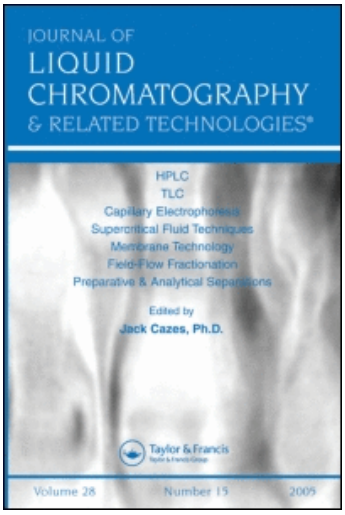

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METHODS OF TLC RESOLUTION OF ENANTIOMERIC AMINO ACIDS AND THEIR DERIVATIVES

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INTRODUCTION

It has been detected that racemization of amino acids in metabolically stable proteins of living mammals takes place (1-4) and as a consequence the protein-structure function relationship may be altered (5). Thus in vitro and in vivo analysis of racemic amino acids is very important. Besides enantiomeric purity becomes very significant when only one of the enantiomers has a potential biological activity for example L-DOPA (3,4-dihydroxyphenyl alanine) is an important drug for treating Parkingson's disease. The measurement of specific rotation is a common and well accepted method for evaluating

the enantiomeric purity of chiral compounds. The determination of enantiomeric excess (ee value) is influenced by the presence of impurities, change in concentration, solvent and temperature (6), and requires the $[\alpha]_D$ value for the pure enantiomer. The availability of a reliable optically pure standard would depend upon the analytical method by which it has been resolved from the enantiomeric or racemic mixture of the compound in question.

The chromatographic separation of enantiomers is an important and rapidly expanding field. There have been a variety of liquid chromatographic approaches utilized successfully to the separation of enantiomers (7-9) but there are only a few reports on thin layer chromatographic separation of enantiomers even though, tlc provides a direct method for resolutions and analytical control of the enantiomeric purity. This may probably be due to the reason that tlc has been applied for such resolutions in recent years. TLC methods/results for the resolution of enantiomers of amino acids and some of their derivatives reported in 1980s have been described in this report.

In general, tlc of enantiomers of amino acids or their derivatives has been carried out using the following approaches :

(i) use of plate impregnated with chiral selector either through mixing the chiral selector with the slurry of the absorbent or by developing the plate of the untreated adsorbent in a solution of the chiral selector prior to sample application, (ii) addition of the chiral selector to the mobile phase, and (iii) pre-derivatization of the enantiomers.

DL-Amino Acids

Yuasa reported (10) tlc separation of D,L-tryptophan on crystalline cellulose coated plates. Gunther et al (11) prepared the plates with hydrophobiated silicic acid (RP 18-TLC), immersed (1 min) in a 0.25% copper (II) acetate solution (MeOH-H_2O), 1:9, v/v), dried and then immersed in a 0.8% methanolic solution of the chiral selector (1 min); the chiral selector was (2S, 4R, 2'RS)-4-hydroxy-1-2'-hydroxydodecyl) proline. They reported the resolution of racemic α-amino acids by developing such plates in MeOH-H_2O-MeCN(50 : 50 : 200; or 50 : 50 : 3, vvv) as shown in Table - 1. Using the same approach Gunther, Martens and coworkers were able to make their systems commercially available as CHIRALPLATE marketed by Machery-Nagel (12). Martens et al (13) and Gunther et al (14) have reported the resolution of DL-methyl DOPA and DL-DOPA on CHIRALPLATE using

Table 1 ENANTIOMERIC RESOLUTION OF AMINO ACIDS BY THIN LAYER CHROMATOGRAPHY (11)

Amino acid	R_f value (configuration)	Eluent
Isoleucine	0.37 (2R,3R)	A
	0.44 (2S,3S)	
Phenylalanine	0.38 (R)	A
	0.45 (S)	
Tyrosine	0.26 (S)	B
	0.34 (R)	
Tryptophan	0.39 (R)	A
	0.45 (S)	
Proline	0.40 (R)	B
	0.59 (S)	
Glutamine	0.37 (S)	A
	0.53 (R)	

Development distance 14 cm.
Saturated chamber A: methanol-water-acetonitrile = 50:50:200 (vvv);
B: methanol-water-acetonitrile = 50:50:30 (vvv).

From Gunther et al. (1984).

MeOH-H_2O-MeCN(50:50:30) as mobile phase and ninhydrin as the locating reagent. The R_f values for L-DOPa and D-Dopa were reported to be 0.47 and 0.61 respectively and the system was capable of resolving enantiomers in trace amounts with the lowest level of detection for the D-enantiomer in L-Dopa samples being > 0.25% (14).

Brinkman and Kamminga (15) achieved success in rapidly separating enantiomers of amino acids on octadecyl modified silicic acid plates containing Cu acetate and using the chiral selector and mobile phase of Gunther et al (11). Reuterbories and Nurok (16) prederivatised amino acids with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and resolved 14 diastereoisomeric pairs on a bonded C_{18} silica gel plate using HOAC-tert Bu_2O as mobile phase.

Bhushan and coworkers (17-20) prepared tlc plates with silica gel mixed with (-) brucine or berberine, the optically pure alkaloids, and resolved various DL-Amino acids as shown in Table - 2. The optically pure chiral base mixed in the silica gel formed diastereoisomeric salts of the corresponding (+) and (-) amino acids which were hydrolysed on the plate by spraying dil HCl after developing the chromatograms in BuOH-HOAc-$CHCl_3$ (3:1:4) or EtOH-HOAc-$CHCl_3$ (3:1:6).

Table 2 RESOLUTION DATA FOR ENANTIOMERS OF AMINO ACIDS

D-L Amino Acid	I		
	hR_f pure	Resolved hR_f	
	L	D	L
1. Alanine	53	18	53
2. 2-Amino butyric acid			
3. Isoleucine	35	16	35
4. DOPA			
5. Leucine			
6. Methionine	29	18	29
7. Norleucine			
8. Phenylalanine	40	27	40
9. Serine	50	12	50
10. Threonine	29	16	29
11. Tryptophan	31	17	31
12. Tyrosine	29	22	29

I: Silica plates impregnated with (-)-brucine, developed in n-butanol-acetic acid-chloroform (3:1:4), upto 10 cm.

Adapted from Bhushan and Ali (1987, 1988).

The colors were developed by usual ninhydrin spray. It has been observed that sometimes resolution on brucine impregnated plates is achieved when ninhydrin sprayed plate is developed again in the same solvent. Table 3 shows a few other systems for the resolution of D,L-amino acids on plates

Table 3 hR_f values OF D,L AMINO ACIDS AS RESOLVED IN VARIOUS SYSTEM (20).

	I		II		III		IV	
	D	L	D	L	D	L	D	L
Leu	-	-	65	90	-	-	-	-
Thr	35	68	44	67	0	18	91	46
Val	37	58	49	67	0	20	14	40
Try	67	87	72	83	0	17	5	41
Ala	38	57	50	65	0	2	41	
Tyr	37	69	46	67	0	19	-	-
Ser	29	87	40	75	0	14	5	39
Thr	31	85	41	77	0	15	6	38
Lys	24	62	-	-	-	-	-	-
Glu	37	76	-	-	0	18	7	48
2-aminobutyric acid	-	-	40	85	-	-	-	-

Samples were applied after developing the activated silica gel plates in (-) Brucine (0.2% in $CHCl_3$)

I : BuOH-HOAC-H_2O (4:1:5) at 19°C followed by Phenol-H_2O (3:1) at 36°C and ninhydrin spray.

II : BuOH-HOAC-H_2O (4:1:5) at 19°C ninhydrin spray, followed by Phenol-H_2O (3:1) at 36°C.

III : $CHCl_3$-MeOH-MeCN (95:5:5), ninhydrin spray followed by $CHCl_3$-MeOH-MeCN (95:5:5), 22°C.

IV : MeOH-MeCN (1:4), ninhydrin spray followed by MeOH-MeCN (1:4) at 22°C.

From Bhushan and Gupta (1988).

first developed in chloroform solution of (-) brucine prior to sample (DL-mixture) application (20).

DERIVATIVES OF AMINO ACIDS

The resolution of enantiomers of α-substituted α-amino acids (21) and racemic mixtures of natural and non natural amino acids, N-methylaled and N-formylated amino acids and various other derivatives of amino acids (22) has been reported by Gunther et al (21) on ready to use CHIRALPLATES, some of the results are presented in Tables 4,5. It is reported that analysis of the enantiomeric purity of a wide variety of α-branched α-amino acids is possible by these methods in less than 2 hr and is more sensitive than NMR techniques (23) and requires no derivatization step.

The adsorption of the chiral metal complex such as Cu(II) complex of N,N-di n-propyl-L-alanine on the reverse phase particles in the HPLC column suggesting selective interactions with the enantiomer at the interface between the solid and mobile phase (24) led Weinstein (25) to impregnate reverse phase tlc plates with chiral metal complex and resolve dansyl amino acids as follows :

Reverse phase tlc plates from Whatman, were developed (prior to application of dansyl amino

Table 4 ENANTIOMERIC RESOLUTION OF RACEMATES BY TLC (21)

Racemate	Rf value (configuration)		Eluent
Valine	0.54(D)	0.62(L)	A
Methionine	0.54(D)	0.59(L)	A
allo-Isoleucine	0.51(D)	0.61(L)	A
Norleucine	0.53(D)	0.62(L)	A
2-Aminobutyric acid	0.48	0.52	A
O-Benzylserine	0.54(D)	0.65(L)	A
3-Chloralanine	0.57	0.64	A
S-(2-Chlorobenzyl)-cysteine	0.45	0.58	A
S-(3-Thiabutyl)-cysteine	0.53	0.64	A
S-(2-Thiapropyl)-cysteine	0.53	0.64	A
cis-4-Hydroxyproline	0.41(L)	0.59(D)	A
Phenylglycine	0.57	0.67	A
3-Cyclopentylalanine	0.46	0.56	A
Homophenylalanine	0.49(D)	0.58(L)	A
4-Methoxyphenylalanine	0.52	0.64	A
4-Aminophenylalanine	0.33	0.47	A
4-Bromophenylalanine	0.44	0.58	A
4-Chlorophenylalanine	0.46	0.59	A
2-Fluorophenylalanine	0.55	0.61	A
4-Iodophenylalanine	0.45(D)	0.61(L)	A
4-Nitrophenylalanine	0.52	0.61	A
O-Benzyltyrosine	0.48(D)	0.64(L)	A
3-Fluorotyrosine	0.64	0.71	A
4-Methyltryptophan	0.50	0.58	A
5-Methyltryptophan	0.52	0.63	A
6-Methyltryptophan	0.52	0.64	A
7-Methyltryptophan	0.51	0.64	A
5-Bromotryptophan	0.46	0.58	A
5-Methoxytryptophan	0.55	0.66	A
2-(1-Methylcyclopropyl)-glycin	0.49	0.57	A
N-Methylphenylalanine	0.50(D)	0.61(L)	A
N-Formyl-tert-leucine	0.48(+)	0.61(-)	A

A: methanol-water-acetonitrile=50:50:200 (vvv);

B: methanol-water-acetonitrile=50:50:30 (vvv)

Development distance 13 cm, saturated chamber.

From Gunther and Schikedanz (1985).

Table 5 ENANTIOMERIC RESOLUTION OF α-DIALKYL AMINO ACIDS BY TLC (22)

Parent amino acid	R^1	R^2	R_f value (configuration		Eluent
Asp	CH_2CO_2H	CH_3	0.52(D)	0.56(L)	A
Glu	$(CH_2)_2CO_2H$	CH_3	0.58(L)	0.62(D)	A
Leu	$CH_2CH(CH_3)_2$	CH_3	0.48	0.59	A
Phe	$CH_2C_6H_5$	CH_3	0.53(L)	0.66(D)	A
Ser	CH_2OH	CH_3	0.56(L)	0.67(D)	B
Trp	CH_2-3-indolyl	CH_3	0.54	0.65	A
Tyr	CH_2-(4-OH-C_6H_4)	CH_3	0.63(D)	0.70(L)	A
Val	$CH(CH_3)_2$	CH_3	0.51	0.56	A
α-Amino butyric acid	CH_2CH_3	CH_3	0.50	0.60	A
Phe	$CH_2C_6H_5$	CHF_2	0.63	0.70	A
Phe	$CH_2C_6H_5$	CH_2-CH=CH_2	0.57	0.63	A
Phe	$CH_2C_6H_5$	$CH_2CH_2SCH_3$	0.57	0.62	A

Eluent A: methanol-water-acetonitrile=1:1:4(vvv); Eluent B: methanol-water-acetonitrile=5:5:3 (vvv); Development distance 13 cm, saturated chamber
From Gunther et al (1986).

acids) in 0.3 M sodium acetate in 40% acetonitrile and 60% water adjusted to pH 7 by acetic acid (Buffer A). After fan drying, the plates were immersed in a solution of 8 mM N,N-di-n-propyl-L-alanine and 4 mM cupric acetate in 97.5% acetonitrile and 2.5% water for one hour and upto overnight and left to dry in air. After applying samples the plates were developed in 'Buffer A' with or without N,N-di-n-propyl-L-alanine (4 mM) and cupric acetate (1 mM) dissolved in it. The enantiomers were located under uv (360 nm) as fluorescent yellow-green spots. Use of 25% acetonitrile was preferred for glutamic and aspartic acids and serine and threonine derivatives. The method is considered to be fast and sensitive and quantitation is possible by densitometry or by measuring the fluorescence or uv absorption of the extracted spots.

Grinberg and Weinstein (26) reported a two dimensional RP-TLC technique for the resolution of complex mixtures of dansyl amino acids. The Dns-derivatives were first separated in a non-chiral mode using 0.3 M sodium acetate in H_2O-MeCN (80 : 20, pH 6.3), to which was added 0.3 M sodium acetate in H_2O-MeCN (70 : 30) to give a final acetonitrile concentration of 38% or 47%. For

the second dimension the mobile phase was 8 mM N,N-di-n-propyl-L-alanine and 4 mM copper (II) acetate dissolved in 0.3 M sodium acetate in H_2O-MeCN (70 : 30, pH 7) and the plates were developed in the second dimension using a temperature gradient. The method is reported to be applicable for the resolution of amino acids in a protein hydrolysate and quantitation by densitometry.

N,N-di-n-propyl alanine can be prepared by following Bowman and Stroud's procedure (27): L-alanine (17.8g) is taken in ethanol (200 ml) and, 10% palladium on activated coal catalyst (3g) and propionaldehyde (43 ml) are added. The mixture is hydrogenated for 48 hr at 40 - 50°C at an initial hydrogen pressure of 50 psi. The catalyst is removed using a sintered glass filter and the filtrate is evaporated to dryness. The reaction product (N,N-di-n-propyl-L-alanine) is crystallized from chloroform, and the purity may be confirmed by tlc, NMR and C,H,N analysis.

Resolution of enantiomers of dansyl amino acids and β-napthylamide amino acids using β-cyclodextrin (β-CD) plates was carried out by Alak and Armstrong (28). The plates were prepared by mixing 1.5 g of β-CD bonded silica gel in 15 mL of 50% methanol (aq) with 0.002 g of binder (ASTE-all

Table 6 SEPARATION DATA FOR ENANTIOMERIC COMPOUNDS ON β-CD BONDED PHASE PLATES (28)

Compound D,L mixture	R_f D	R_f L	Mobile phase*	Detection method
1. Dns-leucine	0.49	0.66	40/60	fluorescence
2. Dns-methionine	0.28	0.43	25/75	-do-
3. Dns-alanine	0.25	0.33	25/75	-do-
4. Dns-valine	0.31	0.42	25/75	-do-
5. Alanine-β-naphthylamide	0.16	0.25	30/70	ninhydrin
6. Methionine-β-naphthylamide	0.16	0.24	30/70	ninhydrin

*Volume ratio of methanol to 1% triethylammonium acetate (pH 4.1)

Adapted from Alak and Armstrong (1986)

solvent binder), and 50/50 MeOH-1% aq triethyl ammonium acetate (pH 4.1) as mobile phase. Some of these results are shown in Table - 6. The resolution of enantiomers of phenyl thio hydantoin (PTH-)amino acids has been carried out by Bhushan and Ali (19) using (+) tartaric acid or (-) ascorbic acid as

Table 7 hR_F OF PURE AND RESOLVED ENANTIOMERS OF PTH-AMINO ACIDS, ON TARTARIC ACID IMPREGNATED PLATE (19)

DL. Mixture of PTH-amino acids	hR_F or pure L	hR_F	
		D	L
Met	83	16	83
Phe	85	15	85
Try	96	-	95
Val	80	21	80
Ile	92	15	92
Tyr	95	16	95
Thr	85	30	85
Ala	55	12	55
Ser	84	10	84

Solvent:chloroform-ethyl acetate-water (28:1:1).
Development time:35 min. Solvent front: 10 cm.
Room temperature 25 ±1°C

Impregnation with (+)-ascorbic acid resolved DL mixtures of PTH-Met,Phe,Val,Thr,ala, Ser,
From Bhusnan and Ali (1987).

the impregnating reagents. The hR_f values for a few derivatives are recorded in Table 7.

The GC resolution of 2-alkyl amino acids is modest (29), the GC and HPLC methods of enantiomeric separations of racemic amino acids and their derivatives are time consuming and require costly equipment. The TLC methods are simple and allow easy adjustment of chromatographic parameters. Besides quantative results may be obtained by densitometry or by measuring the fluorescence or uv absorption of the extracted spots. TLC is being applied for the resolution of more and more compounds including the ones which have been resolved by HPLC (25) and it is believed that enantiomeric resolution by tlc may become a routine method in future.

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