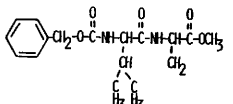
Cbz-Alanyl Valine
Methyl Ester



Cbz-Alanyl-Glycl-Glycine
Methyl Ester



Cbz-Valyl-Valine
Methyl Ester



Cbz-Valyl-Phenylalanine
Methyl Ester

Figure 11. Compounds used to test peak doubling as a function of molecular weight.

doubling as well as for its large molecular weight.

Injections of one, five, ten, fifty, and one hundred microliters of dissolved solute were made on the Partisil-10 column and eluted with an isopropanol-methylene chloride solvent system. The results are summarized in Table I.

Table I. Injection Results for Compounds II-IX in an Isopropanol-methylene Chloride Solvent System.

#	Compound	Mol. Wgt.	Isopropanol	k'	Occurrence of Peak Doubling	Figure
II	Phth-Thr	264	0.15	3.06	No	-
III	Cbz-Val	265	0.15	3.05	No	-
IV	Cbz-Thr	267	1.50	4.31	No	-
V	Cbz-Ala-Ser	325	3.50	4.20	No	-
VI	Cbz-Ala-Val	337	2.00	3.76	Yes	12
VII	Cbz-Ala-Gly-Gly	353	0.50	3.46	Yes	13
VIII	Cbz-Val-Val	365	0.25	3.55	Yes	14
IX	Cbz-Val-Phe	413	2.00	3.25	Yes	15

Note: All compounds are the methyl esters of the above.

The data fails to indicate the source of the peak doubling group present in the compound based merely on the side chain or functional, but it does indicate that there is an apparent relationship between the molecular weight of the peptide and the peak doubling observed; although this observation fails to explain why diastereomer Ia gives only a single peak when Ib exhibits doubling.

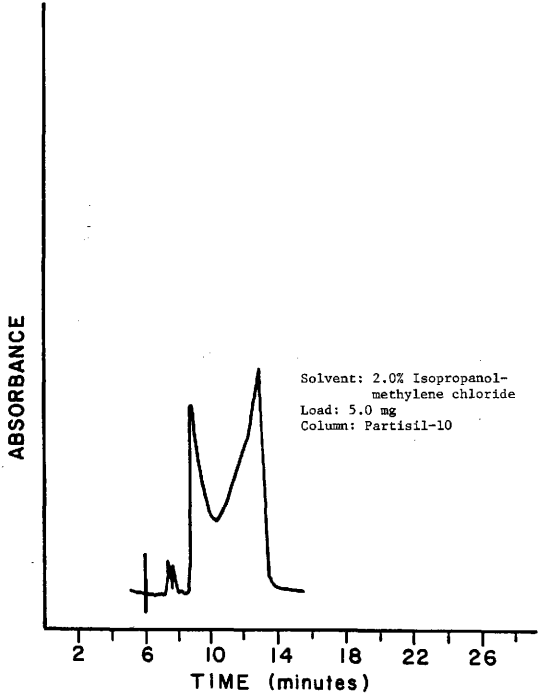


Figure 12. An analytical injection of Cbz-ala-val methyl ester in isopropanol-methylene chloride.

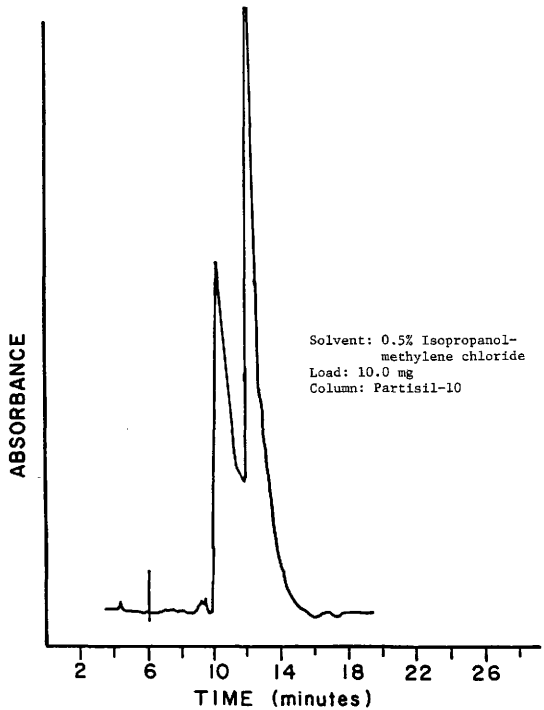


Figure 13. An analytical injection of Cbz-ala-gly-gly methyl ester in isopropanol-methylene chloride

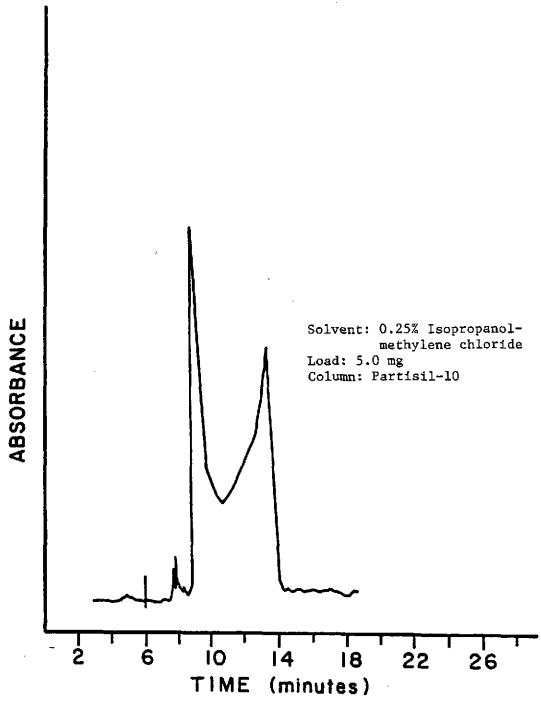


Figure 14. An analytical injection of Cbz-val-val methyl ester in isopropanol-methylene chloride.

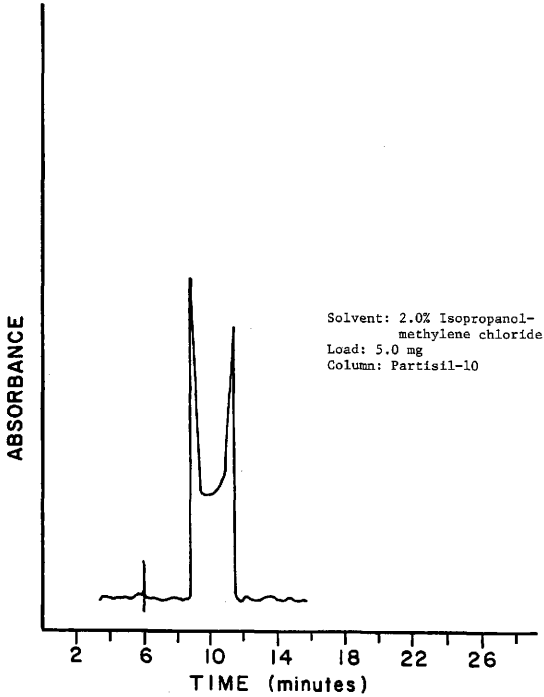


Figure 15. An analytical injection of Cbz-val-phe methyl ester in isopropanol-methylene chloride.

Peak Doubling as a Function of Solvent System

Those peptides which exhibited peak doubling in the isopropanol-methylene chloride solvent system were then eluted from the Partisil-10 column with solvent systems utilizing methanol, *tert*-butanol, octanol, ethylene glycol, and water as cosolvents with methylene chloride to investigate the role of the solvent hydroxyl group in the phenomenon (Table II). The methanol, *tert*-butanol, H_2O and octanol systems exhibited peak doubling while the ethylene glycol system failed to exhibit peak splitting.

Table II. The Occurrence of Peak Splitting in Protic Solvent Systems

Solvent	% Solvent in CH_2Cl_2	Ia	Ib	VI	VII	VIII	IX	Figure
Methanol		No	Yes	Yes	Yes	Yes	Yes	16
t-Butanol	0.12	No	Yes	Yes	Yes	Yes	Yes	17
Octanol	0.25	No	Yes	Yes	Yes	Yes	Yes	18
H_2O	50*	No	Yes	-	-	-	-	19
Ethylene Glycol	<0.1**	-	No	-	-	-	-	20

* H_2O saturated CH_2Cl_2 diluted with an equal volume of dry CH_2Cl_2.

** Ethylene glycol gave no retention for any solvent strength tested.

In looking at the results from these elutions, it was obvious that the protic nature of the solvent system was at least partially responsible for the observed asymmetry. This conclusion was confirmed when aprotic solvent systems of toluene-ethyl acetate [Ib only] (Figure 21)

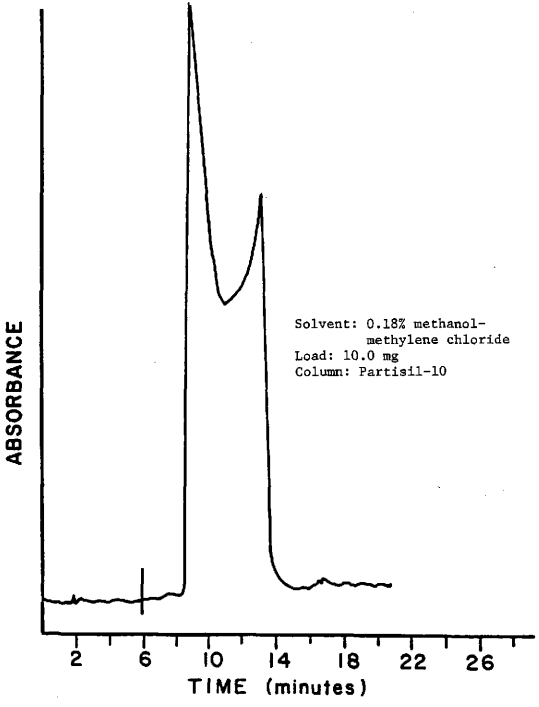


Figure 16. An analytical injection of THPPP(Ib) in methanol-methylene chloride.

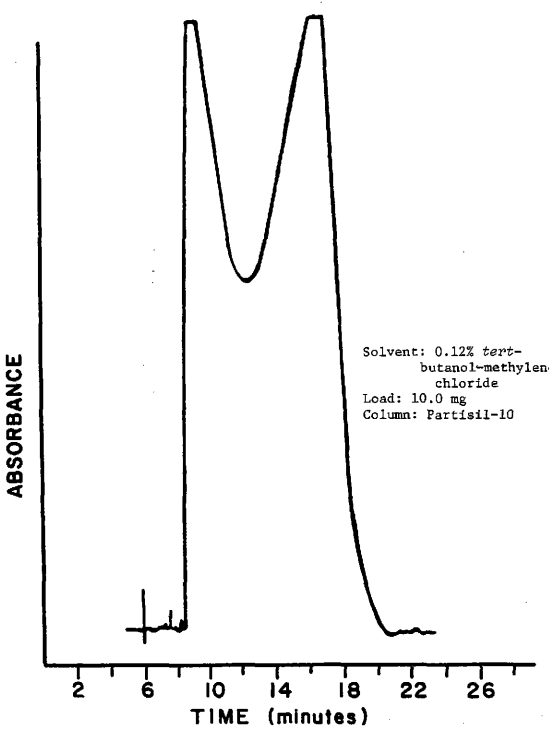


Figure 17. An analytical injection of THPPP(Ib) in *tert*-butanol-methylene chloride.

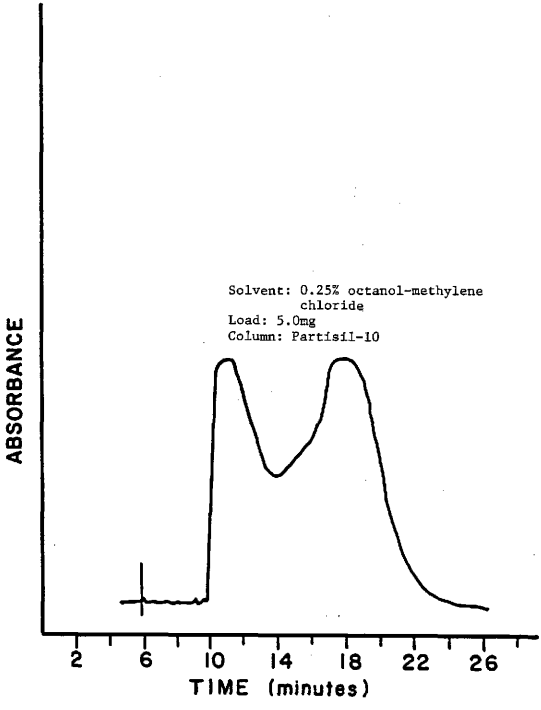


Figure 18. An analytical injection of THPPP(Ib) in octanol-methylene chloride.

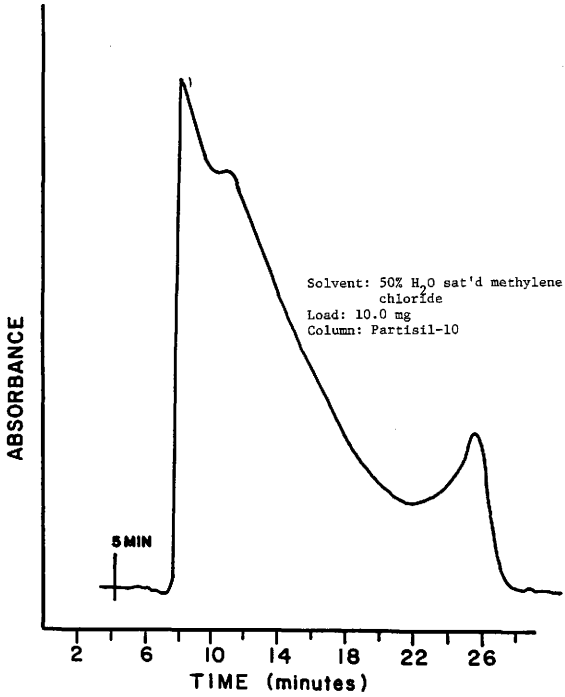


Figure 19. An analytical injection of THPPP(Ib) in H_2O-methylene chloride.

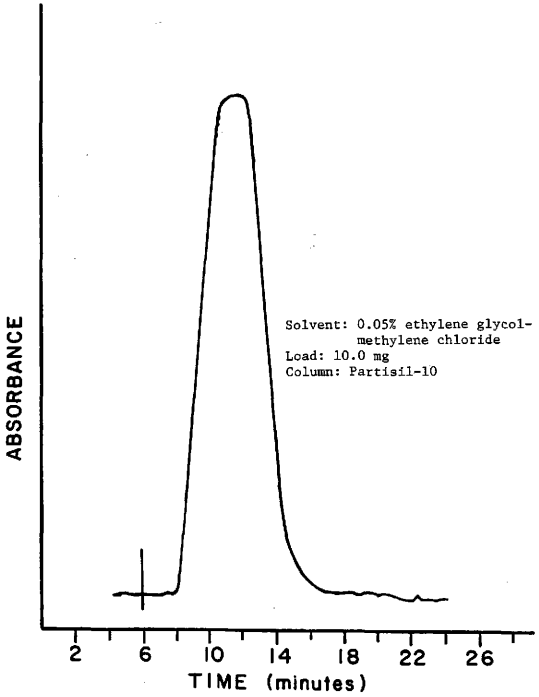


Figure 20. An analytical injection of THPPP(Ib) in ethylene glycol-methylene chloride.

and acetonitrile-methylene chloride (Figure 22) were used to elute those compounds which had previously exhibited peak doubling; the resulting chromatograms showed total suppression of the peak doubling effect, even under column overload conditions.

As a final addition to this section of the procedure, the resolving ability of the acetonitrile-methylene chloride solvent system was checked by the injection of a 1:2 mixture of Ia and Ib. As Figure 22 shows, a ten milligram injection of the mixture upon elution with a 3.0% acetonitrile-methylene chloride solvent gives baseline separation, with two distinct peaks.

Peak Doubling as a Function of Column Packing Material

In order to determine the effect of the column packing pore size of Partisil-10 (60Å) on the peak doubling, columns of Chromasorb LC-6(120 Å pore size) and Vydac 101 TP(330 Å pore size) were prepared and Ib was eluted from the columns with isopropanol-methylene chloride. As can be seen in Figure 23, the peak doubling was exhibited on the Vydac 101 TP column (For column parameters, see Table III).

Peak Doubling as a Function of Hydrogen-bonding Interactions

In an attempt to more clearly elucidate the functional group-molecular weight relationship to peak doubling, it was necessary to synthesize a compound with a molecular weight in the range of those which had exhibited peak doubling in prior experimentation, yet with no protons capable of hydrogen-bonding interactions. 3,5-Dinitrobenzyl 3,4-dinitrobenzoate (Scheme 2) was chosen for this purpose because of

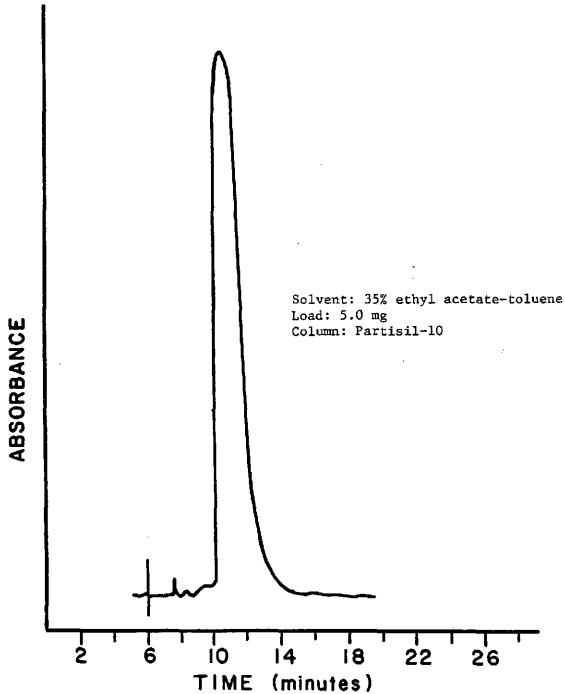


Figure 21. An analytical injection of THPPP(Ib) in toluene-ethyl acetate.

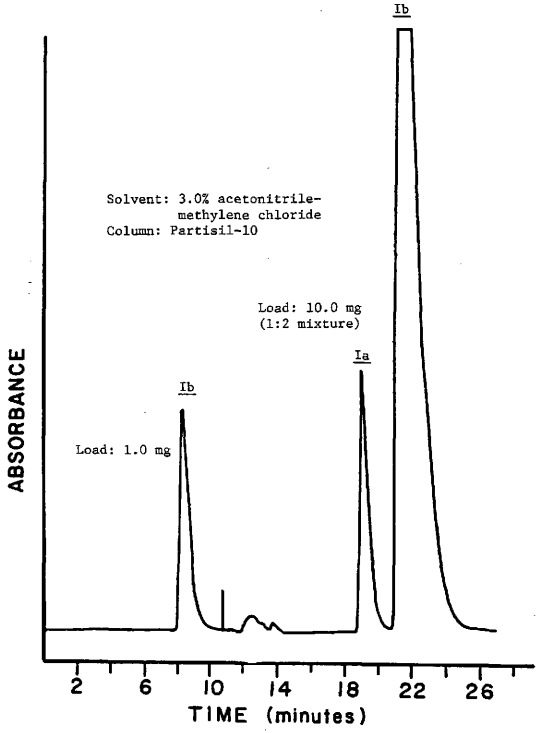


Figure 22. Analytical injections of THPPP(Ib) and a mixture of Ia and Ib in acetonitrile-methylene chloride.

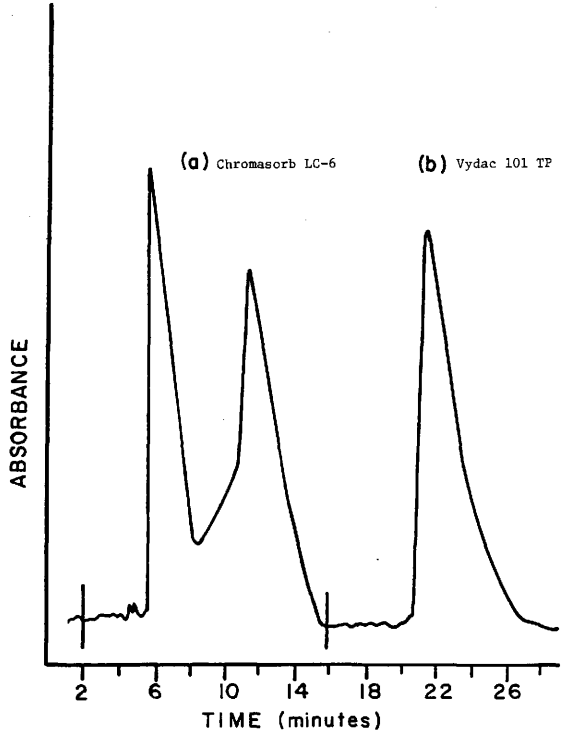


Figure 23. Analytical separation of THPPP (Ib) in isopropanol-methylene chloride on Chromasorb LC-6 and Vydac 101 TP columns.

Table III. Column Parameters for Slurry-Packed Columns.

Packing Materials and Manufacturer	Avg. Particle Size Microns	Avg. Pore Size Angstroms	Column Dimensions	N	N_{eff}	H x 10^2	H x 10^{-3}
Partisil-10 (Whatman)	11	60	4.1mm x 30 cm	5611	3519	5.35	4.86
Chromasorb LC-6 (Johns-Mansville)	10	120	4.1mm x 28cm	5184	2883	5.40	0.54
Vydac 101TP (Separations Group)	10	330	4.1mm x 30cm	7901	5805	3.80	3.80

Refer to pp. 18 for equations.

See Appendix 1 for a qualitative explanation of the column parameters.

its molecular weight (392), its lack of hydrogen-bonding protons, and to its ability to be retained by the column packing due to its four nitro-groups. In an isopropanol-(methylene chloride-hexane 50:50) system [12], the peak of the compound failed to double (Figure 24). This fact, when coupled with the results of the previous determinations, indicates that the hydrogen-bonding proton plays an integral role in the peak splitting phenomenon.

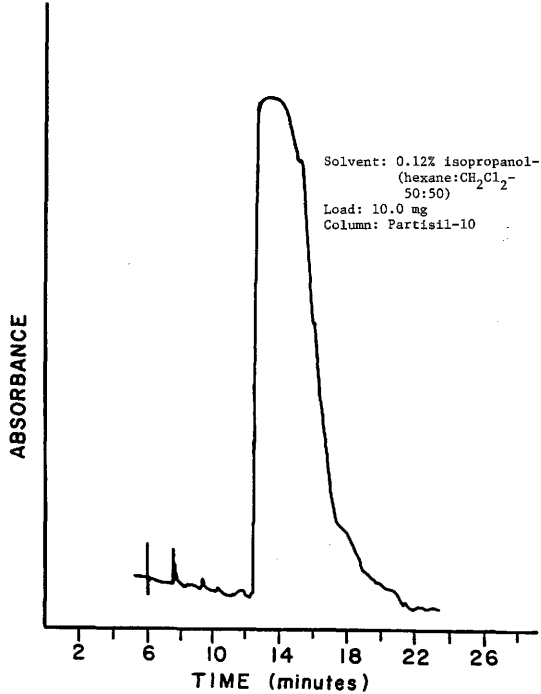


Figure 24. An analytical injection of 3,5-dinitrobenzyl 3,4-dinitrobenzoate in isopropanol-methylene chloride.

CONCLUSION

Our results lead us to conclude that there is an apparent relationship between the molecular weight of a compound and the peak doubling effect, although this relationship by itself fails to explain why the (3S,4S) diastereomer Ib produces a double peak while the (3S,4R) diastereomer Ia does not. A solution for this inconsistency lies in the possibility of intra-molecular hydrogen bonding in Ia that is somehow prevented in Ib. An examination of space-filling models of Ia and Ib (Figure 25) show Ia capable of H-bonding interactions with the carbonyl oxygen of the phthalyl group while both steric and dipole-dipole interactions in Ib prevent effective hydrogen bond formation. Because of the relatively small number of compounds in the study, the results are not definative. However, it would appear that hydrogen bonding protons are required to produce the peak doubling effect. If there is intra-molecular hydrogen bonding in Ia, then the H-bonding proton would be unavailable for whatever extra-molecular role the H-bonding proton has in the peak doubling.

It was noticed during the course of our experimentation that the load capacity of the columns was less than what experience would lead us to predict. We also observed that, in a doubled peak, the size of the right-hand peak became independent of the sample size with increasing column load. Some compounds were observed to be more sensitive to load than others. It was also noticed that the k' for the right-hand peak was that of the original, unsplit peak. Previous chromatograms (Figure 4, p. 6) show that frontal asymmetry leading

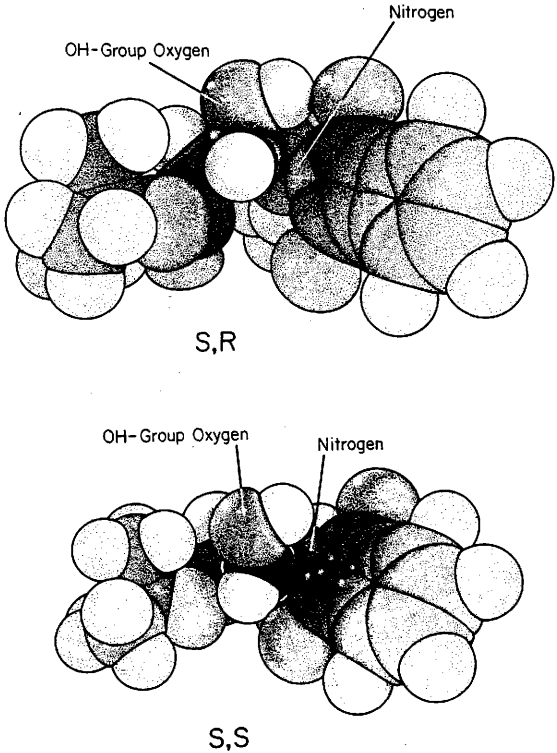


Figure 25. Space-filling models of THPPP diastereomers showing the regions of hydrogen bonding.

to peak doubling can be observed even at lower levels of sample loading.

This being the case, it is possible to theorize that the mechanism of the peak doubling phenomenon might involve partial permeation of the adsorbent pores. This seems a reasonable hypothesis based on the aforementioned observations when coupled with the experimental evidence of molecular weight dependence of the peak doubling.

There exists the possibility that the pore size listed for the column packings used in this study has a large range, such that there is a large percentage of smaller diameter pores and a smaller percentage of larger diameter pores. The protic solvents, possibly by some form of hydrogen bonding within the system, are effectively decreasing the pore diameters. This decrease in pore diameter would have a much more noticeable effect on the smaller diameter pores, possibly giving rise to partial permeation in those pores which are of a small enough size to be affected. As the solute travels through the column, it can equilibrate normally in those pores which are large enough to be relatively unaffected by a decrease in pore diameter due to solvent hydrogen bonding interactions. When these larger pores are completely utilized, the smaller pores, being effectively decreased in diameter, are only capable of being partially permeable, and subsequently give rise to a molecular exclusion-type of effect. This effect would, in turn, produce two elution rates giving rise to two peaks: one at the k' of the original peak, being that of the relatively unaffected permeation of the larger diameter pores as would be predicted by normal adsorption chromatography, and a second peak at a slightly lower k',

caused by the partial permeation of the more hindered, smaller diameter pores. In the area between these two peaks, it is possible that there is a zone in which adsorption and molecular exclusion-type effects will be interacting to give an elution rate that cannot be predicted, but that can be observed under a heavy loading capacity.

The pores of the Vydac 101 TP packing are a great deal larger than either the pores of the Partisil-10 or the Chromasorb LC-6 packings, and will not effectively display partial permeation effects with compounds of the molecular weight used in this study. These compounds were chromatographed on Vydac 101 TP and were found to elute at a single unaffected equilibrium rate to give a single peak.

Of the protic solvents tested, only the ethylene glycol-methylene chloride system failed to show peak splitting. In looking at the chromatogram of Ib in ethylene glycol-methylene chloride (Figure 20, p. 32), it can be seen that only a slight amount of retention is occurring. The k' for this solvent system/solute did not change with a decrease in ethylene glycol concentration, leading us to believe that there is a total lack of solute adsorption due to ethylene glycol being so polar that it is preferentially adsorbed to the exclusion of almost all of the solute molecules.

The aprotic solvent systems, having no interaction with the column packing similar to that of the protic solvents, might be allowing free equilibrium to take place, producing a single peak.

In view of all these findings, it is possible to present an interpretation of the chromatogram of the preparative separation of Ia and Ib as it relates to the peak doubling phenomenon.

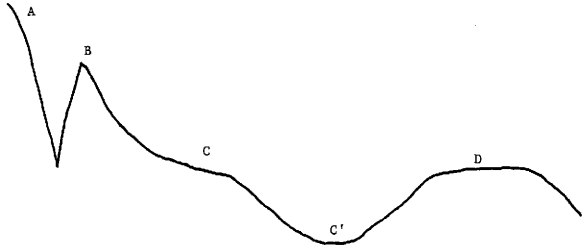


Figure 26. A partial chromatogram of the preparative separation of Ia and Ib in isopropanol-methylene chloride showing the area of peak doubling (from Figure 2, p. 3).

Analysis of the eluent from the peak immediately preceding A showed pure Ia. In the area under B, C, and C', a mixture of Ia and Ib was found. Only under D was pure Ib obtained. B can be identified as that amount of Ib which was eluted based totally on the molecular exclusion-type effect produced by the partial permeation of the smaller diameter pores. In the area under C and C', the combination of molecular exclusion and adsorptive-type phenomena are producing a distorted, poorly resolved "peak" while the solute being eluted at D is that amount of Ib remaining which can freely permeate whatever number of larger diameter pores there are available.

The presence of Ia in B, C, and C' is due largely to the normal tailing of A and not to any involvement of the compound in peak doubling.

A test of an aprotic solvent system in a preparative separation of I (Figure 27) shows baseline separation of Ia and Ib with peak doubling completely suppressed.

In light of these findings, aprotic solvent systems such as toluene-ethyl acetate and acetonitrile-methylene chloride should be tried in cases where peak splitting effects make large-scale separations difficult or impossible. The acetonitrile-methylene chloride system has the advantage, like the alcohol-methylene chloride solvent, of allowing for easy recovery of the methylene chloride by extraction of the acetonitrile with water, drying the methylene chloride with P_2O_5 and distilling the recovered solvent.

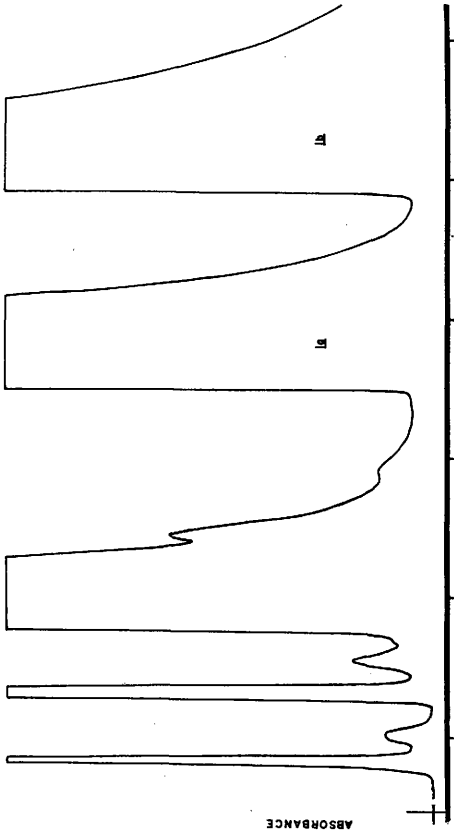


Figure 27. Preparative separation of THPPP in acetonitrile–methylene chloride.

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13. Since it was necessary to use hexane to decrease the polarity of the mobile phase, we first determined that peak doubling was possible in such a system by eluting Ib on a Partisil-10 column with a 0.45% isopropanol solution of 50:50% v/v hexane-methylene chloride.

APPENDIX I

A Qualitative Explanation of Liquid Chromatography Parameters

N — The number of Theoretical plates N is the common expression used to describe the band width. It is approximately constant for different bands in a chromatogram for a given set of operating conditions. Therefore, N is a useful measure of column efficiency: the relative ability of a given column to provide narrow bands and improved separations.

N_{eff} — The effective plate number N_{eff} represents the combined contribution of N and k' (the net retention) to resolution.

H — The height of an equivalent theoretical plate (HETP) given by H is the measure of the efficiency of a column per unit length. Small H values mean more efficient columns or a larger N.

h — The reduced plate height is a dimensionless quantity relating the HETP to the packing particle diameter.

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