Chapter 2

Enantioseparations by Thin-Layer Chromatography

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Abstract

An up-to-date overview of thin-layer chromatography (TLC) techniques for chiral separations of various significant and/or recent examples of enantioresolutions is reported. Furthermore, examples for chiral separations obtained on achiral commercially available C18 TLC plates are described in detail. These include the enantioseparation of methylthiohydantoin-phenylalanine and methylthiohydantoin-tyrosine using hydroxyethyl-β-cyclodextrin as mobile phase additive and the separation of the enantiomers of warfarin and *p*-chlorowarfarin using bovine serum albumin as mobile phase additive.

Key words: Enantioseparation, Chiral thin-layer chromatography, Methylthiohydantoin amino acids, Warfarin, *p*-Chlorowarfarin, Cyclodextrin, Bovine serum albumin

1. Introduction

The direct separation and quantitative determination of optical antipodes by thin-layer chromatography (TLC) can be achieved with different analytical approaches: (1) using commercial (ready-to-use plates) and noncommercial (homemade layers) chiral stationary phases (CSPs) in combination with achiral mobile phases, (2) using commercial and noncommercial achiral TLC plates impregnated with chiral selectors (chiral-coated stationary phases, CCSPs), or (3) achiral TLC plates in combination with chiral additives to the mobile phases (chiral mobile phases additives, CMPAs).

A complete and up-to-date review of these topics was given in a recent book (1) that also encouraged further research in TLC enantioseparations in order to advance this important analytical technique.

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1.1. Commercial and Noncommercial CSPs with Achiral Mobile Phases

1.1.1. Native Cellulose

Homemade and precoated plates of native and microcrystalline cellulose (commercialized under the trade name Avicel™) have been used for direct resolution of highly polar optical antipodes, such as amino acids and dipeptides, mainly using the normal-phase (NP) mode (2–14). In this regard, the work of Lederer should be mentioned (15) describing the resolution of a number of aromatic amino acids using plastic-backed cellulose plates and an aqueous solution of 0.1 M sodium chloride, or a mixture of ethanol–pyridine–water 1/1/1 (v/v/v) as eluents.

1.1.2. Modified Cellulose

Precoated plates of fibrous acetylated cellulose (10 and 20% acetylation degree) are commercially available from Analtech (Newark, DE, USA) and Macherey-Nagel (Düren, Germany). However, only few papers (16, 17) reported enantioseparations using these TLC plates, due to their low chiral resolution related to the low acetylation degree and the lack of a microcrystalline structure. In addition, they are not suitable to be used with eluent mixtures containing a high content of ethanol (e.g., 80% aqueous ethanol) (17). Conversely, more than 75 chiral molecules (18–24) have been resolved on homemade plates of microcrystalline cellulose triacetate (MCTA for HPLC, Sigma-Aldrich, Milwaukee, IW, USA). Table 1 summarizes enantioresolutions obtained using such MCTA material. Among the numerous separations achieved on MCTA, the ones concerning uncharged enantiomers with a carbonyl group in the α- or β-position with respect to the stereogenic center highlight the fundamental role of dipole–dipole interactions between the carbonyl group of the solute and the ester carbonyl group of MCTA. Racemates with a stereogenic center on a rigid ring structure (e.g., oxiranes) as well as alcohols and amines containing one or more aromatic groups (e.g., Tröger's base, 1,1′-binaphthyl-2,2′-diamine, 7,8,9,10-tetrahydro-benzo(a)pyren-7-ol, 1,1,2-triphenyl-1,2-ethandiol) can also be resolved on MCTA layers using aqueous–alcoholic mixtures as eluents even if the compounds do not have a carbonyl group. This separation is based on the formation of inclusion complexes between the solute and the chiral cavities of cellulose, the dimensions of which change as a consequence of the different swelling due to the different solvents employed. Homemade plates of cellulose tribenzoate and cellulose tricarbamate have been also used for the resolution of a number of aromatic alcohols, Tröger's base, aromatic ketones, and β-blockers, by eluting with either aqueous–alcoholic or *n*-hexane/propan-2-ol mixtures (25–29).

1.1.3. Molecularly Imprinted Polymers with Chiral Cavities

In 1994, Kriz et al. (30) used homemade plates of molecularly imprinted polymers (MIPs) with chiral cavities for the separation of phenylalanine anilide enantiomers for the first time. Lateron, Suedee et al. (31–34) and Enein et al. (35) prepared MIPs using biologically active chiral molecules for the resolution of racemic α-adrenergic

Table 1
Retention (hR_{f1}, hR_{f2}), separation selectivity (α), and resolution (R_S) data of recently investigated racemic solutes, baseline resolved on noncommercial MCTA/silica gel 60GF$_{254}$ plates

Racemate	hR_{f1}[a]	hR_{f2}[a]	α[b]	R_S[c]	Ratio MCTA/silica gel	Eluent (v/v)	References
Aminoglutethimide	23	29	1.36	3.0	3:1	Ethanol/water (50/50)	(17)
Fmoc-proline	34(D)	40(L)	1.29	2.0	3:1	2-propanol/water (60/40)	(17)
Alphamethrin	23	29	1.37	1.7	3:1	Ethanol/water (80/20)	(18)
Fenpropathrin	30	34	1.20	2.0	3:1	Ethanol/water (80/20)	(18)
Fenoxaprop-ethyl	36(R)	46(S)	1.52	2.2	3:1	2-propanol/water (80/20)	(18)
Flurbiprofen	18	24	1.44	2.0	3:1	Ethanol/water (40/60)	(17)
Carprofen	36	41	1.23	1.6	3:1	Ethanol/water (40/60)	(20)
MTH-proline	33	37	1.19	1.0	3:1	2-propanol/water (60/40)	(20)
MTH-phenylalanine	43	49	1.27	1.7	3:1	2-propanol/water (80/20)	(20)
MTH-tyrosine	42	45	1.13	1.0	3:1	2-propanol/water (60/40)	(20)
PTH-proline	13	25	2.23	2.5	3:1	2-propanol/water (60/40)	(20)
PTH-phenylalanine	31	36	1.25	1.6	3:1	Ethanol/water (80/20)	(22)
PTH-tyrosine	53(L)	64(D)	1.58	1.6	3:1	2-propanol/water (80/20)	(22)
N-benzoyl-leucine-βNA	61	66	1.24	1.1	3:1	2-propanol/water (80/20)	(22)
N-benzoyl-phenylalanine-βNA	38	46	1.39	1.1	3:1	2-propanol/water (80/20)	(22)
2-methyl-1-indanone	50	57	1.33	1.8	3:1	Ethanol/water (80/20)	(18)

(continued)

Table1
(continued)

Racemate	hR_{f1}[a]	hR_{f2}[a]	α[b]	R_S[c]	Ratio MCTA/silica gel	Eluent (v/v)	References
2-ethyl-1-indanone	58	66	1.38	2.0	4:1	Ethanol/water (80/20)	(24)
2-butyl-1-indanone	64	68	1.19	1.4	4:1	Ethanol/water (80/20)	(24)
2-bromo-1-tetralone	31	44	1.76	4.2	4:1	Ethanol/water (80/20)	(24)
4-methoxybenzhydrol	41	46	1.23	1.0	4:1	2-propanol/water (70/30)	(24)
4-chlorobenzhydrol	41	49	1.38	1.7	4:1	2-propanol/water (70/30)	(24)

[a] $hR_f = R_f \times 100$
[b] $\alpha = [(1/R_{f1}) - 1]/[(1/R_{f2}) - 1]$
[c] $R_S = 2 \times$ (distance between the centers of two adjacent spots)/(sum of the widths of the two spots in the direction of development)
MTH methylthiohydantoin, *PTH* phenylthiohydantoin, β-*NA* β-naphthylamine

agonists and β-blockers such as ephedrine, pseudoephedrine, norephedrine, epinephrine, isoproterenol, salbutamol, propranolol, oxprenolol, pindolol, nadolol, timolol, and atenolol.

1.2. Commercial and Noncommercial Chiral-Coated Stationary Phases with Achiral Mobile Phases

1.2.1. Commercially Impregnated Chiral Plates

The only plate commercially available chiral-coated stationary phase (CCSP) is Chiralplate™ (Macherey-Nagel, Düren, Germany) which is based on silanized silica gel permanently coated with the copper (II) complex of (2*S*,4*R*,2*RS*)-*N*-(2-hydroxydodecyl)-4-hydroxyproline. These plates have been successfully used for the resolution and control of the enantiomeric purity of proteinogenic and non-proteinogenic amino acids, dipeptides, and heterocyclic compounds using mixtures of water–methanol–acetonitrile in different ratios as eluents and ninhydrin as visualization reagent (Table 2). These separations are based in the principle of chiral ligand-exchange chromatography (CLEC) in the reversed-phase mode (36). This mechanism involves the formation of ternary diastereomeric complexes between the copper ion, the chiral selector on the plate, and the ligands of opposite configuration (e.g., the complex of the D-enantiomer of α-amino acids and α-hydroxy acids is more stable than that of the L-enantiomer). However, it should be noted that racemic threonine and basic amino acids were not resolved on Chiralplate™.

1.2.2. Home-Impregnated Commercial Layers

Ready-to-use RP-18 plates commercially available from Whatman (Maidstone, UK) and Merck (Darmstadt, Germany) can be impregnated with the copper(II) complex of various chiral selectors (e.g., *N*,*N*-di-*n*-propyl-L-alanine, poly-L-phenylalaninamide, L-decylhistidine). These layers can be used for the enantioresolution of racemic dansyl amino acids (37–42). Further studies were carried out under normal-phase (NP) conditions on precoated plates of silica gel G (Merck, Darmstadt, Germany) impregnated with copper(II) complexes of amino acid enantiomers (e.g., L-proline and L-arginine) for the resolution of racemic aromatic amino acids, histidine, and β-blockers such as propranolol, atenolol, and metoprolol (43–46). Even in this case, ternary mixtures of water–methanol–acetonitrile were used as eluents. Very interesting results were obtained by Kowalska et al. (47, 48) who used precoated plates of silica gel 60 F_{254} (Merck, Darmstadt, Germany) impregnated with L-arginine (and not with its copper complex) for the resolution of racemic ibuprofen and propranolol. The mechanism governing these separations will be discussed in the following paragraph.

1.2.3. Home-Impregnated Noncommercial Layers

A number of studies have been performed on homemade plates coated with a slurry of silica gel 60 G containing an appropriate chiral selector, such as acidic or basic enantiomeric compounds, amino acids, macrocyclic antibiotics, and *N*-(3,5-dinitrobenzoyl) derivatives of amino acids (Pirkle-type selectors) (49–68). With this analytical approach, the enantiomers of neutral and basic amino

Table 2
Retention (hR_{f1}, hR_{f2}) and separation selectivity (α) data for amino acids baseline resolved on commercial plates of silanized silica gel impregnated with copper(II) complex of (2*S*,4*R*,2*RS*)-*N*-(2-hydroxydodecyl)-4-hydroxyproline (Chiralplate™) (36)

Amino acid	hR_{f1} [a]	hR_{f2} [a]	α [b]	Eluent (v/v/v)
Alanine	69(D)	73(L)	1.22	Acetone/methanol/water (5/1/1)
Serine	73(D)	76(L)	1.17	Acetone/methanol/water (5/1/1)
Valine	54(D)	62(L)	1.39	Methanol/water/acetonitrile (1/1/4)
Norvaline	49(D)	56(L)	1.32	Methanol/water/acetonitrile (1/1/4)
Leucine	53(D)	63(L)	1.51	Methanol/water (1/8)
Isoleucine	47(D)	58(L)	1.55	Methanol/water/acetonitrile (1/1/4)
Norleucine	53(D)	62(L)	1.44	Methanol/water/acetonitrile (1/1/4)
allo-Isoleucine	51(D)	61(L)	1.50	Methanol/water/acetonitrile (1/1/4)
tert-Isoleucine	40(D)	51(L)	1.56	Methanol/water/acetonitrile (1/1/4)
Methionine	54(D)	59(L)	1.23	Methanol/water/acetonitrile (1/1/4)
Proline	41(D)	47(L)	1.27	Methanol/water/acetonitrile (1/1/4)
Hydroxyproline	41(L)	59(D)	2.08	Methanol/water/acetonitrile (1/1/4)
Phenylalanine	49(D)	59(L)	1.49	Methanol/water/acetonitrile (1/1/4)
Tyrosine	58(D)	66(L)	1.40	Methanol/water/acetonitrile (1/1/4)
3,4-dihydroxyphenylalanine	47(L)	58(D)	1.55	Methanol/water/acetonitrile (5/5/3)
Tryptophan	51(D)	61(L)	1.50	Methanol/water/acetonitrile (1/1/4)
Aspartic acid	50(D)	55(L)	1.22	Methanol/water/acetonitrile (1/1/4)
Glutamic acid	54(D)	59(L)	1.22	Methanol/water/acetonitrile (1/1/4)
7-methyl-tryptophan	51	64	1.70	Methanol/water/acetonitrile (1/1/4)
5-bromo-tryptophan	46	58	1.61	Methanol/water/acetonitrile (1/1/4)
α-Methyl-serine	56(L)	67(D)	1.59	Methanol/water/acetonitrile (5/5/3)
α-Methyl-valine	51	56	1.22	Methanol/water/acetonitrile (1/1/4)
α-Methyl-leucine	48	59	1.55	Methanol/water/acetonitrile (1/1/4)
α-Methyl-methionine	56(D)	64(L)	1.39	Methanol/water/acetonitrile (1/1/4)
α-Methyl-phenylalanine	53(L)	66(D)	1.72	Methanol/water/acetonitrile (1/1/4)
α-Methyl-tyrosine	63(D)	70(L)	1.37	Methanol/water/acetonitrile (1/1/4)
α-Methyl-tryptophan	54	65	1.58	Methanol/water/acetonitrile (1/1/4)

[a] $hR_f = R_f \times 100$
[b] $\alpha = [(1/R_{f1}) - 1]/[(1/R_{f2}) - 1]$

acids, derivatized amino acids, alkaloids, β-blockers (e.g., atenolol, propranolol, and metoprolol), and 2-arylpropionic acids have been resolved by eluting with ternary organic or aqueous–organic mixtures. The success of these separations depends on the optimization of the eluent mixture as well as on a number of additional parameters such as (1) the specific ratio between silica gel and the chiral selector, (2) pH of the slurry used for coating the plate, and (3) the chromatographic development temperature. Critical aspects of this technique are related to the particular mechanism of chiral recognition that involves the formation of diastereomeric ion pairs within the pores of the silica gel, while diastereomers formed outside the pores due to the excess of the chiral selector do not give enantioselective interactions, thus, negatively influencing the chiral resolution.

1.3. Commercial Achiral Plates in Combination with Chiral Mobile Phase Additives

1.3.1. β-Cyclodextrin and Its Derivatives as Mobile Phase Additive

β-Cyclodextrin and modified cyclodextrins (e.g., methyl, hydroxyethyl, and hydroxypropyl derivatives) are chiral mobile phase additives mostly used for RP enantioresolutions on commercially available silanized silica gel plates, such as the DC RP-18W/UV254 and the HPTLC Nano-Sil C18-50/UV254 plates from Macherey-Nagel (Düren, Germany), the KC2F and the KC18F plates from Whatman (Maidstone, UK), and the HPTLC RP-18W/F254 plate from Merck (Darmstadt, Germany).

Under these experimental conditions, many different classes of chiral compounds were resolved including dansyl amino acids, flavanones, amino acids, oxazolidinones, aromatic amino acids and alcohols, and budesonide in pharmaceuticals (19, 21, 69–76). It is assumed that inclusion complexes are formed between the analytes and β-cyclodextrins in a 1:1 ratio. These complexes are much stable in aqueous solutions than in organic solvents, and therefore, in order to increase the solubility of β-cyclodextrin in water, solutions containing high concentrations of urea were used. Conversely, β-cyclodextrin derivatives have a higher solubility in water so that the addition of urea is not required.

1.3.2. Bovine Serum Albumin as Mobile Phase Additive

The protein bovine serum albumin (BSA) has been used as chiral mobile phase additive for the resolution of more than 80 racemates, most of them being amino acids and their derivatives, specific drugs, and uncharged compounds such as benzoin, 2-hydroxy-flavanone, homoeriodictyol, and oxazolidinones (21, 77–84). These separations have been achieved using RP mode on commercially available plates of silanized silica gel (DC RP-18W/UV254, HPTLC Nano-Sil C18-50/UV254 from Macherey-Nagel, Düren, Germany; HPTLC RP-18W/F254 from Merck, Darmstadt, Germany) using aqueous solutions with different pH values containing 2–6% of 2-propanol and 4–8% of BSA as eluents. Table 3 summarizes examples of enantioseparations of amino acid derivatives obtained under these experimental conditions.

Table 3
Retention (hR_{f1}, hR_{f2}), separation selectivity (α), and resolution (R_S) data for dansyl (Dns) amino acids and other selected racemates on commercial $RP_{18}W/UV_{254}$ plates

Solute	hR_{f1} [a]	hR_{f2} [a]	α [b]	R_S [c]	Mobile phase	% BSA	References
Dns-serine	39(D)	46(L)	1.33	1.1	a	7	(82)
Dns-threonine	34(L)	43(D)	1.47	1.2	b	6	(82)
Dns-aminobutyric acid	34(L)	56(D)	2.47	2.1	b	5	(82)
Dns-valine	20(L)	33(D)	1.97	1.6	b	5	(82)
Dns-norvaline	25(L)	73(D)	8.13	5.5	b	5	(82)
Dns-leucine	6(D)	15(L)	2.77	1.7	b	5 + 1% NaCl	(82)
Dns-norleucine	38	50	1.63	1.5	b	6	(82)
Dns-methionine	32(L)	50(D)	2.12	2.2	b	6	(82)
Dns-phenylalanine	24(L)	45(D)	2.59	1.7	b	6	(82)
Dns-tryptophan	37(D)	62(L)	2.78	2.2	b	5	(82)
Dns-aspartic acid	68(D)	79(L)	1.77	1.6	b	5	(82)
Dns-glutamic acid	45(D)	65(L)	2.26	2.8	b	5	(82)
dl-Amethopterin	9(L)	19(D)	2.34	2.3	a	8	(77)
Dinitrophenyl-norvaline	40	89	12.19	5.0	b	4	(78)
N-carboxybenzyl-tryptophan	44(D)	88(L)	9.33	3.0	a	5	(83)

[a] $hR_f = R_f \times 100$
[b] $\alpha = [(1/R_{f1}) - 1]/[(1/R_{f2}) - 1]$
[c] $R_S = 2 \times$ (distance between the centers of two adjacent spots)/(sum of the widths of the two spots in the direction of development)
Mobile phase: (a) 0.5 M acetic acid (pH = 3.40) or (b) 0.1 M acetate buffer (pH = 4.72) solutions containing BSA (fraction V, pH = 5.2) and 2% 2-propanol

1.4. Selected Enantioseparations

Since homemade layers have a lower quality and reproducibility than those commercially available TLC plates, below experimental details of the separation of enantiomers in combination with β-cyclodextrin derivatives and BSA as mobile phase additives are reported. The analytes include methylthiohydantoin-D,L-phenylalanine (MTH-D,L-Phe) and methylthiohydantoin-D,L-tyrosine (MTH-D,L-Tyr) which can be resolved using hydroxyethyl-β-cyclodextrin (76) and racemic warfarin and *p*-chlorowarfarin using BSA as mobile phase additive (77).

2. Materials

2.1. Equipment

1. A thermostated TLC chamber such as the Desaga thermostated TLC Thermo Box 230 V provided with a flat-bottom developing chamber (22×22×6 cm) (Sarstedt, Nümbrecht, Germany).
2. A UV-viewing box with UV lamps for 254 and 366 nm such as Camag UV Cabinet 3 (Camag, Muttenz, Switzerland).
3. A Hamilton 10-µl syringe model 7001 (Bonaduz, Switzerland).
4. HPTLC Nano-Sil C18-50/UV_{254} plates (10×10 cm) (Macherey-Nagel, Düren, Germany).
5. DC RP-18W/UV_{254} plates (10×10 cm) (Macherey-Nagel, Düren, Germany).

2.2. Chemicals

1. MTH-D,L-Phe (Sigma-Aldrich, St. Louis, MO, USA).
2. MTH-D,L-Tyr (Sigma-Aldrich, St. Louis, MO, USA).
3. Racemic warfarin (Sigma-Aldrich, St. Louis, MO, USA).
4. Racemic *p*-chlorowarfarin (Sigma-Aldrich, St. Louis, MO, USA).
5. Hydroxyethyl-β-cyclodextrin, average molar substitution 1.6 (Sigma-Aldrich, St. Louis, MO, USA).
6. Bovine serum albumin (BSA), fraction V, pH 7.0, assay ≥98% (Serva, Heidelberg, Germany) (see Note 1).

3. Methods

For all solutions, use HPLC grade solvents and ultrapure water (conductivity not higher than 0.055 µS/cm at 25°C). Store all standard solutions at 4°C. Follow all appropriate safety regulations for the chemicals and solvents as well as waste disposal regulations.

3.1. Resolution of MTH-Labeled Amino Acids

1. Sample solution: Dissolve 10 mg MTH-D,L-Phe or MTH-D,L-Tyr in 10 mL water/acetonitrile 1/1 (v/v).
2. Mobile phase: Dissolve 8.15 g hydroxyethyl-β-cyclodextrin in 50 mL water/acetonitrile/acetic acid 45/4/1 (v/v/v) using a magnetic stirrer (the concentration of hydroxyethyl-β-cyclodextrin is 0.1 M) (see Note 2).
3. Fill TLC developing chamber with the mobile phase.
4. Set thermostat to 25°C.
5. Equilibrate chamber for 60 min.

Table 4
Retention (hR_{f1}, hR_{f2}), separation selectivity (α), and resolution (R_S) data of the enantioseparation of MTH-D,L-Phe and MTH-D,L-Tyr on precoated HPTLC Nano-Sil C18-50/UV_{254} plates (10 × 10 cm) eluted with a 0.1 M solution of hydroxyethyl-β-cyclodextrin in water/acetonitrile/acetic acid (45:4:1, v/v/v). Development for 8.0 cm

Solute	hR_{f1} [a]	hR_{f2} [a]	α [b]	R_S [c]
MTH-D,L-Phe	45	52	1.32	2.2
MTH-D,L-Tyr	72	78	1.37	2.0

[a] $hR_f = R_f \times 100$
[b] $\alpha = [(1/R_{f1}) - 1]/[(1/R_{f2}) - 1]$
[c] $R_S = 2 \times$ (distance between the centers of two adjacent spots)/(sum of the widths of the two spots in the direction of development)

6. Spot 0.5 mL of the sample solution on the HPTLC Nano-Sil C18-50/UV254 plates using a 10-mL Hamilton syringe at a distance of 1 cm from the bottom of the plate.
7. Develop plate for 8 cm.
8. Remove TLC plate from the chamber and immediately mark the solvent front.
9. Visualize spots by UV detection at 254 nm, and mark each spot with a pencil.
10. Calculate retention (R_f), separation selectivity (α), and resolution (R_S) factors (see Note 3).

The best separation data are reported in Table 4 (see Note 4).

3.2. Resolution of Racemic Warfarin and p-Chlorowarfarin

1. Sample solution: Dissolve 10 mg warfarin or p-chlorowarfarin in 5 mL methanol.
2. Mobile phase: Dissolve 4 g of BSA in 49 mL of a 0.5 M sodium acetate solution using a magnetic stirrer (see Note 5), and add successively 1 mL propan-2-ol, drop by drop, in order to eliminate the foam (see Note 6). The pH of 7.3 of the resulting solution is measured by a glass electrode (see Note 7).
3. Fill TLC developing chamber with the mobile phase.
4. Set thermostat to 25°C.
5. Equilibrate chamber for 60 min.
6. Spot 0.5 mL of the sample solution on DC RP-18W/UV254 plates using a 10-mL Hamilton syringe at a distance of 1 cm from the bottom of the plate.
7. Develop plate for 7 cm.

Table 5
Retention (hR_{f1}, hR_{f2}), separation selectivity (α), and resolution (R_S) data of the enantioseparation of warfarin and *p*-chlorowarfarin on precoated DC RP-18W/UV$_{254}$ plates (10 × 10 cm) eluted with a 8% BSA solution in 0.5 M sodium acetate/propan-2-ol (49:1, v/v). Development for 7 cm

Solute	hR_{f1}[a]	hR_{f2}[a]	α[b]	R_S[c]
Warfarin	18	25	1.51	1.3
p-Chlorowarfarin	11	16	1.54	1.1

[a] $hR_f = R_f \times 100$
[b] $\alpha = [(1/R_{f1}) - 1]/[(1/R_{f2}) - 1]$
[c] $R_S = 2 \times$ (distance between the centers of two adjacent spots)/(sum of the widths of the two spots in the direction of development)

8. Remove TLC plate from the chamber, and immediately mark the solvent front.
9. Visualize spots by UV detection at 254 nm, and mark each spot with a pencil.
10. Calculate retention (R_f), separation selectivity (α), and resolution (R_S) factors (see Note 3).
    The best separation data are reported in Table 5 (see Note 8).

4. Notes

1. BSA fraction V, pH = 7 is stored at 4°C. BSA can be used as mobile phase additive in neutral or alkaline eluents.
2. The eluent is prepared with 100% acetic acid. The measured pH of the eluent is 2.7.
3. The retention factor (R_f) is calculated by manually measuring the distance traveled by the center of the spot (d_S) and the eluent front (d_E) and using the eq. (1)

$$R_f = \frac{d_S}{d_E} \tag{1}$$

The separation factor is calculated according to the following eq. (2):

$$\alpha = \frac{\frac{1}{(R_{f1} - 1)}}{\frac{1}{(R_{f2} - 1)}} \tag{2}$$

The resolution factor is calculated according to eq. (3):

$$R_S = 2 \times \frac{\text{distance between the centres of two adjacent spots}}{\text{sum of the widths of the two spots in the direction of development}} \quad (3)$$

4. After the development of the chromatogram, two solvent fronts can be observed. The lower one has an R_f of about 0.83. Round and compact spots are obtained which can be used for the quantitative determination of the two enantiomers by UV densitometry.
5. Sodium acetate solution 0.5 M was prepared by dissolving 10.25 g of the anhydrous salt in ultrapure water in a 250-mL calibrated flask.
6. A concentration of 8% BSA in the eluent is the maximum concentration that can be obtained.
7. Enantiomer resolution cannot be achieved using 0.1 M phosphate buffer (pH = 6.8) or more acidic solutions (e.g., 0.1 M sodium acetate buffer or 0.5 M acetic acid) as eluents. Stronger alkaline eluents (e.g., ammonia buffer or sodium hydroxide solutions) cannot be used with the plates used in the experiments.
8. Round and compact spots are obtained and can be used for the quantitative determination of the two enantiomers by UV densitometry.

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