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Article

Thin Layer Chromatographic Resolution of Some β-adrenolytics and a β2-Agonist Using Bovine Serum Albumin as Chiral Additive in Stationary Phase

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Abstract

Direct enantiomeric resolution of commonly used five racemic β-adrenolytics, namely, bisoprolol, atenolol, propranolol, salbutamol and carvedilol has been achieved by thin layer chromatography using bovine serum albumin (BSA) as chiral additive in stationary phase. Successful resolution of the enantiomers of all racemic β-adrenolytics was achieved by use of different composition of simple organic solvents having no buffer or inorganic ions. The effect of variation in pH, temperature, amount of BSA as the additive, and composition of mobile phase on resolution was systematically studied. Spots were visualized in iodine vapors. Native enantiomers for each of the five analytes were isolated and identified and their elution order was determined. The limit of detection was found to be 0.7, 1.2, 0.84, 1.6 and 0.9 μg (per spot) for each enantiomer of bisoprolol, atenolol, propranolol, salbutamol and carvedilol, respectively.

Introduction

The two enantiomers of a pharmaceutically active compound should, in fact, be considered as different drugs because of significant differences in their pharmacodynamics and pharmacokinetic profiles. With such an increasing awareness of these issues among those involved in the drug development, marketing and law enforcement the importance of developing simple methods of enantioseparation and control of enantiomeric purity of such pharmaceuticals cannot be overemphasized. Thus, there continues a strong need to develop rapid and reliable methods that can be used for verification of enantiomeric purity or to monitor stereoselective synthesis. In general, the correctness of the *ee* reported for an enantioselective synthesis should be considered as authentic only if it is determined via enantioseparation soon after the step of synthesis and prior to any purification step by "normal" chromatography (1).

Liquid chromatography (LC) has been extensively employed for chiral separation and detection of the products of organic synthesis, especially enantioselective synthesis, and biological molecules and racemic drugs in the areas of pharmaceutical and biotechnological research & development. Among the techniques used, thin layer chromatography (TLC) has advantages, such as low cost, simplicity of the method and ease of control of experimental strategies and optimization with the advantage that the chromatogram is photographed as a clearly visible evidence of separation. Therefore, it constitutes and could be the method of choice for routine analysis. Sherma (2, 3) reviewed literature on application of TLC for enantioresolution in his regular biennial reviews along with advantages of modern TLC in pharmaceutical and drug analysis, comparing to high-performance liquid chromatography (HPLC) and HPTLC as well.

Though the methods of enantioseparation are generally classified as direct and indirect, the direct approach may further have different strategies, e.g., (i) use of a stationary phase which is chiral for its structural feature, (ii) use of a chiral additive in the mobile phase with achiral stationary phase, and (iii) use of '*chiral additive in achiral stationary phase*' (CAASP) is another strategy under direct approach (involving non-covalent interactions) and is thus suitable for TLC, specially. Both in (i) and (iii) the mobile phase remains achiral because there is no external chiral additive in it.

"β-adrenolytics" (the analytes chosen; commercial racemic bisoprolol, atenolol, propranolol, salbutamol and carvedilol):

There are three different prescription drugs that are included in the group β-adrenolytics, for selectively "blocking" the effects of adrenaline for one of the three known types of beta receptors designated β_1, β_2 and β_3 receptors. They belong to a group of synthetic chiral hydroxyl amine-containing compounds. In general, β-adrenolytic drugs are commonly used in the treatment of hypertension and for controlling acute panic symptoms in anxiety-provoking situations, besides in controlling certain other diseases. Atenolol is a selective β_1 receptor antagonist and is used in the treatment of hypertension and is one of the most widely used β-adrenolytics. Bisoprolol and propranolol are the cardioselective β_1-adrenergic blocking agents used for secondary prevention of myocardial infarction. Salbutamol is a β_2 agonist; (*R*)-(−)-Salbutamol causes smooth muscle to relax whereas (*S*)-(+)-salbutamol causes smooth muscle to contract. Carvedilol is a mixed alpha/beta adrenergic antagonist and is used for treating mild to severe congestive heart failure. Generally, the (*S*)-(−)-enantiomer of β-adrenolytics shows a few hundred fold higher pharmacological activity than the (*R*)-(+)-enantiomer but most of them are clinically practiced in racemic form. Use of β-adrenolytics is illegal as a sports enhancing drug.

Bovine Serum Albumin

Bovine serum albumin (BSA) is a giant globular protein; it has a molecular weight of about 66,000 D and contains about 607 amino acid residues in a single polypeptide chain and no carbohydrates (4). At pH 5–7 it contains 17 intrachain disulfide bridges and one sulfhydryl group. The isoelectric point of the protein in water at 25°C is 4.7. BSA is easily available at low costs. BSA is a serum protein that binds mostly acidic and neutral drugs. It behaves as a chiral complexing agent. Direct enantioseparation by LC based on enantioselective properties of a protein, particularly BSA, has been found to be useful with diversity and variety in analytical applications. BSA has been used as a chiral mobile phase additive for TLC separation of a variety of enantiomers such as amino acids and their derivatives, specific drugs, uncharged compounds like benzoin, 2-hydroxyflavanone, homoeriodictyol, and oxazolidinones (5–7), warfarin and *p*-chlorowarfarin (8). About 12 dansyl amino acids have been enantioseparated on RPTLC plates using BSA as chiral complexing agent in mobile phase (9). BSA bonded chiral stationary phase (CSP) was used for HPLC separation of 19 racemic dansyl α-amino acids (10). BSA modified silica nanoparticles were prepared and used as a chiral adsorbent for enantioseparation of propranolol and tryptophan (11).

Present Work

Literature survey on enantioresolution of pharmaceutically important compounds, including β-adrenolytics, by TLC (2, 8, 12, 13) and our work published on TLC enantioresolution of the chosen β-adrenolytics (14–16), and the literature cited therein, clearly shows that BSA has not been used as chiral additive in stationary phase (CASP) in a non-covalent mode for enantioresolution of racemates of any kind by direct approach. Though, there are reports on application of BSA-based CSPs for enantioresolution of a variety of chiral compounds by HPLC or CE (17). Therefore, we were prompted to develop simple sensitive TLC method for direct enantioresolution of certain commonly used β-adrenolytics using BSA. To the best of authors' awareness, the novelty of the present paper is the first time application of BSA as CASP in planar chromatography to achieve direct enantioresolution of certain racemic β-adrenolytics.

Experimental Section

Chemicals and reagents

(*RS*)-"Atenolol" (Atl), (*RS*)-"propranolol" (Prl) and BSA (Cohn fraction V, pH 5.2, assay ≥96–99%) were obtained from Sigma-Aldrich (St Louis, MO, U.S.A.). Tablets of (*RS*)-"bisoprolol" (Bpl) as *Concor* (Merck Ltd, Waluj, Aurangabad, India), "carvedilol" (Cdl) as *Carca* (Intas Pharma, Ahmedabad, India) and of "salbutamol" (Sbl) as *Asthalin-SA* (Cipla LTD, Mumbai, India), containing their racemic forms, were purchased from the pharmaceutical shops in the local market. Solvents employed, i.e., ethanol (EtOH) and glacial acetic acid (HOAc) of analytical reagent grade, and dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), acetonitrile (CH_3CN) and methanol (MeOH) of HPLC grade, were obtained from E. Merck (Mumbai, India). Silica gel G (pH 7.0) having 13% calcium sulfate as binder and 0.02% iron, chloride and lead impurities in a 10% aqueous suspension, was purchased from Merck (Mumbai, India).

Instrumentation

The equipment/instrument used for the present experiments consist of a UV-2450 spectrophotometer (Shimadzu, UV-2450 spectrophotometer), a pH meter (Cyberscan 510, Singapore), an FT-IR spectrometer (Nicolet-6700, Thermo scientific, USA), an ^{1}HNMR spectrometer 500 MHz (Bruker, Germany), Milli-Q system from Millipore (Bedford, MA, USA) to obtain purified water (18.2 MΩ cm^3), an elemental analyzer Vario EL III (Hanau, Germany), a polarimeter (Krüss model P3001RS, Germany).

Isolation and purification of racemic analytes from commercial formulations

The coating of 10 "Concor" tablets (each containing 5 mg of (*RS*)-bisoprolol, for example) was scratched out and these were finely powdered in mortar. The powder was suspended in 20 mL methanol and was sonicated for about 10 min at room temperature. It was filtered and the residue obtained was further extracted with methanol. Both combined filtrates were concentrated and kept in the refrigerator until crystals appeared. The crystals were washed with diethyl ether and dried in a vacuum desiccator. The remaining analytes were also extracted, purified and characterized by following the same procedure. These compounds were used as standard reference for experiments of enatioresolution.

Preparation of standard solutions

Stock solutions of (*RS*)-Bpl, Atl, Prl, Sbl, and Cdl in MeOH (each 10 mM) were prepared and further diluted with MeOH (5×10^{-2} M) for required working solutions. All solutions were filtered through a 0.45 μm filter. The solutions were scanned for determination of λ_{max}. Six solutions in the range 1×10^{-4}–5×10^{-4} M were prepared by dilution. Their absorbance was recorded and a calibration plot was constructed.

Preparation of TLC plates

The TLC plates were prepared in the laboratory as described earlier (18) except that BSA was used as chiral additive in the stationary phase and the concentration of BSA was varied in the silica gel slurry from 0.2 to 0.5 mM, at an interval of 0.1 mM. For this

purpose, at first solutions of different molarities of BSA were prepared in water containing 0.20% glacial acetic acid and the slurry of silica gel (25 g) was prepared in these solutions. The slurry was adjusted at four different pH, i.e., 3, 4, 5 and 6 for each concentration of BSA. The slurry of was applied on glass plates (10 × 5 cm × 0.5 mm) with a Stahl-type applicator. Thus, 16 sets of TLC plates were prepared. The thin silica gel plates were kept overnight in oven (at 50 ± 2°C). About 10 µL solution of each of the racemic analytes were spotted on TLC plates with a 25 µL Hamilton syringe.

Development of chromatograms and isolation of enantiomers

Chromatograms were developed in a completely dried, pre-equilibrated, paper-lined rectangular glass chamber and then dried in an oven. Experiments were performed with binary, ternary and quaternary mixtures of solvents such as chloroform, acetic acid, ethanol, methanol, dichloromethane and acetonitrile to achieve enantiomer separation. The chromatographic chambers were placed inside an incubator to maintain each specific temperature (15, 20, 25 or 30°C) before development. The chamber was pre-equilibrated for nearly 15 min at each temperature.

About 10 TLC plates were run by applying four spots in parallel on a single plate for one racemic analyte using final optimized mobile phase. Chromatograms were dried at 40°C in an oven for 10 min and cooled to room temperature. The spots were located in an iodine chamber. The spots were marked and left at room temperature for iodine to evaporate from the TLC plates. The silica gel of each marked spot was scraped and extracted with ethanol. The combined extracts, pertaining to each of the enantiomers, were centrifuged at 2,500 rpm for 5 min and the supernatant was concentrated in vacuum. The same procedure was followed for all the analytes. Each of these solutions was examined by UV spectrophotometer and polarimeter to ascertain their concentration (using the standard plot as described above) and to calculate specific rotation.

Results

Recovery of the active pharmaceutical ingredients obtained from the commercial formulations was of the order of 96–99% and the purity of the crystals was confirmed by recording melting point, λ_{max} and IR spectra; the m.p. data was found in agreement with the literature values (19). Since the focus of the paper is on enantioresolution by direct approach using BSA the characteristic IR peaks or the λ_{max} values are not being included (Figure 1).

TLC enantioresolution

Only the solvent systems, i.e., the combinations of different solvents, enabling successful resolution are reported in Table I along with the hR_F ($R_F \times 100$) values. hR_F values are averages of at least five runs on different plates under identical conditions on the same day and on different days. The resolution was calculated by dividing the distance between two spots by the sum of the two spot radii. The resolution (R_S) varied from the lowest 1.3 for (*RS*)-Bpl to 2.6 for (*RS*)-Atl. Representative photographs of actual chromatograms are shown in Figure 2. Specific rotation values were calculated and were found to be $[\alpha]_D^{25} = +22.9°$ ($c = 0.7$, MeOH), + 11.0°, +26.1°($c = 1.0$, MeOH), + 24.0°($c = 0.5$, MeOH), + 14.1°($c = 1.0$, MeOH) for the upper spots of Bpl, Atl, Prl, Sbl and Cdl, respectively.

Effect of pH, temperature and amount of chiral additive on enantioseparation

The effect of varying pH, temperature and the concentration of the impregnating reagent were studied for a large number of solvent systems tried for enantioresolution.

pH: As mentioned before, resolution studies were conducted on plates with four different pH. Two clear spots were observed on the plates prepared at pH 4.0 (approximately). The increase in pH caused a loss of resolution. The results clearly indicated that very good enantioresolution of all the racemates was obtained at acidic pH close to the isoelectric point of BSA.

Temperature: As given in Table I, it was observed that (*RS*)-Bpl, Atl, and Prl got resolved at 28°C, and Sbl was resolved at 25°C while Cdl resolved at 22°C. There was poor resolution or no resolution as indicated by the observation of tailing or elongated spots outside this temperature range.

Amount of chiral additive: The best separation for all the analytes was obtained at 0.3 mM of BSA (Table I). At lower concentration there was no resolution and at concentration higher than 0.3 mM there was observed long tailing of spots.

Method validation

Different solutions of known concentration (300, 500 and 1,000 µg mL^{-1}) of each of the racemic analytes were applied three times on the TLC plates having BSA as a chiral additive and determined repeatability of the method. Relative standard deviation (RSD) was found to be 0.95%. Recovery of the enantiomers was in the range 96–99%. The results indicate that TLC with BSA as chiral additive can be used for detection of very small amounts of each enantiomer as per the detection limits of 0.7, 1.2, 0.84, 1.6 and 0 0.9 µg (per spot) for each enantiomer of Bpl, Atl, Prl, Sbl and Cdl, respectively.

Discussion

Chiral additive and native enantiomers

The TLC plates with "chiral additive in stationary phase" (CASP) were successful in resolving the racemic analytes. When the TLC plates having no CASP were spotted with the racemic analytes and developed under the identical experimental conditions each of the racemates gave a single spot. This confirmed that presence of BSA was necessary for resolution of the enantiomers.

Since BSA is insoluble in ethanol the (+)- or (−)-isomer of the corresponding racemate present in the scrapped silica gel, pertaining to each spot, went into ethanol when the said silica gel was extracted. Results from polarimetric experiments and spectrophotometric determination of concentration of the isolated enantiomers of all the analytes were used to calculate specific rotation. The polarimetric measurements also showed that the two isomers were in the ratio of 1:1 and the (+)-isomer had R_F higher than the (−)-isomer and thus eluted first. These results also confirmed the elution order. The specific rotation values so determined were in agreement with literature values (5, 19). The enantiomers isolated in this manner were taken as reference samples for all five β-adrenolytics and were used in a second set of TLC experiments in which they were applied to the plate adjacent to the racemic mixture, for comparison of R_F values with those separated from the mixture (Figure 3). Thus the isolation of native enantiomers characterized by their specific rotation values confirms direct resolution of all the β-adrenolytics. Though there was a very good resolution of all the β-adrenolytics resolution of atenolol was better in comparison to other analytes.

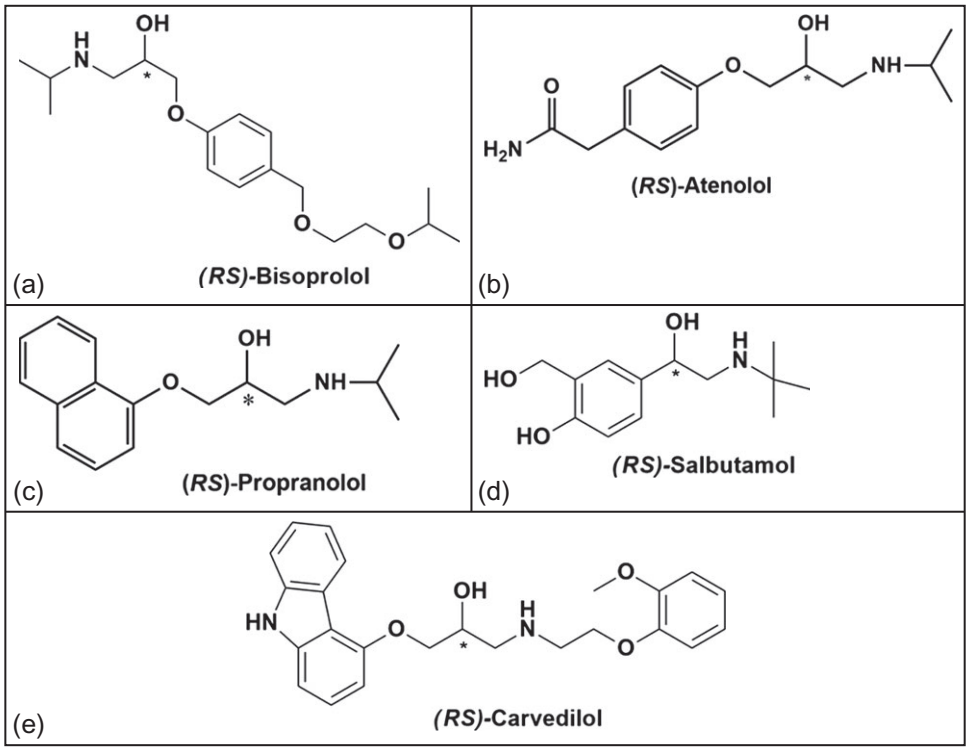

Figure 1. Structures of the racemic analytes: (a) Bisoprolol, (b) Atenolol, (c) Carvedilol, (d) Propranolol, (e) Salbutamol; *represent the stereogenic center in each analyte.

Table I. TLC Experimental Conditions for Successful Resolution of the Five (*RS*)-β–Adrenolytics using BSA as Chiral Additive in the Silica Gel along with hR_F Values and Resolution Data

(*RS*)-Analyte	Mobile phase	Solvent ratio (*v*/*v*)	Temp (°C)	hR_F values		R_S
				The enantiomer		
				(*S*)	(*R*)	
Bisoprolol	CH_3CN-$CHCl_3$-EtOH	2.5:2:3	28	65	52	1.3
Atenolol	CH_3CN-CH_2Cl_2-CH_3OH	3:2:3	28	37	21	2.6
Propranolol	CH_3CN-CH_2Cl_2-$CHCl_3$-CH_3OH	2:2:2:1.5	28	57	44	2.4
Salbutamol	CH_3CN-CH_2Cl_2-CH_3OH	5:2:1.5	25	63	48	2.3
Carvedilol	CH_3CN-CH_2Cl_2-EtOH	3:1:3	22	65	44	2.0

*R*s: resolution; hR_F: retardation factor × 100 (R_F × 100); Development time: 10–15 min; Detection: iodine vapors; Temp: temperature; BSA concentration: 0.3 mM.

The five β-adrenolytics can be arranged as Atl>Prl>Sbl>Cdl>Bpl in decreasing resolution (*R*s) order.

Effect of temperature: Experiments were performed in a range of temperature systematically until its effect was noted in terms of either tailing or figure-of-eight shaped spots or clear resolution. The racemates, under study, resolved well into their enantiomers in a temperature range between 22 and 28°C (Table I) when BSA was used as a chiral additive in the silica gel used for making TLC plates. A change in temperature might be affecting the formation and/or mobility of the transient diastereomers, resulting into poor resolution or no resolution.

Mobile phase: Addition of MeOH in different combinations of CH_3CN-CH_2Cl_2 was successful in resolving (*RS*)-Atl and (*RS*)-Sbl while addition of EtOH was required in different combinations of the same two solvents [CH_3CN-CH_2Cl_2] for successful resolution of (*RS*)-Bpl and (*RS*)-Cdl (Table I). Though all the racemates resolved well into their enantiomers the resolution of enantiomeric pair (*RS*)-Atl was the best among the analytes investigated.

Enantioselective recognition using BSA

Proteins are chiral in nature due to their chemical composition and three-dimensional shape/structure and different spatial arrangements of the functional groups, and thus show stereoselective binding to chiral molecules. Although the mechanism of chiral recognition by proteins, e.g., BSA, is largely unknown some empirically found correlations between retention behavior and mobile phase composition give a general idea of the main types of solute-protein interactions involved (20, 21). The three-dimensional structure of proteins can have various kinds of interactions, e.g., electrostatic interaction,

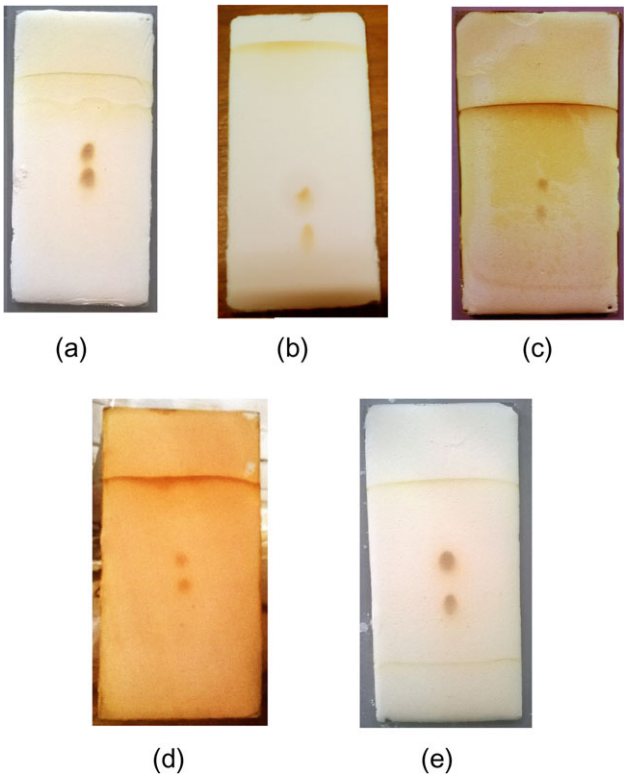

Figure 2. Actual photographs representing separation of racemic β-adrenolytics using BSA as chiral additive in stationary phase, (a) bisoprolol, (b) atenolol, (c) propranolol, (d) salbutamol and (e) carvedilol.

Figure 3. Photographs of chromatograms showing resolution of (*RS*)-Bisoprolol by use of BSA as chiral additive in stationary phase. From left to right: Spot 1: lower spot for (*R*)-enantiomer and the upper spot for (*S*)-enantiomer resolved from the racemate; Spot 2: pure (*R*)-isomer and Spot 3: pure (*S*)-isomer (both were isolated and characterized during this experiment, as described in the text).

dipole interaction, hydrophobic interaction, π–π interaction, steric interaction, complex formation, and cavity inclusion between protein and analytes (22, 23); such interactions including hydrogen bonding between –OH of the β-adrenolytics and $–NH_2$ of the chiral selectors may be held responsible for enantioresolution using BSA in the present case in accordance with the three point interaction as explained by Dalgliesh (24). These interactions favored formation of *transient* diastereomers *in situ* and, hence, enantiomer resolution, as evidenced by the isolation of individual enantiomer(s) from the two spots on the TLC plate.

BSA has a pI of *ca* 5.4 and good resolution was obtained at pH 4.0 and the selectivity of BSA is due to the presence of a large number of amino and carboxylic groups. BSA contains nearly 60 lysine residues (7) which exist in the hydrophobic regions of the macromolecular moiety. The primary interaction between the chiral selector (BSA) and the analytes for enantioseparation seems to involve steric and hydrophobic interactions for the large size of BSA. It is proposed that separation of the enantiomers could be a result of the formation of a hydrophobic pocket resulting in a selective interaction for inclusion of enantiomeric molecules from the racemic mixtures of β-adrenolytics.

Literature (25, 26) reveals that high polarity or high ionic strength of the mobile phase is not favorable for enantioresolution because it reduces the electrostatic interaction or hydrogen bonding between the BSA-silica stationary phase and analyte and thus affects stereoselectivity and retention; the success of the mobile phase in resolution of the analytes, in the present studies, is in agreement with literature explanation it is not a very polar system and also does not contain any buffer to provide any kind of ions for interactions during resolution.

In a study of BSA adsorption at the hydrophilic silica/water interface Su *et al.*, (27) reported that BSA had a high surface affinity since adsorption reached a plateau at a very low BSA bulk concentration at pH 5, close to its pI. Adsorption was found to be irreversible with respect to changes in BSA concentration but reversible with respect to solution pH at low BSA concentrations and BSA formed a uniform layer between 30 and 40 Å thick (28). These findings suggest that there was a uniform irreversible layer of BSA on the silica gel surface on the TLC plate and in this manner there was available a very good CSP without any covalent linkage in comparison to the reports where covalently bonded BSA-silica CSP was synthesized (29) and was used for resolution of tryptophan enantiomers (17) and enantioresolution of different chiral compounds at small scale HPLC (26).

Comparison of *R*s and LOD with literature reports (30–41)

A comparison of the present results with those reported in literature on chromatographic separation parameters (*R*s and LOD) using different CSPs in HPLC or chiral selectors in TLC with respect to the β-blockers under study has been given in Table II. It shows that the present results are superior in terms of *R*s and LOD values. It has been observed that higher *R*s (as mentioned in Table I) and lower LOD (as shown under "Method Validation") for TLC resolution of enantiomers of racemic β-adrenolytics are obtained than early literature reports whether the enantioseparation was done using different types of CSPs or by using expensive instrumental techniques like RPHPLC or gas chromatography.

Conclusion

There have been used various synthetic resin matrices and covalently bonded BSA-silica CSP for successful resolution of a wide variety of chiral compounds by HPLC or CE but the present method provides a first time approach to use BSA as chiral additive in stationary phase for direct enantioresolution of β-adrenolytics by TLC. The method is very simple, direct, fast, sensitive (with very low LODs) and economical for the resolution of the enantiomers of all the selected pharmaceutical analytes. The method is successful in obtaining native enantiomers for further use. The method may be worked

Table II. Comparison of Literature Reports with Present Study on TLC Separation (in terms of *Rs* and LOD) of Enantiomers of (*RS*)-β-Adrenolytics using Different CSPs/Chiral Selectors/CDRs/CIR

Sr. no.	CSP/chiral selector/CDR/CIR	Technique used	LOD	*Rs*	Reference
1.	Chirobiotic V vancomycin	i	NA	0.8	(30)
2.	Chirobiotic T (Teicoplanin)	i	15 μg/L	1.3	(31)
3.	Cellulose tris(3,5-dimethylphenyl-carbamate)	i	NA	0.6	(32)
4.	Amylose tris(3,5-dimethoxyphenylcarbamate)	i	10 μg/mL	1.80	(33)
5.	(−)−α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride	ii	NA	1.44	(34)
6.	1-Fluoro-2,4-dinitrophenyl-L-alanine	ii	NA	1.11	(34)
7.	(−)−α-Methoxy-α-(trifluoromethyl) phenylacetyl chloride	ii	NA	1.30	(34)
8.	1-Fluoro-2,4-dinitrophenyl-(*R*)-(−)-1-cyclohexylethylamine	ii	NA	1.05	(34)
9.	Cu(II)-L-phenylalanine	iii	NA	1.7	(35)
10.	Cu (II)-L-Arginine complex	iii	NA	1.20	(16)
11.	L-Lysine	iii	2.6 μg	NA	(36)
12.	Vancomycin	iii	1.3 and 1.5 μg	NA	(37)
13.	Cellulose tris(4-chlorophenylcarbamate)	i	NA	2.18	(14)
14.	Chiralpak AD-H	i	NA	0.7	(38)
15.	Chirobiotic V2	i	NA	1.48 and 2.21	(39)
16.	Chirobiotic V	i	NA	1.11 and 2.10	(40)
17.	CHIRALPAK IA; tris (3,5-dimethyl phenyl-carbamate)	i	NA	1.97	(40)
18.	Amylose tris(2-chloro-5-methylphenylcarbamate)	i	NA	2.2	(41)
19.	BSA	iii	0.7–1.6 μg/mL	1.3–2.6	Present work

CDR, chiral-derivatizing reagent; CIR, chiral-inducing reagent; NA, not available in the paper cited.
i, ii and iii represent the techniques used as direct HPLC, indirect HPLC and direct TLC separation, respectively.

out for successful resolution of a variety of pharmaceuticals and other organic racemic mixtures along with isolation of native enantiomers which is otherwise not feasible in other approaches or becomes very expansive using preparative chiral HPLC. Use of BSA in very small amount as chiral additive with simple silica gel provides a very good CSP for application in planar mode. Nevertheless, selection of the appropriate matrix or CSP or chiral additive in stationary or mobile phase required for resolution of a given pair of enantiomers is difficult and usually empirical.

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