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Different approaches of impregnation for resolution of enantiomers of atenolol, propranolol and salbutamol using Cu(II)-L-amino acid complexes for ligand exchange on commercial thin layer chromatographic plates

R. Bhushan*, Shivani Tanwar

Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee – 247667, India

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ABSTRACT

Atenolol and propranolol (the β-blocking agents) and salbutamol (broncho- and vasodilator) were resolved into their enantiomers by adopting different modes of loading/impregnating the Cu(II) complexes of L-proline (L-Pro), L-phenylalanine (L-Phe), L-histidine (L-His), *N*,*N*-dimethyl-L-phenylalanine (*N*,*N*-Me_2-L-Phe), and L-tryptophan (L-Trp) on commercial precoated normal phase plates. The three different approaches were (A) using the Cu(II)-L-amino acid complex as chiral mobile phase additive, (B) ascending development of plain commercial plates in the solutions of Cu complex, and (C) using a solution of Cu(II) acetate as mobile phase additive for the commercial TLC plates impregnated with ascending development of plates in the solutions of amino acid. Spots were located using iodine vapour. The results obtained for the three methods have been compared for their efficiency and the issue of involvement of the Cu(II) cation for the best performance of the three methods has been discussed with respect to the same mobile phase. The detection limit is 0.18 μg for each enantiomer.

1. Introduction

The title compounds belong to commonly known group of β-blockers (or β-adrenergic antagonists) and β-agonist. These are amino alcohols. Atenolol belongs to commonly known group of β-blockers and is used to treat hypertension, sinus tachycardia, arrhythmias, coronary heart disease and myocardial infarction where it acts preferentially upon the β-adrenergic receptors in the heart [1]. (*S*) enantiomer possesses much greater affinity for binding to the β-adrenergic receptors than the (*R*) antipode [2]. (*R*)-salbutamol and (*S*)-salbutamol cause smooth muscle to relax and contract, respectively; the two isomers act on different receptors and thus on different pathways resulting in the opposing effects. (*S*)-propranolol is an important therapeutic against oxidative stress [3] and its β-blocking potency is *ca.* 40 times greater than that of (*R*)-(+)-enantiomer; in humans, the bioavailability of (*S*)-propranolol exceeds that of the (*R*)-isomer. In spite of the fact that nearly complete therapeutic activity resides in (*S*)-enantiomers [4], most of the β-blockers are marketed as racemic mixtures and applied in therapy as such. The two enantiomers should be considered as different drugs as there are differences in their stereo selective mechanism. To develop analytical methods for their enantiomeric resolution and control of enantiomeric purity has become an important field of investigation.

Indirect enantiomeric separation of β-blockers, including atenolol, in the form of diastereomers has been reported by RP-HPLC using a variety of chiral derivatizing reagents [5–10]. β-Blockers have been enantioseparated by ligand-exchange capillary electrophoresis using copper(II)-*N*-(2-hydroxyoctyl)-L-4-hydroxyproline [11] and Cu(II) complexes of L-tartaric acid or L-threonine [12] as chiral selector. Application of ligand exchange and use of different chiral selectors as impregnating reagents has been well described in literature [13–16] for TLC resolution of enantiomers of a variety of compounds. Chiral separation of β-blockers by TLC using both direct and indirect modes has been reviewed by Agbaba and Ivković [17]. Ligand exchange TLC has been reported from this laboratory for resolving racemic atenolol, propranolol and metoprolol on plates impregnated with Cu(II)-L-arginine complex [18] and for resolving a few DL-amino acids on plates containing copper(II)-L-proline complex [19]. Direct TLC resolution of enantiomers of three β-blockers by ligand exchange on home-made plates by mixing of chiral selector in the silica gel slurry and using a large number of different solvent systems has been reported [20]. Mixing of chiral impregnating reagent for enantiomeric resolution with the inert support has been one of the introducing approaches from this laboratory [21]. TLC enantiomeric separations based on ligand exchange were published by Günther et al. [22] and Weinstein [23] in 1984, for the first time. Though the procedures differed in their choice of chiral selector and range

* Corresponding author. Tel.: +91 1332 285795; fax: +91 1332 286202.
E-mail addresses: rbushfcy@iitr.ernet.in, rbushfcy@gmail.com (R. Bhushan).

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of applicability, they had a very similar methodology. Separation models developed for ligand exchange HPLC [24,25] are also valid for TLC; the diastereomeric complex formed with the metal ion (e.g., Cu^{2+}) and the chiral adsorbent have different stabilities for the different antipodes which lead to chromatographic separation. Davankov [26] observed that the stability of the diastereomeric complexes formed in ligand exchange chromatography is higher than the stability of the diastereomeric adducts formed by other chiral selectors. This consideration has been described in literature to explain the separation of enantiomers of α-amino acids and α-hydroxy acids and is well satisfactory for the separation of enantiomers of the β-blocking molecules, under study, as suggested by their structures.

Schmid et al. [11] proposed formation of ternary mixed metal complexes between the selector and the analyte in ligand exchange chromatography. The formation of a five membered ring results in the best chance to obtain a ternary complex (of chiral selector, copper ion, and the enantiomer) of sufficient stability.

In the present studies, separation of three β-blockers into their enantiomers has been achieved adopting the following three different approaches, (A) using the Cu(II)-L-amino acid complex as chiral mobile phase additive with plain plates, (B) ascending development of plain silica gel plates in the solutions of Cu complex, and (C) ascending development of plain silica gel plates in the solutions of amino acid and using a solution of Cu(II) acetate as mobile phase additive. In all the three approaches commercial pre coated silica gel plates were used. Use of Cu(II) complexes of five L-amino acids (L-Pro, L-Phe, L-Trp and L-His, and *N*,*N*-Me_2-L-Phe) has been made for ligand exchange seaparation. To the best of authors' knowledge this is a first report adopting these approaches of impregnation of the commercial plates. The performance of the three methods was compared by choosing one mobile phase to discuss the issue of involvement of Cu(II) for the best resolution.

2. Experimental

2.1. Apparatus and chemicals

UV–vis spectrophotometry was performed with Hitachi U 2001 spectrometer and pH was measured with a pH meter (Cyberscan 510, Singapore). Racemic atenolol and propranolol were obtained from I.C.I. (Madras, India) while salbutamol acetate was obtained from Vamsi Labs Ltd. (Solapur, Maharashtra, India). Optically pure isomers of both atenolol and propranolol and optically pure L-amino acids were obtained from Sigma–Aldrich (St. Louis, MO, USA). Normal Phase Plates (Alugram®, SIL G/UV_{254}) precoated (20 cm × 20 cm × 0.15 mm) were from Macherey-Nagel (Düren, Germany). All other reagents and chemicals used were of analytical reagent grade and were obtained from SISCO Research Laboratory (Mumbai, India).

2.2. Extraction and purification

The extraction and purification of atenolol and propranolol was done from their tablets as per procedure described earlier [10]. The samples were recrystallized from methanol–water, melting point, yield, λ_{max} and IR spectra were recorded.

3. TLC

3.1. Preparation of solutions

Solutions (25 mM) of purified (±)-atenolol, (±)-propranolol, and (±)-salbutamol were prepared in methanol; solutions of optically pure (*S*)-isomers were also prepared at the same concentration.

3.2. Preparation of Cu(II)-L-amino acid complex

The L-amino acids used were, L-Pro, L-Phe, L-His, and *N*,*N*-Me_2-L-Phe. Solution of copper(II) acetate (2 mM) and L-amino acid (4 mM) were first prepared in water–methanol (95:5); the two were mixed in a ratio of 1:2 and the final pH 7 was maintained by addition of a few drops of ammonia.

3.3. Impregnation and development of chromatograms

(A) The chromatograms pertaining to commercial precoated plates were developed using the solutions of LER [Cu(II)-L-amino acid(s) complexes] as mobile phase additive.
(B) The commercial precoated plates were impregnated with each of the four LERs by ascending development of the blank commercial plates in their solutions for 15–20 min. The plates were then air dried and used for enantioseparation.
(C) Plain plates were impregnated by ascending development of the blank commercial plates in solution of L-amino acid (100 mL of 2 mM). The plates were dried at 60 °C. The amino acids used for preparing these plates were L-Pro, L-Phe, L-Trp and L-His, and *N*,*N*-Me_2-L-Phe. The plates were developed using solution of Cu(II) acetate as the mobile phase additive since these were impregnated with L-amino acid only.

Solution of racemic atenolol and its pure (*S*)-isomer (2 mM) were spotted (5–10 μl) side by side with the help of 25 μL Hamilton syringe. The chromatograms were developed in cleaned, dried and paper lined rectangular glass chambers. These were pre equilibrated with the mobile phase at 20 ± 2 °C for 20–30 min. Binary and ternary mixtures of acetone, methanol, acetonitrile, dichloromethane, and water were used as mobile phase to achieve enantiomeric resolution. Chromatograms were dried at 40 °C in an oven and then cooled to room temperature; spots were located in an iodine chamber. Similar experiments were carried out using propranolol for enantiomeric resolution.

3.4. Effect of mole ratio of Cu(II) to amino acid

The ratio of Cu(II) to L-amino acid was optimized by using all the three β-blockers and *N*,*N*-dimethyl-L-phenylalanine (for impregnation) and Cu(II) in the mobile phase (i.e., approach C). For this purpose, the plates were prepared by using 2, 4, 6 and 8 mM concentrations of *N*,*N*-dimethyl-L-phenylalanine for impregnation. At first, Cu(II) concentration was kept at 2 mM in the mobile phase [MeCN–MeOH–Cu(II) (3:5:4)] while the concentrations of L-amino acid changed from 1 to 8 mM to provide a ratio of Cu(II)/L-amino acid at 1:1, 1:2, 1:3, and 1:4. Secondly, the mobile phase [MeCN–MeOH–Cu(II) (3:5:4)] used for each of these plates had a different concentration of Cu(II), i.e., 1, 2, 3 and 4 mM, respectively. This provided different combinations viz., 1 mM Cu(II)/2 mM L-amino acid, 2 mM Cu(II)/4 mM L-amino acid), 3 mM Cu(II)/6 mM L-amino acid and 4 mM Cu(II)/8 mM L-amino acid.

4. Results and discussion

The melting points of the compounds isolated from the commercial tablets were in agreement with the reported values (SciFinder Scholar 2007) and also the λ_{max} [27]. The melting points and IR characterization peaks of the purified compounds are not included here in view of the focus of the paper being on enantiomeric resolution.

Incorporation of a suitable reagent with the adsorbent without covalently affecting its inert character, prior to development of chromatogram, is termed as impregnated TLC. Methods used for impregnation include mixing the impregnating reagent with the

Table 1
Different combinations of MeCN–MeOH–H_2O showing successful resolution of atenolol, propranolol and salbutamol along with hR_F.

Analyte	Chiral selector	Approach (A)			Approach (B)			Approach (C)		
		Solvent ratio	hR_F		Solvent ratio	hR_F		Solvent ratio	hR_F	
			(*R*)	(*S*)		(*R*)	(*S*)		(*R*)	(*S*)
Atenolol	L-Phe	1:2:4	36	28	–	–	–	2:2:5	38	23
	L-His	0.5:3:6	38	30	1:5:4	43	32	2:2:4	30	20
	N,*N*-Me_2-L-Phe	1:2:5	28	17	–	–	–	3:4:5	36	16
	L-Pro	1:3:5	37	29	–	–	–	–	–	–
	L-Trp	–	–	–	–	–	–	3:2:5	37	27
Propranolol	L-Phe	1:2:4	36	27	2:5:5	40	27	2:2:5	38	23
	L-His	0.5:3:6	33	25	1:5:8	33	26	2:2:4	32	16
	N,*N*-Me_2-L-Phe	1:2:5	38	26	1:1:1	34	25	3:4:5	34	17
	L-Pro	1:3:5	31	26	–	–	–	–	–	–
	L-Trp	–	–	–	–	–	–	3:2:5	37	26
Salbutamol	L-Phe	1:2:4	34	26	1:5:1	26	18	2:2:5	32	25
	L-His	0.5:3:6	31	25	1:5:5	42	32	2:2:4	31	16
	N,*N*-Me_2-L-Phe	1:3:5	35	23	–	–	–	3:4:5	35	19
	L-Pro	1:3:5	31	26	–	–	–	–	–	–
	L-Trp	–	–	–	–	–	–	3:2:5	38	28

Solvent system: MeCN–MeOH–H_2O^*; H_2O^*, the volume of water in the ternary mobile phase represents the volume of aq. solution of the corresponding Cu(II)-amino acid complex. The three racemic β-blockers were also resolved under the following conditions:

1. *Approach A*: mobile phase CH_3COCH_3–MeOH–aq. solution of Cu(II) L-Pro complex (5:1:1).
2. *Approach B*: mobile phase MeCN–MeOH–CH_2Cl_2 (1:1:1) using the plate impregnated with Cu(II) complex of *N*,*N*-Me_2-L-Phe.
3. *Approach B*: mobile phase MeOH–CH_2Cl_2 (4:6) for resolution of atenolol only using the plate impregnated with Cu(II) complex of L-Phe.
4. *Approach C*: mobile phase of MeOH–H_2O–aq. Cu(II) 2 mM (2:1:10) using the plate impregnated with L-Pro.

inert support at the time of plate preparation, spraying it onto the plate, exposing the layer to the vapours of impregnating reagent, immersing or dipping the plate in the solution of the reagent, or allowing the solution to ascend or descend in a normal manner of development [14]. The reagents or the methods used for impregnation are different than those used as spray reagents or for locating the spots.

Different solvent combinations were found successful using the approach (A), (B) and (C). Based on these results, the performance of the three methods was compared by choosing one mobile phase to examine the involvement of Cu(II) for the best resolution. Nevertheless, one particular ratio of the three solvents (MeCN, MeOH and H_2O) was hardly successful in all the situations; a variation in the ratio of the three solvents was to be made to achieve resolution; Table 1 shows different successful combinations of MeCN–MeOH–H_2O and the R_F values obtained using them. In all the cases (*R*)-isomer was eluted before (*S*)-isomer. A representative photograph of the actual chromatogram is shown in Fig. 1. Resolution (R_S) of two adjacent spots was calculated by dividing the distance between the two spot centers with the sum of their radii; the two spots were considered to have reasonably separated when $R_S = 1.2$ [28].

A comparison of resolution data from the three methods, as given in Table 2, shows that the three methods can be arranged in the decreasing order (C > B > A) of resolution of enantiomers. In the approach (A) and (B) the replacement of one of the amino acid molecules is taking place in the already existing complex by the enantiomer in the racemic analyte while in approach (C) the formation of ternary complex is probably taking place *in situ*, during the chromatographic development, as the Cu(II) is available at the

Table 2
Comparison of resolution (R_S) using three approaches with common solvent system of varying composition (as per Table 1).

Amino acid	R_S								
	(±)-Atl			(±)-Prl			(±)-Sal		
	(A)	(B)	(C)	(A)	(B)	(C)	(A)	(B)	(C)
L-Phe	1.7	–	3.0	1.7	2.9	3.0	1.7	1.8	2.4
L-His	2.3	2.5	2.1	1.7	1.6	3.1	1.8	2.3	3.1
N,*N*-Me_2-L-Phe	1.7	–	3.5	2.6	1.4	3.2	2.5	–	4.0

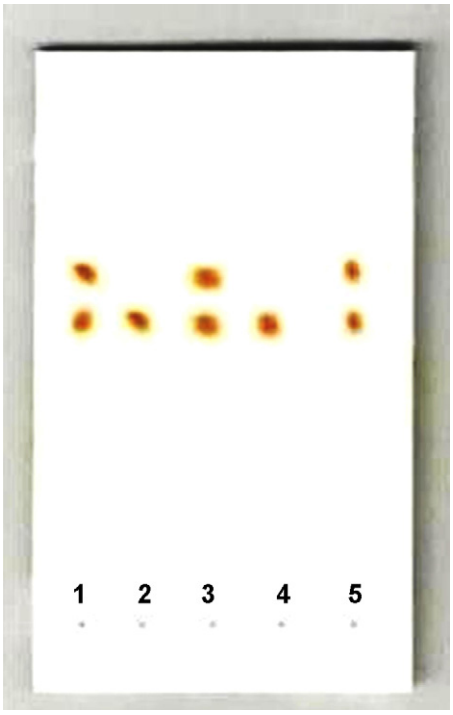

Fig. 1. Photograph of the chromatogram showing resolution of racemic atenolol, propranolol and salbutamol using the plate impregnated with *N*,*N*-Me_2-L-Phe (approach C), run time: 12 min, detection: iodine vapour, solvent front – 4 cm. Solvent system: MeCN–MeOH–aq. Cu(II) 2 mM (3:4:5), left to right: in track 1 the lower spot is that of (*S*)-atenolol and the upper one is that of (*R*)-atenolol; track 2 is that of pure (*S*)-atenolol; track 3 lower spot is (*S*)-propranolol and the upper one is (*R*)-propranolol and track 4 is that of pure (*S*)-propranolol; in track 5 lower spot is that of (*S*)-salbutamol and the upper spot is that of (*R*)-salbutamol.

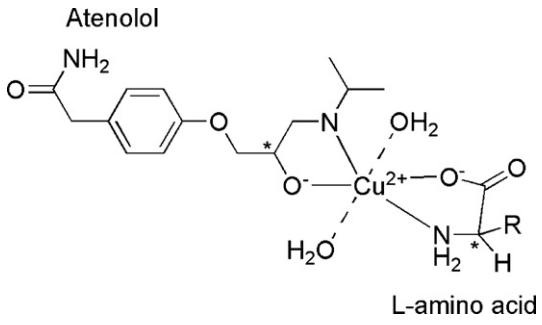

Fig. 2. Proposed structure of the ternary complex of atenolol and amino acid with Cu(II).

same time to the L-amino acid (present in the impregnated form) and to the analyte molecule spotted on the plate. Therefore, there are differences in resolution and retention. From the contents of Table 2 it is evident that approach (C) almost in each presented case outperforms approaches (A) and (B).

As mentioned in the experimental, with the increasing molar concentrations of both the amino acid and the Cu(II) the available amount of the complex also increased slightly (approach C) though the ratio of Cu(II) to amino acid remained at 1:2 and a slight increase in *R*s was observed though the best resolution (R_S) was at a ratio of Cu(II) 2 mM and L-amino acid 4 mM(i.e. a ratio of 1:2). Proposed structure of the ternary complex of atenolol and amino acid with Cu(II) is shown in Fig. 2.

4.1. Effect of temperature on separation

Additional experiments, with successful solvent systems, were carried out between 15 and 35 °C. For this purpose the chromatographic chambers were placed inside an incubator to attain the specific temperature. The best resolution for all the three racemates was obtained at 20 ± 2 °C with any of the chiral selectors used herein. Increase of temperature to 25 or 30 °C resulted in tailing of spots and a decrease in temperature to 15 °C showed no resolution (with all the three approaches).

5. Precision and limit of detection (LOD)

Solutions of known concentration (10 mM) of racemic atenolol, propranolol and salbutamol were applied on the TLC plate six times to determine the repeatability of the proposed method. The relative standard deviation (RSD) was between 0.25 and 0.50% for all the three β-blockers. To establish detection limits, different concentrations of the three β-blockers (0.1–1.0 μg per spot) were spotted. Secondly, different concentrations of (*S*)-enantiomers of atenolol and propranolol were spiked into fixed concentration of (*R*)-enantiomer in the range 0.1–5% using standard solutions of the two isomers. The chromatograms were developed using approach (C) and the mobile phase MeCN–MeOH–aq. Cu(II) 2 mM (3:4:5, v/v) followed by visualization with iodine vapours. The LOD was found as 0.18 μg per enantiomer.

6. Conclusion

The modern TLC laboratories almost exclusively use the commercial precoated TLC plates for better reproducibility of the results with the better standardized adsorbents. The methods presented herein provide a simple, rapid and effective approach in planar mode for chiral control of optical purity of pharmaceuticals which can be realized even in a small laboratory. The three approaches are successful for enantioseparation via the same ligand exchange mechanism for which the method of impregnation and successive formation of ternary complex is different.

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