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Nonequivalence behavior studies for the direct determination of enantiomeric purity and absolute configuration of timolol by NMR

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Direct determination of both the enantiomeric purity and absolute configuration of timolol was accomplished utilizing ^{1}H NMR (400 MHz) spectroscopy with fast diamagnetic chiral solvating agent to dissimilarly perturb the spectra of enantiomeric solutes. Nonequivalence behavior was studied for all variables that affect populations and intrinsic spectra of the diastereomeric solvates. Optimization of the experimental conditions in terms of probe temperature, substrate concentration and solvating agent to substrate molar equivalents provided resolved enantiomeric signals suitable not only for chiral recognition but also for quantification. Enantiomeric impurity was determined on the basis of relative intensities of the tert-butyl methyl protons resonances; the assignment of enantiomeric configuration was based on the relative field positions of these resonances. The analysis of synthetic mixtures of the enantiomers by the proposed NMR method resulted in assay values which agreed closely with the known quantities of each enantiomer in mixtures tested. The mean ±SD recovery values for the (*R*)-(+)-enantiomer was 100.0 ± 1.6% of added antipode (n = 8). The optically pure enantiomers were used to establish the minimum detection limits of 0.1%. The developed methodology represents a rapid and powerful tool for regulatory analysis.

1. Introduction

Timolol, (*S*)-(−)-1-(*tert*-butylamino)-3-[(4-morpholino-1, 2, 5-thiadiazol-3-yl) oxy]-2-propanol, is a nonselective active β-adrenoceptor-blocking drug in that it blocks β_1 and β_2 receptors.

Timolol is marketed as a maleate and only in the levorotatory form. The (+)-enantiomer is four times less potent in reducing intraocular pressure in man, forty nine times less potent as a β_2-adrenoceptor antagonist in animals, thirteen times less potent as bronchoconstricor in normal subjects, and considerably less effective in reducing the heart rate of exercising human subjects (Tocco et al. 1976; Share et al. 1984, Richards and Tattersfield 1985, 1987, Rotmensch et al. 1993). In addition to the different pharmacological activities, several studies have shown that absorption, metabolism and disposition kinetics of timolol also depend greatly on molecular chirality's (Nathanson 1988; Walle et al. 1988; Karhuvaara et al. 1989). Therefore, the development of facile specific and accurate analytical methods capable of separating identifying and quantifying the enantiomers for purity assessment is an important aspect of quality regulatory purposes.

Two fundamentally different approaches for the resolution of enantiomers are categorized as: the indirect technique involving the formation of non-transient diastereoisomeric derivatives by reaction with chiral derivatizing reagents and the direct method using the formation of transient diastereoisomers. The frequently used approach for the resolution of β-blockers appears to be via covalent diastereomer-formation using a chiral derivatizing agent followed by chromatographic resolution of the resulting products. Chiral derivatizing reagents such as 2,3,4,6-tetra-*O*-acetyl-beta-D-gucopyranosyl isothiocyanate (Zhou 1990), 4-(3-isothiocyanatopyrrolidine-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl, 1)-2,1,3-benzoxadiazole (Toya'oka et al. 1997), or 1,3-diacetoxy-1-(4-nitrophenyl)-2-propylisothiocyanate (Gyeresi et al. 2000; Peter et al. 2001) have been employed for the conversion of timolol enantiomers to chromatographically resolvable diastereomers. Although extensively used, this technique must be carefully controlled to avoid errors caused by different reaction rates of enantiomers with a chiral reagent (Adams et al. 1982), or by racemization during derivatization. Furthermore, chiral reagents are susceptible to problems such as racemization and instability during storage (Silber and Riegelman 1980). Optical resolution of â-amino alcohols including β-blockers was obtained by HPLC using chiral columns. Chiral stationary phases derived from (*R*)-*N*-(3,5-dinitrobenzoyl) phenylglycine (Pirkle-type) was employed for the resolution of β-blockers. Nevertheless, the prior derivatization of the amine function was required in order to reduce its basicity and shorten elution time. In that way, Pirkle et al. (1981) resolved enantiomers as lauroyl amide derivatives to reduce the basicity of the nitrogen and shorten the elution times, Weiner et al. (1984) assayed the enantiomers as oxazolidin-2-one derivatives, and Yang et al. (1988) carried out the enantiomeric separation of β-amino alcohols as α-naphthylurea derivatives. Other chiral stationary phases have been used for the chromatographic

resolution of β-blockers with required derivatization: *N,N′*-(3,5-dinitrobenzoyl)-*trans*-1,2-diaminocyclohexane (Gasparrini et al. 1991), α_1-acid glycoprotein (Hermansson 1985; Enquist and Hermansson 1989; Marit and Hermansson 1990), bovine serum albumin, or ovomucoid in which chiral recognition both enantioselectivity and retention was found to be altered by change in eluent pH and/or solvents (Haginaka et al. 1992; Mano et al. 1994). Although all of the HPLC methods have provided reasonable separation of the enantiomers of β-blockers including timolol, they require restricted conditions and/or prior derivatization. These methods are laborious, time consuming, demanding the reaction mixture to be cooled and stirred or refluxed for a long period. They did not seem sufficiently rugged for analysis. More direct enantioselective methods using cellulose tris (3,5-dimethylphenylcarbamate) stationary phase column, the enantiomers of a number of β-blockers have been resolved without resorting to derivatization (Ching et al. 1992; Krstulovic et al. 1988; Ekelund et al. 1995). Although direct, these methods require the use of the expensive pure enantiomers as reference standards. Furthermore, the use of HPLC may cause sample racemisation during the separation of an enantiomer on an optically active sorbent (Mannschreck et al. 1998).

The NMR spectroscopic approach enables the discrimination on the differential interactions of each enantiomer with a chiral probe. This laboratory has proposed a simple, specific, and quantitative ^{1}H NMR spectroscopic method using a Chiral Pr(III) shift chelate for the determination of the enantiomeric composition (optical purity) of timolol maleate (Hanna and Lau-Cam 1995). Salient favorable features of this method were economy of the reagents and procedural steps, non-reliance on reference standards, freedom from potential racemization, and the possibility of simultaneously obtaining positive proof on the identity of the drug. However, it requires the use of absolutely anhydrous reagents, solvents of high purity, and completely anaerobic working environment. The purpose of this report is to describe a further simplification of previous approach, an alternative ^{1}H NMR spectroscopic method free from the stringent requirements imposed by the earlier method. The required resolution of the enantiomeric resonance lines is accomplished through interaction of the enantiomeric mixture with a chiral solvating agent. The NMR signals observed in these binary selector-solute solutions are the time-averaged signals of both the complexed and uncomplexed substances. Optimization of experimental conditions can give rise to the shift displacements coupled with shift nonequivalence. In addition to permitting further simplification of the analytical procedure, this approach is also suitable for establishing the absolute configuration of the enantiomers.

2. Investigations, results and discussion

The nonequivalence in the NMR spectra of enantiomers may be induced by a chiral solvating agent (CSA) through combination of several factors. First, the diastereomeric solvates may have intrinsically nonidentical spectra. Second, if a chemical shift perturbation occurs upon solvation, a difference in equilibrium constants such that one solute enantiomer is solvated to a greater extent than the other can also result in nonequivalence. Since solute-solute association is a second order in solute concentration, whereas solute-CSA association is a first order in solute concentration, the extent to which these two processes compete depends on the concentration of solute enantiomers. In this study, the effect of solute-solute interaction was kept to a minimum by the combined use of an excess of solvating agent with a concentration of solutes that was just enough to produce adequate signal strength.

Nonequivalence was not observed in the ^{1}H NMR spectra of 0.04 M enantiomeric mixture of timolol and its (*R*)-(+)-antipode, Fig. 1. The effect of varying CSA molar equivalents on the chemical shift, induced shift Δδ and the differential induced shift ΔΔδ of the *tert*-butyl methyl proton signals of timolol and its (*R*)-(+) antipode diastereomeric solvates is shown in Table 1. The induced shift Δδ and the differential induced shift, ΔΔδ, continued to increase with increasing CSA molar equivalents and then tended to level out at higher values. The ΔΔδ between the diastereomeric solvates might arise from at least two, (probably mutually dependent) interactions: (a) the differences in equilibrium constants for formation of the various possible diastereomeric solvates between enantiomeric so-

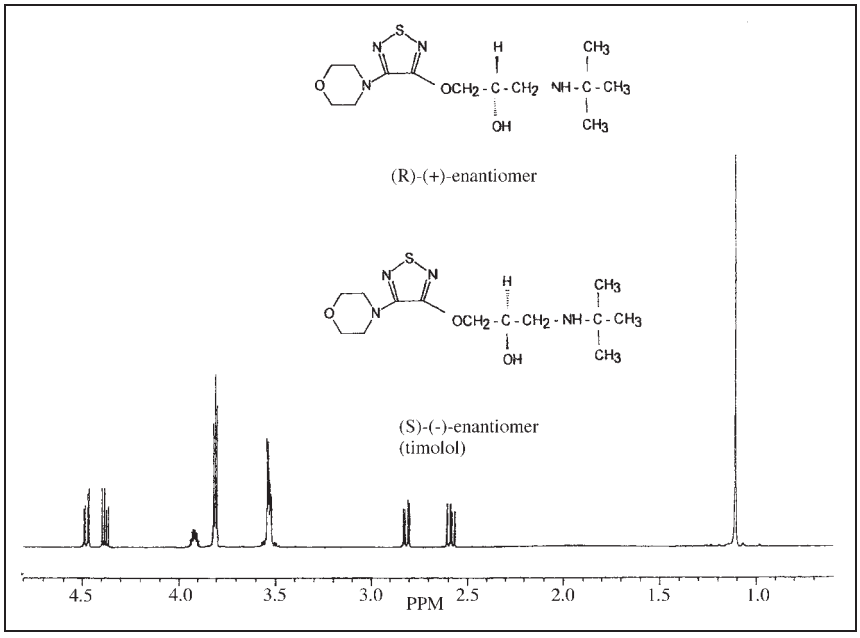

Fig. 1:
^{1}H NMR spectra of timolol and its (R)-(+)-antipode in $CDCl_3$

Table 1: Influence of CSA[a] molar equivalents on chemical shift, δ, induced shift, Δδ, and differential induced shift, ΔΔδ, of the tert-butyl methyl protons of timolol and its (R)-(+)-antipode[b] at 28 °C

CSA molar equivalents	(S)-(−)-Enantiomer		(R)-(+)-Enantiomer		
	δ	Δδ	δ	Δδ	ΔΔδ
0	1.1053	0	1.1053	0	0
1	1.0888	0.0165	1.0817	0.0236	0.0071
1.8	1.0777	0.0267	1.0656	0.0397	0.0130
2.6	1.0684	0.0369	1.0520	0.0533	0.0164
3.3	1.0600	0.0453	1.0396	0.0657	0.0204
4.0	1.0518	0.0535	1.0280	0.0773	0.0238
7.0	1.0277	0.0776	0.9934	0.1119	0.0343

[a] Chiral solvating agent (S)-(+)-TFAE
[b] Total enantiomeric mixture concentration in $CDCl_3$ was 0.04 M

Table 2: Shift data of the tert-butyl methyl protons signals of the solvates of timolol and it (R)-(+)-antipode with 4 molar equivalent of CSA at 28 °C

Solvating agent	(R)-(+)-Enantiomer		(S)-(−)Enantiomer, timolol		
	δ	Δδ	δ	Δδ	ΔΔδ
(S)-(+)-TFAE	1.052	0.054	1.028	0.078	0.024
(R)-(−)-TFAE	1.028	0.078	1.052	0.054	0.024

[a] Total enantiomeric mixture concentration in $CDCl_3$ was 0.04 M

lutes and the CSA and (b) the distinct geometry of resulted solvates. The data in Table 1 provide a qualitative support to conclude that there was only one main contribution of the two types of interactions to the observed ΔΔδ values. The signal for the *tert*-butyl methyl proton of the (*R*)-(+)-enantiomer was shifted upfield to a greater extent than that of the (*S*)-(−)-enantiomer after salvation with (*S*)-(+)-TFAE. The nonequivalence was definitely a reflection of difference in the geometries of the solvates formed and not simply the result of difference in equilibrium constants. The sense of nonequivalence for this particular CSA-solute combination was mainly dependent on the configuration of each component. As demonstrated in Table 2, reversed senses of nonequivalence were observed for the (*S*)-enriched enantiomeric mixture upon solvation with the antipode of the CSA.

The enantiomeric purity of the CSA was found to affect only the magnitude of spectral nonequivalence but not the relative size of the signals stemming from the diastereomeric solvates. No nonequivalence was observed when a racemic CSA was used. Since the CSA need not to be enantiomerically pure for nonequivalence to arise, enantiomeric purity determination by this method was found to be absolute in the sense that no reference to standard of known optical purity was required. Using more enantiomerically pure CSA than the one used, $>98\%$, will contribute only a negligible effect to Δδ or ΔΔδ. Because the amount of intermolecular hydrogen bonding to the CSA-solute interaction is an important factor, dilution induced a dramatic effect on Δδ and ΔΔδ. Slopes of all curves were

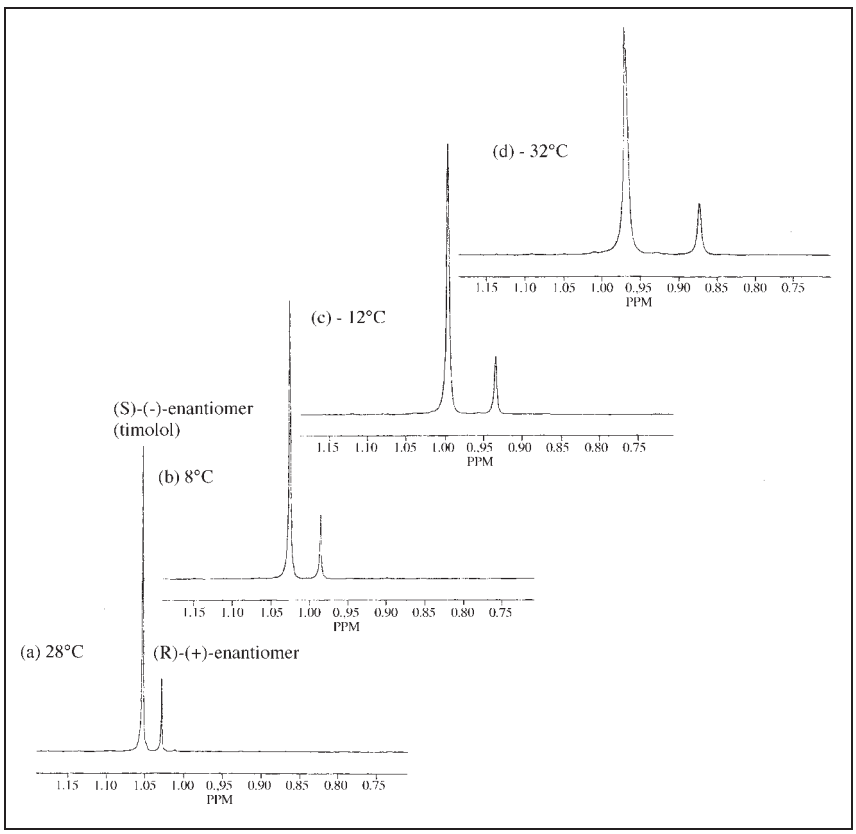

Fig. 2:
Upfield regions of the ^{1}H NMR spectra of the tert-butyl methyl protons of timolol and its (R)-(+)-antipode (0.04 M total)) solvated with (S)-(+)-TFAE (0.16 M) at temperatures: (a) 28 °C; (b) 8 °C; (c) −12 °C; (d) −32 °C

Table 3: Influence of probe temperature on the chemical shift, δ, the induced shift, Δδ, and the differential induced shift, ΔΔδ, of the tert-butyl methyl protons of timolol and its (R)-(+)-antipode[a] solvated with 4.0 molar equivalents of (S)-(+)-TFAE

Temperature, °C	(S)-(−)-Enantiomer, timolol		(R)-(+)-Enantiomer		
	δ	Δδ	δ	Δδ	ΔΔδ
28	1.0518	0.0535	1.0280	0.0773	0.0238
8	1.0246	0.0754	0.9850	0.1150	0.0396
−12	0.9947	0.0996	0.9325	0.1618	0.0622
−32	0.9669	0.1220	0.8721	0.2168	0.0948

[a] Total enantiomeric mixture concentration in $CDCl_3$ was 0.04 M

Table 4: Effect of probe temperature on differential induced shift, ΔΔδ, for the tert-butyl methyl protons of timolol and its (R)-(+)-antipode[a] solvated with various molar equivalents of (S)-(+)-TFAE

Molar equivalent	ΔΔδ			
	28 °C	8 °C	−12 °C	32 °C
0.0	0.0	0.0	0.0	0.0
1.0	0.007	0.012	0.021	0.034
1.8	0.012	0.021	0.035	0.057
2.6	0.016	0.028	0.046	0.074
3.3	0.020	0.034	0.055	0.086
4.0	0.024	0.040	0.062	0.095
7.0	0.034	0.053	0.076	0.103

[a] Total drug concentration in $CDCl_3$ was 0.04 M

different at low CSA concentrations for different solute concentrations despite equal CSA/solute ratios. Addition of a small quantity of a polar material such as dimethyl sulfoxide or methanol severely reduced and eliminated nonequivalence. Obviously, the polar material competed with the solute for CSA and probably altered conformations of the solvates that give rise to nonequivalence

The effect of varying the temperature on the enantiomeric separation was evaluated. with a mixture of 0.04 M (*S*)-(−)- and (*R*)-(+)-enantiomers, solvated with 0.16 M (*S*)-(+)-TFAE in $CDCl_3$. As shown in Fig. 2, the degree of Δδ and ΔΔδ of the *tert*-butyl methyl proton signals of the diastereomeric solvates increased in a proportional manner by decreasing the temperature. However, at lower temperatures than −12 °C these signals started to broaden. As it can be seen in Table 3, sufficiently large ΔΔδ values were obtained in the temperature range between 28 °C and −12 °C. The effect of probe temperature on the differential induced shift, ΔΔδ, for the tert-butyl methyl protons of timolol and its (*R*)-(+)-antipode solvated with various molar equivalents of (*S*)-(+)-TFAE is shown in Table 4. The plots of the induced shift Δδ and differential induced shift, ΔΔδ, for the *tert*-butyl protons signals of timolol and its (*R*)-(+)-antipode versus molar equivalents of (*S*)-(+)-TFAE at various temperatures are presented in Fig. 3 and Fig. 4, respectively. Although the increase in nonequivalence magnitude with reduction of temperature can be attributed to an increase in the equilibrium constants for CSA-solute association, such an enhancement was observed even when the CSA molar equivalents was at 7 as shown in Table 4. In other words, the CSA was present in such excess to cause essentially complete solvation. In this case, temperature reduction increased spectral difference of the diastereomeric solvates, by increasing the populations of specific conformations that gave rise to nonequivalence.

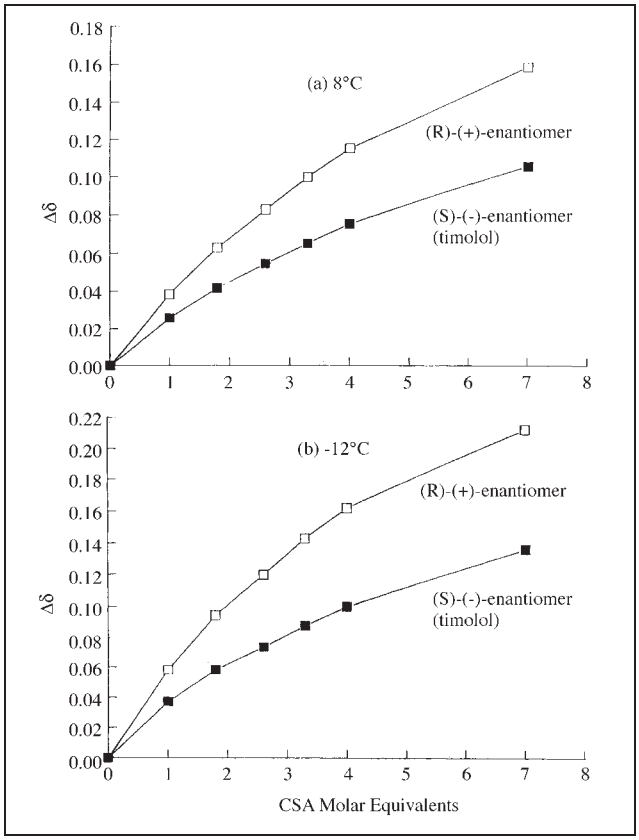

Fig. 3: Plots of the induced shift, Δδ, for the tert-butyl methyl protons of timolol and its (R)-(+)-antipode, 0.04 M in $CDCl_3$ versus molar equivalents of (S)-(+)-TFAE at (a) 8 °C and (b) −12 °C

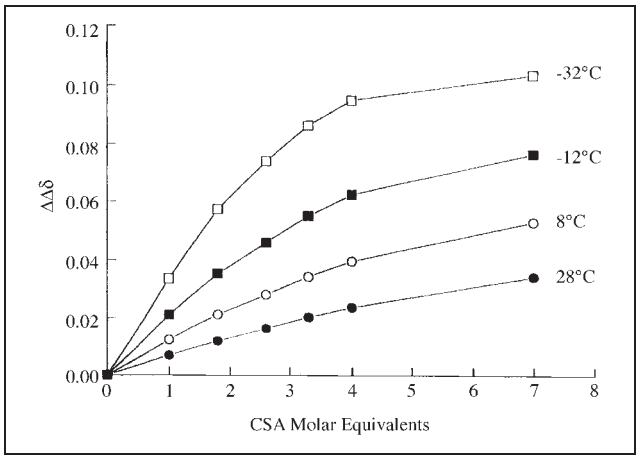

Fig. 4: Plots of the differential induced shift, ΔΔδ, for the tert-butyl methyl protons of timolol and its (R)-(+)-antipode, 0.04 M in $CDCl_3$ versus molar equivalents of (S)-(+)-TFAE at different probe temperatures

^{1}H NMR spectra of 0.04 M solutes and 0.1 6M CSA in $CDCl_3$ provided sufficient strength and well separated signals at probe temperature 28 °C convenient for chiral recognition and optical purity determinations. Using (*S*)-(+)-TFAE under the stated conditions, the enantiomeric *tert*-butyl methyl proton signals of the timolol and (*R*)-(+)-antipode were clearly resolved into two singlets: the downfield signal was assigned to the *t*-butyl methyl protons of timolol and the upfield signal was assigned to the corresponding *t*-butyl methyl protons of (*R*)-(+)-enantio-

Table 5: Assay results of synthetic mixtures of timolol and its (R)-(+)-antipode[a] by ^{1}H NMR spectroscopy[b] using solvating agent[c]

Mixture	Timolol, mg	(R)-Antipode, mg	(R)-(+)-Antipode, %		
			Added	Found	Recovered[d]
1	1.86	9.05	83.0	81.4	98.1
2	3.20	6.36	66.5	65.6	98.6
3	4.34	7.36	62.9	62.2	98.9
4	5.35	4.88	47.7	47.5	99.6
5	5.97	3.06	33.9	43.3	101.8
6	6.69	2.76	29.2	29.8	102.1
7	0	10.91	100.0	99.9	99.9
8	7.77	1.42	15.5	15.6	100.6
Av					100.0
SD					1.6

[a] Total concentration of timolol and its (R)-(+)-antipode was 0.04 M in $CDCl_3$
[b] Probe temperature 28 °C
[c] (S)-(+)-TFAE concentration was 0.16 M
[d] Recoveries were calculated from (amount found X 100)/amount added

mer. It can be seen from Fig. 2 that the degree of nonequivalence of the *tert*-butyl methyl signals of the diastereomeric solvates at all temperatures were sufficiently large and usable for enantiomeric purity determination.

The absolute configuration of stereogenic centers are difficult to determine. The readily practical technique such as NMR analysis is preferable. The construction and examination of suitable ball-and-stick molecular models of the diastereomeric solvates formed can facilitate the assignment of the absolute configuration of the enantiomers of timolol. It can be seen that the spatial orientation of the *t*-butyl methyl group of each of the solvates that will determine if this group will be shielded or less shielded by the anthryl moiety of the CSA. For example, the chemical shift will occur at a higher field if this group is located approximately above or below the ring current, in other words, cis to the anthryl current. The resonance for the methyl proton will appear at lower field because this group will be oriented approximately with the plane of the anthryl ring that is trans to the anthryl. These predictions were confirmed by separately studying the interactions with a specific enantiomer of the CSA as shown in Table 2. As expected, the chemical shift of the *tert*-butyl methyl group of the (*S*,*S*) solvate is observed downfield to that of the (*R*,*S*) solvate and the reversed is observed for (*R*,*R*) and (*S*,*R*) solvates due to the shielding effect of the anthryl.

The advantages of using ^{1}H NMR spectroscopy for measuring enantiomeric composition lie in the high sensitivity of the ^{1}H nucleus and in the fact that relative signal intensities directly reflect relative number of resonating nuclei and hence relative enantiomeric populations. Eight mixtures of timolol and (*R*)-(+)-antipode, made up in proportions shown in Table 5 were mixed with the specific amounts of chiral solvating agent (*S*)-(+)-TFAE, and dissolved in $CDCl_3$, to yield solutions with ca. 0.04 M and 0.16 M solute and CSA concentrations, respectively. The results of enantiomeric composition calculated based on the integrals of the methyl protons were found in close agreement with the known values. Average recovery ± SD for the (*R*)-(+)-enantiomer was 100.0 ± 1.6%. The optically pure timolol was found to contain 0.1% of (*R*)-(+)-enantiomer.

3. Experimental

3.1. Apparatus

^{1}H NMR spectra were obtained on a Brŭker AMX-400 spectrometer equipped with a carbon/proton 5 mm probe (Brŭker Instruments, Inc., Billerica, MA, USA). The ^{1}H NMR spectra were obtained under the following conditions: acquisition time, 2.03 s; data point resolution, 0.492 Hz/point; pulse width, 7.0 μs and tip angle 30°; relaxation delay, 2.0 s; number of scans, 32. Chemical shifts were referred to $CHCl_3$ (δ 7.26).

3.2. Materials

Deuterochloroform ($CDCl_3$, 99.8 atom% D, stabilized with Ag foil), and (*R*)-(−)- and (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (TFAE; > 99% chemical purity and > 98% optical purity) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

The (*R*)-(+)-, (*S*)-(−)-, and (+)-1-(tert-butyl amino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl) oxy]-2-propanol maleate (1 : 1) salts were obtained from Merck, Sharp & Dome, West Point, PA, USA. The samples were analyzed by polarimetry and the proposed NMR method.

3.3. Sample preparation

The maleate salt of was converted to the free-base form as follows: a quantity of the drug, accurately weighed, was dissolved to the extent possible in 1.5 mL of D_2O. One drop of 0.5 M sodium deuteroxide was added to $CDCl_3$ (0.75 mL). Both solutions were bubbled with N_2 and then combined. The $CDCl_3$ layer was removed using a separator. A second extraction was performed and $CDCl_3$ fractions were combined and evaporated to dryness. The sample was dried in vacuum at 50 °C for approximately 30 min and weighed.

3.4. Nonequivalence behavior studies

Conditions for the determination of the enantiomeric composition were explored by observing the tert-butyl methyl proton signal and study: (a) the effect of varying the chiral solvating agent, CSA, molar equivalents; (b) the effect of probe temperature; and (c) combined effect of both variables, CSA molar equivalents and probe temperature, on chemical shift δ, induced chemical shift Δδ, and differential induced chemical shift ΔΔδ.

The required changes in CSA molar equivalents were obtained by first preparing stock solutions of sample (95.0 mg/mL) and (*S*)-(+)-TFAE (ca.110.5 mg/mL) in $CDCl_3$. A 0.1 mL of total timolol and its antipode (9.5 mg) solution and the appropriate amount of (*R*)-(−)-TFAE solution were added to a 5-mm NMR tube. The final volume was adjusted with $CDCl_3$ to 0.75 mL. The NMR tube was capped with a Teflon cap, its contents were mixed by inversion, allowed to stand for 10 min, and then placed in the spectrometer to obtain the ^{1}H NMR spectrum. The additions and spectral recording were repeated until an appropriate number of spectra were available for properly defining the effects of CSA molar equivalents on the enantiomeric spectral lines.

3.5. Determination of enantiomeric purity

A quantity of timolol maleate sample of approximately 13.0 mg was converted to the free base as described above. The dry residue was dissolved in 0.5 mL $CDCl_3$, and the solution was transferred to a dry NMR tube containing approximately 33.0 mg of (*S*)-(+)-TFAE. The final volume was adjusted to 0.75 mL and then the tube was capped, inverted several times to effect solution, allowed to stand for 10 min, and then used to obtain the ^{1}H NMR spectrum. The intensities of enantiomeric tert-butyl methyl protons enantiomeric signals centered at approximately δ 1.052 and δ 1.028 corresponding to timolol and (*R*)-(+)-antipode, respectively, were measured and the percentage of each enantiomer was calculated based on the contribution of each resonance to the sum of both resonances as follows:

$$\%(\mathrm{R})-(+)\text{-enantiomer} = \frac{100 \times A_{(-)}}{A_{(-)} + A_{(+)}}$$

$$\%\ \mathrm{Timolol} = \frac{100 \times A_{(+)}}{A_{(+)} + A_{(-)}}$$

where $A_{(+)}$ = area of the resonance signal for the tert-butyl proton of the (*R*)-(+)-enantiomer, and $A_{(-)}$ = area of the resonance signal for the tert-butyl proton of the (*S*)-(−)-enantiomer (timolol).

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