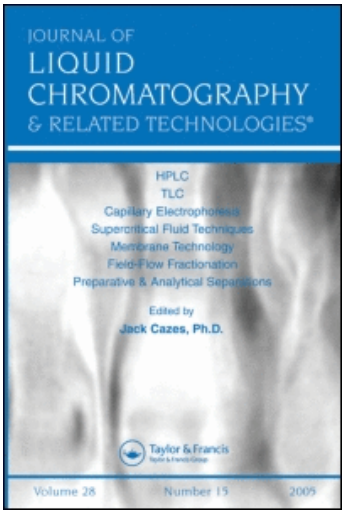

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Separation of Optical Isomers as Diastereomeric Derivatives by High Performance Liquid Chromatography

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SEPARATION OF OPTICAL ISOMERS AS DIASTEREOMERIC DERIVATIVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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1. INTRODUCTION

Optical isomers occuring widely in nature, display different biochemical and pharmacological effects against their enantiomeric isomers. Therefore, it is very important to distinguish or resolve enantiomers, especially in biochemical and pharmaceutical applications.

The classical methods reviewed by Raban et al. (1) for the detection of enantiomeric compositions are accurate only for samples of relatively low optical purity. For quantitative purposes, chromatographic methods offer higher sensitivity of detection. The advantages of chromatographic methods are small sample size, independence from the magnitude of specific rotation and independence from other optically active species initially present in the sample.

The chromatographic separation of optical isomers was first reported by Kotake (2) for the resolution of racemic amino acids

using paper chromatography. Subsequently, Dalgliesh (3) studied the structural features of amino acids necessary for the resolution to occur and proposed the concept of " three point attachment " required for the resolution of optical isomer.

In recent years, a significant number of advances have been made in gas chromatographic separation techniques for the resolution of enantiomers (4-6). Liquid chromatographic methods have been widely used for the separation of enantiomers, giving a moderate-scale separation of synthetic intermediates and final products. For analytical purposes, however, high performance liquid chromatographic (HPLC) method has been shown to be much more efficient because of its high resolution, high sensitivity and versatility.

The resolution of optical isomers by HPLC have been investigated by two different methods. The first approach is the use of chiral solvents or sorbents where a diastereomeric complex is formed by solute-solvent interactions not involving the formation of a covalent bond. The second approach is the alteration of the solubility of enantiomers by derivatization with suitable chiral reagent, where the diastereomers separate on an optically inactive stationary phase.

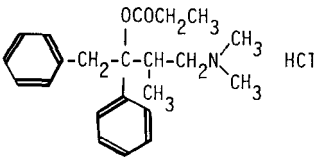
The first method has been investigated by Baczuk (7), Davankov (8-14), Klemm (15), Mikes (16-19), Rebatkem (20), Newman (21) Sogah (22), Lefebvre (23) and their co-workers. The method is in a development stage and is quite complicated by the fact individual antipodes differ only in their chirality and not in solubilities, Further studies are necessary before it can be put into practical use.

However, at present, the second method appears to be gaining popularity. This review deals with the method, i.e. the resolution of optical isomers as diastereomeric derivatives by HPLC.

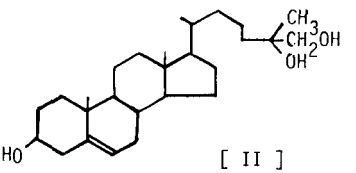
2. ALCOHOL COMPOUND

The α-d- and α-l-isomers of 1,2-diphenyl-2-propionoxy-3-methyl-4-dimethylaminobutane hydrochloride [I] possess analgesic and antitussive activities respectively, while the β-d- and β-l-isomers are almost inactive. Souter (24) described the resolution and quantitative analysis of these diastereomeric isomers by HPLC using a Micropak NH_2 column. The method was valuable to determine the diastereomer content of crude carbinol intermediates.

The resolution of a mixture of (25R)- and (25S)-26-hydroxycholesterol [II] was achieved as 3,26-diacetate derivatives by recycling using two columns packed with μ Porasil and n-hexane containing 2.5 % (v/v) of ethyl acetate as a mobile phase (25).



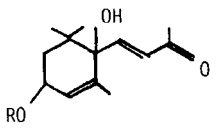
[I]

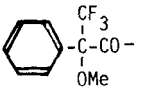


[II]

Koreeda et al. (26) demonstrated preparative-scale separation of the enantiomers of intermediate diol [IIIa] for the synthesis of (+)-abscisic acid. The diol IIIa was converted to a diastereomeric ester [IIIb] with (+)-α-methoxy-α-trifluoromethylphenylacetyl (MTP) chloride (27). The ester was separated by four recycles through a HPLC column of Porasil T with 1 % iso-PrOH in n-hexane. MTP chloride was also used for the determination of the absolute configuration of juvenile hormone [IVa]. Diastereomeric esters [Va] formed from the spiked diols of IVa, were separated by HPLC using three columns packed with Corasil II connected in series (28). The enantiomeric purity of the intermediate diol ethyl ester [Vb] for the synthesis of juvenile hormone was determined using a column of Hitachigel #3040 and 15 % ethyl ether in n-hexane as a mobile phase, after conversion of Vb into the MTP ester [Vc]. As well as Vb, the purity of the diol methyl ester was determined after conversion into the 3β-acetoxyetienyl ester (29).

Acetalization of the 16α-, 17α-dihydroxy group in 16α-hydroxyprednisolone leads to the introduction of a new chiral center at C_{22} of steroid skeleton. Wikby et al. (30) investigated the HPLC



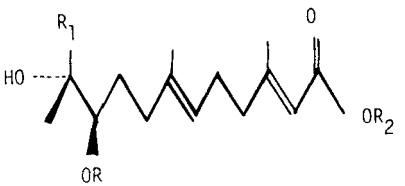


[IIIa] ; R = H

[IIIb] ; R = MTP

[MTP]

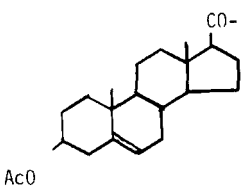
[IVa] ; R_1 = Me or Et



[Va] ; R_1 = Me or Et, R_2 = Me, R = MTP

[Vb] ; R_1 = Me R_2 = Et, R = H

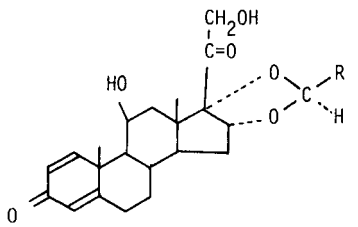
[Vc] ; R_1 = Me R_2 = Et, R = MTP



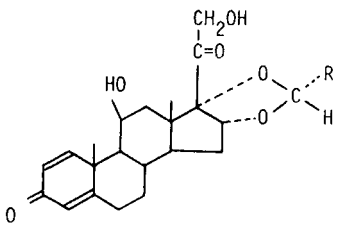
[3β-acetoxyetienyl]

condition for the separation of these epimeric homologues, [VIa] and [VIb] in straight phase system (on μ Bondapak -NH_2, -CN and SiOH with n-hexane • ethanol) and reversed phase system (on μ Bondapak -C_{18}, -alkylphenyl and -CN with water • ethanol). The separation of epimers on straight phase supports increases with increasing polarity of packing materials, i.e. in the order -CN, -SiOH, -NH_2. This suggests that the solute stationary phase interactions contribute to the separation. The length of the alkyl chain at C_{22} has only a marginal effect on the separation of the epimers. On the other hand, the separation of the

epimers on reversed phase supports increases strongly with the carbon number in the substituent at C_{22}. The selectivity increaes with increasing lipophilic charactoer of staionary phase, i.e. in the order -isopropylcyano < -alkylphenyl < $-C_{18}$.



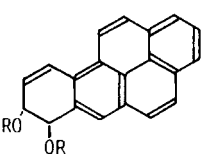
[VIa]



[VIb]

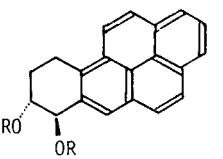
R = $-CH_3$, $-CH_2-CH_3$, $-(CH_2)_2-CH_3$, $-(CH_2)_4-CH_3$

Benzo[a]pyrene, an environmental pollutant, is metabolically converted into (-)-r-7,t-8-dihydroxy-7,8-dihydrobenzo[a]pyrene, which is further metabolized and exerts mutagenicity in mammalian cells. Young et al. (31) described the HPLC method for the separation of the optical isomers of synthetic r-7,t-8-dihydroxy-7,8-dihydrobenzo[a]-pyrene [VIIIa] and their synthetic precursor [VIIa]. The compounds VIIa and VIIIa were converted to diastereomers [VIIb and VIIIb] with di-(-)menthoxyacetyl chloride and then were resolved by using a Zorbax SIL column and methylene chloride containing 0.3 % (v/v) ethyl acetate as a mobile phase.



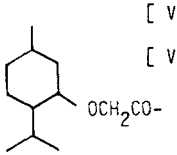
[VIIa] ; R = H

[VIIb] ; R = (-)menthoxyacetyl



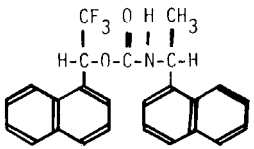
[VIIIa] ; R = H

[VIIIb] ; R = (-)menthoxyacetyl



Racemic 1-(1-naphthyl)-2,2,2-trifluoroethanol, a chiral NMR solvent, was separated on an acidic alumina column as the diastereomers [IX] with (+)-1-(1-naphthyl)ethylisocyanate. This chiral isocyanate also reacts readily with most alcohols to give a mixture of diastereomeric carbamates.

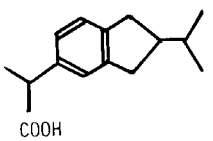
[(-)menthoxyacetyl]



3. CARBOXYLIC ACID COMPOUND

Helmchen et al. (33-34) reported that the resolution of carboxylic acids and amines could be generally achieved by forming diastereomeric secondary amides and separating them by liquid chromatography on silica gel. Later Helmchen and co-workers (35) separated the enantiomer of carboxylic acid, $R_1R_2HCCOOH$ as the diastereomers of amines R_1R_2NH by HPLC on silica gel with n-hexane • ethyl acetate as a mobile phase.

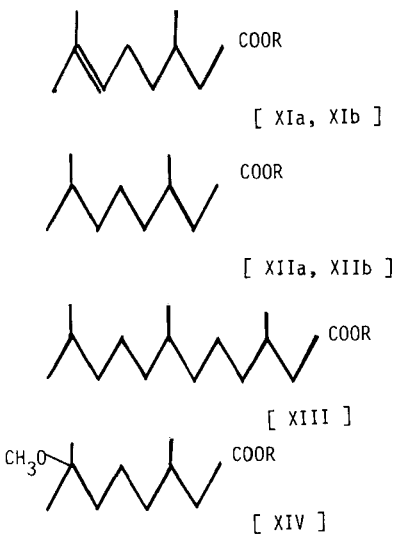
Naruto et al. (36) also separated the diastereomeric isomers of 2-(2-isopropylindan-5-yl)propionic acid [X] with α-1-phenylethylamine by preparative HPLC using μ-Porasil as a stationary phase and ethyl acetate • chloroform • n-hexane (15:15:70) as a mobile phase.



[X]

Valentine,Jr. (37), Scott (38), Bergot (39) and their co-workers described the HPLC method for the separation of enantiomeric isoprenoid acids as the diastereomers with optically pure S(-)- or R(+)-α-methyl-p-nitrobenzylamine. Citronellic acid and related substances

were converted to acid chlorides, and the crude acid chlorides were reacted with R(+)-α-methyl-p-nitrobenzylamine. The crude amines [XIa, XIIa and XIII], thus formed were then separated on a Partisil column with 20 % (v/v) tetrahydrofuran in n-hexane. R/S ratio at C_3 in these acids were calculated from the relative peak areas. Results were in good agreement with the data obtained by the NMR method. Citronellic acid and related substances were also separated as diastereomeric amides [XIb, XIIb and XIV] with R(+)-1-(1-naphthyl)ethylamine, readily available in high optical purity. The corresponding crude amides were purified by preparative thin layer chromatography (TLC) and subjected to HPLC on a Zorbax SIL column with ethyl acetate in n-pentane saturated with water.

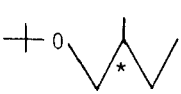


XIa, XIIa, XIII :
R = R(+)-α-methyl-p-nitrobenzylamine
XIb, XIIa, XIV :
R = R(+)-1-(1-naphthyl)ethylamine

Scott et al. (38) demonstrated that isoprenoid acids could be separated as the diastereomeric amides of S(-)- or R(+)-α-methyl-p-nitrobenzylamine by HPLC using a Partisil column and 20 % (v/v) tetrahydrofuran in n-heptane. The capacity factor, k', and selectivity coefficient, α, for the pair of diastereomers obtained by the reaction with α-methyl-p-nitrobenzylamine are given in TABLE 1. There is progressive decrease in α from 2.2 for the C_2-position to 1.0 for the C_5-position.

Recently, it has become clear that arylaliphatic acids, i.e. α-methylfluoren-2-acetic acid (40) and 2-(4-isobuthylphenyl)propionic acid

TABLE 1 Resolution of enantiomeric isoprenoid acids

Acid		k' R	k' S	α
	1)	3.13	6.92	2.21
	2)	7.79	9.22	1.18
	2)	6.52	8.04	1.23
	2)	8.25	7.83	1.05
	2)	7.67	7.67	1.00
	2)	9.00	11.00	1.22
	2)	12.80	8.71	1.47
	2)	9.25	9.50	1.03
	2)	7.58	8.71	1.15

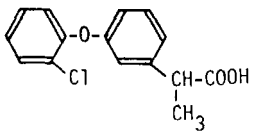
1) 50 cm column, S(-)-α-methyl-p-nitrobenzylamine
2) 50 cm X 2 column, R(+)-α-methyl-p-nitrobenzylamine

(41), are capable of undergoing stereospecific inversion from l-enantiomer to d-enantiomer in vivo. Tamegai et al. (42) applied the HPLC method to the resolution of dl-2-[3-(chlorophenoxyphenyl)]propionic acid [XV] as the diastereomeric amides obtained by treatment with various optically active amine compounds. The resolution factor, R , and minimum detectable quantities of enantiomers of XV are shown in TABLE 2. Tamegai et al. (43) also reported the HPLC method for the identification of optical isomers of XV in rat plasma. The compound XV was converted to the diastereomeric amide by treatment with (+)-2-aminobutane and was then determined by HPLC using a Nucleosil NH_2 column and cyclohexane • ethyl acetate (5:1) as a mobile phase. Time course of the optical isomers of XV in rat plasma after oral administration was measured and a facile epimerization of the l-isomer to d-isomer was proved.

TABLE 2 Resolution of enantiomeric 2-[3-(chlorophenoxyphenyl)]propionic acid

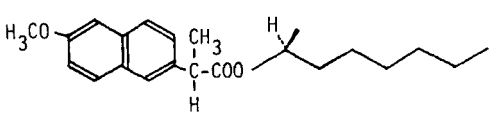
Amino compound	Resolution factor	Minimum detectable quantities (ng)
(+)-2-aminobutane	1.25	100
(+)-2-aminoheptane	2.71	100
D-(+)-1-phenylethylamine	2.27	15
(+)-1-(1-naphthyl)ethylamine	4.11	5
(-)-leucine methylester	1.09	100
(-)-phenylalanine methylester	4.25	50
(-)-glycine methylester	1.00	100

Conditions of HPLC
Column ; Nucleosil NH_2, 10μm, 4.6 mm I.D. X 250 mm
Mobile phase ; cyclohexane • ethyl acetate (5:1)
Detector ; UV_{280} , Flow rate ; 1.5 ml/min.



[XV]

Carboxylic acids can be also separated as diastereomeric esters. Johnson et al. (44) developed the HPLC method for the determination of the enantiomeric purity of naproxen. Naproxen was reacted with S(+)-2-octanol in the presence of concentrated sulfuric acid and the diastereomeric ester [XVI], thus formed was separated on a Spherisorb silica column with 0.5 % (v/v) ethyl acetate in n-hexane.

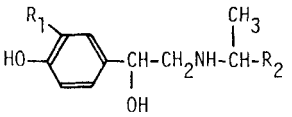


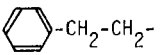
[XVI]

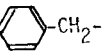
4. AMINO COMPOUND

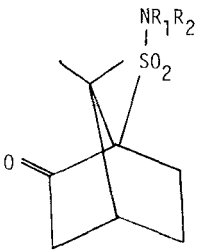
Diastereomers of arylaliphatic amines have been successfully resolved as the corresponding amides. Labetalol [XVIIa], which contains two asymmetric centers and normally consists of two (R)(S)+(S)(R) and (R)(R)+(S)(S) racemic mixtures,was separated into each racemate by Selby et al. as N,O-bis-(dansyl)-derivatives on a Partisil 10 column (45). Tamegai et al. (46) also separated two racemic mixtures of 1-[(4'-hydroxy-3'-methoxymethylphenyl]-2-N-(p-methoxybenzylisopropyl)aminoethanol [XVIIb] as N,O-bis-(benzyl)-derivatives by HPLC on a Zorbax SIL column. The diastereomeric ratio of two racemic mixture was determined.

Souter (47) applied the HPLC method to the separation of amines as the diastereomeric (+)-10-camphorsulfonamide derivatives. [XVIII] Diastereomeric amides were prepared by the procedure of Furukawa et al. (48) from amines by treatment with (+)-10-camphorsulfonyl chloride and were separated on Micropak NH_2. The data are shown in TABLE 3, in which the



[XVIIa] ; R_1= NH_2CO- , R_2 = 

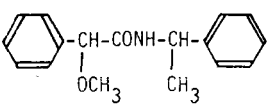
[XVIIb] ; R_1= CH_3OCH_2- , R_2= CH_3O- 



[XVIII]

degree of separation is shown as α (uncorrected) in each case.

α-Phenylethylamines can be separated as the (S)-o-methylmandelylamides [XIX]. Helmchen et al. (49) treated optically active amines with (S)-o-methylmandelyl chloride and resolved the amides by HPLC using a Merckosorb SI 60 column and isooctane • ethyl acetate (4:1) as a mobile phase. The ratio of diastereomers, which corresponds to the optical purity of the amines, was determined by comparing the area of two peaks. The lower limit for detection of (S,S) isomer of XIX in (R,S) was 0.1 % and (R,S) isomer in (S,S) was 0.2 %.



[XIX]

TABLE 3 α-Value for (+)-10-camphorsulfonoamide separation

Amine	(A)	(B)	(C)
α-methylbenzyl	1.60	1.56	1.59
p-methoxy-α-methylbenzyl	1.54	1.56	1.00
m-methoxy-α-methylbenzyl	1.00	1.71	1.73
o-methoxy-α-methylbenzyl	1.31	1.28	1.13
α-methylphenethyl	1.24	1.04	1.10
o-methyl-α-methylphenethyl	1.19	1.00	1.05
p-methoxy-α-methylphenethyl	1.22	1.00	1.03
p-chloro-α-methylphenethyl	1.25	1.08	1.15
3,4-methylenedioxy-α-methylphenethyl	1.24	1.07	1.12
α-ethylphenethyl	1.12	1.00	1.00
1-methyl-3-phenylpropyl	1.13	1.08	1.05
1-methyl-2-phenoxyethyl	No separations		

(A) 8 % (9:1 CH_2Cl_2-isopropanol) in isooctane at 2 cm^3min^{-1}(2000 psi)
(B) 5 % (9:1 CH_2Cl_2-ethanol) in n-hexane at 1.5 cm^3min^{-1}(1100 psi)
(C) 15 % (9:1 buthyl chloride-isopropanol) in n-hexane at 2 cm^3min^{-1}(1500 psi)

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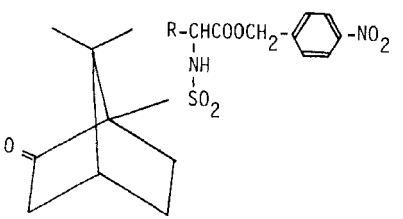
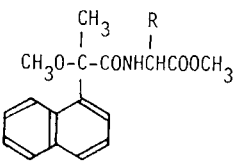
5. AMINO ACID COMPOUND

Amino acids can be converted to a mixture of diastereomers by derivatizing with suitable chiral reagents at either amino or carboxylic radical. Usually, the α-amino group is reacted with chiral reagents and carboxylic group protected by esterification.

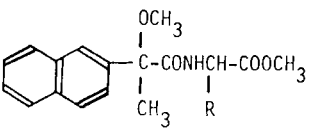


[XX]

Furukawa et al. (48) for the first time reported the HPLC method for the separation of optical isomers of four amino acids. Amino acids were treated with (+)-10-camphorsulfonyl chloride, and then with p-nitrobenzylbromide as the chromophor for detection. The N-(+)-camphorsulfonyl-p-nitrobenzyl esters [XX] thus formed were separated by HPLC on Micropak Si-5. Later they further carried out resolution of methionine, glutamic acid, tryptophane, tyrosine, isoleucine, leucine, phenylalanine and alanine by HPLC on Micropak NH_2 in the same manner (50). Efficient separation of amide derivatives of all amino acids was made under the condition using isooctane • dichloromethane • isopropanol as the eluting solvent. The change of the ratio of isooctane and dichloromethane in the solvent affected the absolute retention time of each amino acid derivative but not the relative retention times of corresponding D- and L-amino acids.



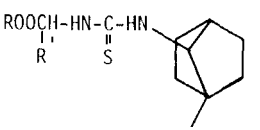
[XXI]



[XXII]

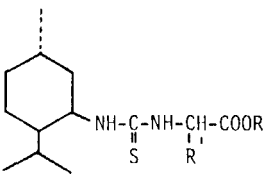
Amino acid can also be converted to diastereomers by derivatizing with (-)-α-methoxy-α-methyl-1-naphthalene acetic acid, (-)-α-methoxy-α-methyl-2-naphthalene acetic acid, (-)-1,7-dimethyl-7-norbornyl isothiocyanate and (+)-neomenthyl isothiocyanate, Goto et al. (51) treated amino acid methyl esters with (-)-α-methoxy-α-methyl-1-naphthalene acetic acid or (-)-α-methoxy-α-methyl-2-naphthalene acetic acid in the presence of N,N'-dicyclohexylcarbodiimide and separated

the corresponding crude amide, [XXI] or [XXII], on a reversed phase column (μ-Bondapak C_{18}) and on a normal phase column (μ-Porasil). All the pair of diastereomeric XXI derived from alanine, valine and proline methyl esters, were resolved on a normal phase column much more efficently than those from XXII. The limit of detection for the amides by fluorometric monitoring was one tenth less than that obtained by UV absorption monitoring. Results of the liquid chromatographic analysis of XXI on reversed phase and normal phase columns are shown in TABLE 4. Amides are more efficiently resolved on a normal phase column than on a reversed phase column. Nambara et al. (52) converted enantiomeric methyl or tert-butyldimethylsilyl ester of amino acid to the diastereomeric thiourea derivatives with two chiral derivatization reagents, (-)-1,7-dimethyl-7-norbornyl isothiocyanate and (+)-neomenthyl isothiocyanate. The corresponding thiourea derivatives, [XXIII] and [XXIV], thus formed were separated on a μ-Porasil column with cyclohexane·ethyl acetate as a mobile phase. The k and R of the diastereomeric thiourea derivatives, [XXIIIa and XXIVa] are listed in TABLE 4. The data suggest that derivatization of amino acids into XXIIIa was much more effective for optical resolution than into XXIVa.



[XXIIIa] ; $R' = CH_3$

[XXIIIb] ; $R' = Si(CH_3)_2$-t-Bu



[XXIVa] ; $R' = CH_3$

[XXIVb] ; $R' = si(CH_3)_2$-t-Bu

TABLE 4 Resolution of enantiomeric amino acid

chiral reagent	XXI						XXIIIa			XXIVa		
Amino acid	Reversed phase k'			Normal phase k'			Normal phase k'			Normal phase k'		
	D	L	R	D	L	R	D	L	R	D	L	R
Alanine	a 1.83	1.83	0.00	c 1.91	3.10	5.84	h 4.96	4.57	1.10	h 3.49	3.04	1.73
Valine	a 3.06	2.66	0.92	c 1.76	1.06	9.17	i 8.06	7.25	1.30	i 6.07	5.71	0.75
Norvaline	a 3.33	2.95	0.75	c 2.17	1.22	5.90	i 9.13	8.00	1.48	i 6.49	6.49	0.00
Leucine	a 3.49	3.20	0.50	c 1.83	0.78	6.14	i 8.62	7.95	1.00	i 3.70	3.91	0.70
Norleucine	a 4.80	3.87	1.00	c 1.92	0.96	6.00	i 6.49	5.86	1.47	i 5.18	5.46	0.66
Isoleucine	a 4.36	3.81	0.95	c 1.53	0.88	4.26	i 7.44	6.55	1.05	i 3.70	3.91	0.63
Proline	a 3.30	3.00	0.50	c 1.42	2.38	2.48	h 5.91	6.64	1.69	h 4.13	4.72	1.79
Phenylglycine	a 3.73	3.57	0.27	c 2.56	1.32	6.22	i 9.38	8.96	0.45	i 7.30	7.53	0.36
Phenylalanine	a 4.00	3.67	0.56	c 2.41	1.52	4.35	i 8.81	7.50	1.77	i 5.23	5.47	0.62
Serine				e 2.52	7.32	8.63	d 4.10	4.20	0.07	g 7.00	6.46	0.59
Threonine	b 2.80	2.64	0.23	f 1.20	1.85	2.98	d 3.14	3.25	0.36	g 5.75	5.54	0.39
Tyrosine	b 4.60	4.60	0.00	d 2.02	1.74	1.02						
DOPA				e 2.32	2.04	0.67						
Cystein				e 2.08	0.98	2.80						
Methionine	b 7.53	6.42	0.97	d 1.60	1.24	1.61						
Aspartic acid				c 5.61	7.00	2.52	h 7.87	8.13	0.48	h 5.72	5.66	0.15
Glutamic acid				c 6.46	6.81	0.62	h 16.3	15.4	0.94	h 11.3	10.1	1.43
Ornitine				f 2.00	3.52	3.33						
Lysine				f 1.60	2.20	1.97						
Tryptophane	b 11.2	11.0	0.15									
Histidine	b 2.50	2.34	0.28	f 1.36	1.96	1.47						

mobile phase ; methanol water (2:1)[a],(3:2)[b]

cyclohexane ethyl acetate (4:1)[c], (2:1)[d], (1:1)[e], (2:3)[f]

cyclohexane (3:1)[g], (10:1)[h], (20:1)[i]

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Scott et al. (38) and Goto et al. (51) strongly implied that the efficiency of separation of diastereomers on a normal phase column would depend largely on the rigidity of conformation. Introduction of a bulky group, i.e. the tert-butyldimethylsilyl residue, into the ester moiety may make the conformation of the molecule more rigid. In fact, derivatization of amino acids to the diastereomeric (+)-neomenthylthiourea silyl esters [XXIVb] was more effective for the separation than derivatives to the (+)-neomenthylthiourea methyl esters [XXIVa].

Penicillins and cephalosporins contain diastereomeric counterpart when acylating agent of 6-aminopenicillanic acid, 7-aminodeacetoxycephalosporanic or 7-aminocephalosporanic acid is a mixture of optical isomers. The separation of such diastereomers of β-lactam antibiotics has been performed using a μ-Bondapak C_{18} column and mixture of appropriate buffers and methanol as a mobile phase.

Salto (53) apllied the HPLC method to the resolution of ampicillin and α-phenoxyethylpenicillin. With 5 % methanol in the mobile phase content, the resolution of ampicillin diastereomer was almost constant between PH 2 to 5, but decreased for PH >5. For each PH level, the resolution efficiency was decreased with the increase in methanol content of the mobile phase. On the other hand , the resolution of α-phenoxyethylpenicillin isomers by HPLC was decreased as the PH and methanol content increased. Meanwhile, Yang (54) and Breuer et al. (55) determined the diastereomeric purity of 7-ureidoacetamide and 7-ureidoacetyl cephalosporins by HPLC also.

6. PEPTIDE COMPOUND

Enantiomers of amino acids can be separated as [L-Leu]dipeptide diastereomers (56). Free D- and L-amino acids were reacted with tert-butyloxycarbonyl-L-leucine-N-hydroxysuccinimide, followed by acidic cleavage of the tert-butyloxycarbonyl group. The resulting [L-Leu]-dipeptide diastereomers were separated on a standard amino acid analyzer using sulfonated polystyrene column. For chromatography of [L-Leu]-dipeptide containing Tyr, Phe or Lys, a long column was used, while a short column sufficed to resolve [L-Leu]dipeptide containing His or Arg. Diastereomers of short chain peptides have been separated by column chromatography. Feltkamp et al. (57) resolved L,L- and D,L-isomer of Ala-Leu using a column packed with silicagel and a eluting solvent containing n-butanol, water and acetic acid. Wieland et al. (58)

separated the diastereomers of Ala-Tyr, Val-Tyr, Ala-Phe and Met-Ala on Sephadex G-25 or G-50 column with pyridine • water (1:1). Diastereomers of tetrapeptide, Leu-Ala-Gly-Val were separated on a standard amino acid analyzer using a sulfonated polystyrene column with 0.2 N sodium citrate buffers (59). The single D-amino acid diastereomers, L-Leu-D-Ala-Bly-L-Val and D-Leu-L-Ala-Bly-L-Val, were separated from one another and from the all L-amino acid tetrapeptide.

Kroeff et al. (60) described the HPLC factor which influences the retention of diastereomeric di- and tripeptide. HPLC was performed on a bonded reversed stationary phase of C_8 type (Lichrosorb RP 8), with the eluting agent made from ethanol and phosphate salts (or HCl) at μ = 0.1. The k' for di- and tripeptide diastereomers are shown in TABLE 5 and 6. In dipeptide in which there is a significant difference in the size and polarity of the amino acid subunits, the difference in the k' values for diastereomers is large enough to permit their separation. Also, retention differences for the given set of diastereomers are enhanced by using a lower PH and/or reducing the amount of ethanol in the eluting mixture.

TABLE 5 Capacity Factor for Di- and Trialanine Diastereomers

Peptide \ PH *	2.10	2.32	5.75	7.89
L-Ala-L-Ala	0.28	0.17	0.00	0.00
D-Ala-D-Ala	0.29	0.18	0.00	0.09
L-Ala-D-Ala	1.13	0.65	0.16	0.21
D-Ala-L-Ala	1.11	0.64	0.17	0.20
L-Ala-L-Ala-L-Ala	0.68	0.62	0.09	0.26
D-Ala-D-Ala-D-Ala	0.69	0.63	0.09	0.29
L-Ala-L-Ala-D-Ala	1.59	1.14	0.22	0.59
L-Ala-D-Ala-L-Ala	3.32	2.58	0.69	1.18

* using 5 % EtOH ÷ 95 % H_2O with phosphate buffer or HCl

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TABLE 6 Capacity Factor for di- and tripeptide diastereomers

Peptide \ PH	3.45*	5.98*	6.14**
L(D)-Ala-L(D)-Val	0.30	0.03	
L(D)-Ala-D(L)-Val	0.69	0.21	
L-Ala-L-Leu	1.37	0.30	
D-Ala-L-Leu	2.51	0.88	
L-Ala-L-Phe	1.65	0.61	
D-Ala-D-Phe	1.65	0.61	
L-Ala-D-Phe	2.83	1.48	
D-Ala-L-Phe	2.84	1.46	
L-Leu-L-Tyr	1.74	0.62	
D-Leu-L-Tyr	2.63	1.38	
L-Val-L-Val	0.59	0.12	
D-Val-D-Val	0.58	0.11	
D-Val-L-Val	3.50	1.55	
D-Leu-D-Leu	7.35	2.71	0.92
D-Leu-L-Leu	>10.0	>10.0	4.79
L-Leu-L-Leu			0.92
L-Leu-D-Leu			4.78
L(D)Leu-L(D)Phe			2.16
L(D)Leu-D(L)Phe			6.94
L(D)Leu-Gly-L(D)Phe			1.12
L(D)Leu-Gly-D(L)Phe			1.58

* using 10 % EtOH + 90 % H_2O with phosphate buffer

** using 20 % EtOH + 80 % H_2O with phosphate buffer

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In dipeptide, the L-L and D-D enantiomers have an identical k' values which is less than a identical k' values found for the L-D and D-L enantiomers. The fact can be applicable to tripeptide diastereomers. In the L-D and D-L forms of Ala-Ala, the methyl groups for the two Ala

subunits may be on the same side of the peptide bond and in close proximity to one another. This should produce a higher overall hydrophobic surface area and increase the retention of the L-D and D-L enantiomers, in comparison to the L-L and D-D enantiomers, where the methyl groups are on oppsite side.

Furthermore, Larsen et al. (61) showed efficient separation of diastereomers of oxytocin and its analoges by HPLC, using two μ-Bondapak C_{18} column connected inseries and an eluant composed of 0.1 M ammonium acetate (PH 4.0) and acetonitrile.

7. CONCLUSIONS

This promising field of research has been just begun and has in fact some problems to overcome. The chiral reagents used in this method are not always available in optically pure form. Futhermore, a change of the original enantiomeric ratio may occur during derivatization to the diastereomers. These two erroneous factors may be quite troublesome, if a high accuracy is required, when the sample nearly contains enantiomeric impurity. In addition, the approach indicated in this review is only applicable to the compounds containing hydroxy, carboxylic and amino groups.

Further investigation is necessary to establish the usefulness of the method in high performance liquid chromatography.

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