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^{1}H-NMR and HPLC analysis on the chiral discrimination of β-blockers using (*S*)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid

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Abstract: In the group of commonly prescribed β-blocker drugs, one of the enantiomers is generally relatively more active than the others. This study aims to develop a technique for the chiral analysis of select β-blockers based on proton nuclear magnetic resonance (^{1}H-NMR) spectrometry. (*S*)-2-*Tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid ((*S*)-TBMB) was synthesized and utilized as a chiral derivatizing agent. Pure β-blocker enantiomers were isolated from racemates by semi-preparative liquid chromatography prior to derivatization. The reaction time and concentration of (*S*)-TBMB were controlled to improve the derivatization procedure. No racemization was found during the analysis. High-performance liquid chromatography (HPLC) analysis was also performed for comparative purposes. High agreement between the NMR and HPLC methods was achieved in the determination of (*R*)-metoprolol in a standard solution of the (*S*) isomer.

Key words: β-blockers, Chiral derivatizing agent, ^{1}H-NMR, HPLC

1. Introduction

Chiral research has become crucial to drug development because the living system is chiral in nature and the pharmacodynamics/pharmacokinetics of each enantiomer of a drug might be stereospecific.[1] In 2001, the Nobel Prize in Chemistry honored a study on chirally catalyzed hydrogenation and oxidation reactions.[2] Thus far, various techniques have been applied to chiral synthesis and analysis in the pharmaceutical industry. The US Food and Drug Administration (FDA) also recommended the assessment of the activity of each enantiomer of racemic drugs in the body and acceleration of the development of chiral pharmaceuticals as single enantiomers.[3,4]

β-blockers, also known as β-adrenergic antagonists, are commonly used in the treatment of hypertension. These drugs act by preventing the attachment of catecholamines to β-adrenoreceptors.[5] In the past few years, β-blockers have earned distinction as one of the most commonly prescribed drugs. A large number of β-blockers is available on the pharmaceutical market as racemates. However, the pharmacological properties of the enantiomers differ considerably. With the exception of labetalol for which the active enantiomer is (*R*,*R*), the (*S*) enantiomers are generally

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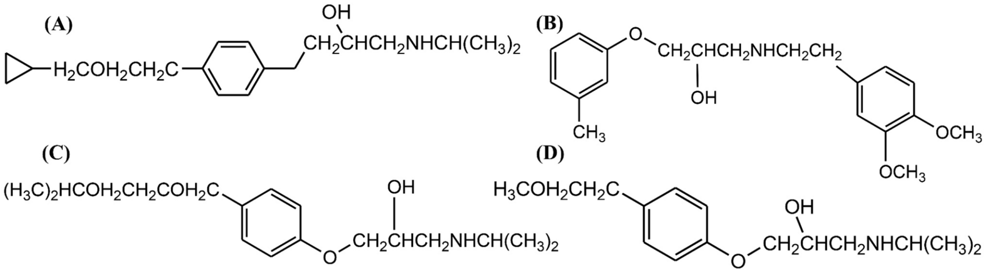

Fig. 1. Structures of β-blockers: (A) betaxolol, (B) bevantolol, (C) bisoprolol, (D) metoprolol.

more active, or sometimes are the only active compounds in the racemates.[6] Therefore, over the previous decades, extensive effort has been made on the enantio-separation of β-blockers for clinical study or drug quality control purposes.

Various chromatographic methods for the chiral analysis of β-blockers have been developed based on the separation of diastereomeric derivatives using chiral reagents (indirect approaches),[7-11] using mobile phase additives and application of novel chiral stationary phases (direct approaches).[9-15] Capillary electrophoretic methods were also reported for the same purposes.[16-20] However, to date, only a limited number of studies have reported the application of nuclear magnetic resonance (NMR) spectrometry to the chiral discrimination of β-blockers, with focus on the earliest generation: propranolol, timolol, and nadolol.[21-24] The development of NMR methods might be challenging, but can provide valuable structural information that cannot obtained from chromatographic and electrophoretic methods. In chiral analysis by NMR, it is necessary to use chiral auxiliaries such as chiral derivatizing agents, chiral solvating agents, or chiral lanthanide shift reagents.[25] Among the auxiliaries, (*S*)-2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid ((*S*)-TBMB), a derivatizing agent, has been proven to have notable properties such as showing strong and stable fluorescence, strong ion peaks in mass spectrometry detection, and strong singlet peaks in ^{1}H-NMR detection.[26]

Therefore, in this study, we present an NMR-based analytical method using (*S*)-TBMB for the enantioseparation of typical β-blockers (betaxolol (BETX), bevantolol (BEVA), bisoprolol (BISO), and metoprolol (METO); *Fig.* 1). High-performance liquid chromatography (HPLC) analysis using (*S*)-TBMB is also performed for comparative purposes.

2. Experimental

2.1. Chemicals and reagents

Bevantolol hydrochloride was obtained from Ildong Pharmaceutical (Seoul, Korea), betaxolol hydrochloride was procured from Bukwang Pharmaceutical Company (Seoul, Korea), bisoprolol hemifumarate and metoprolol tartrate were purchased from Yuhan Company (Seoul, Korea). HPLC grade methanol, acetonitrile, and other analytical grade reagents were obtained from Duksan Pure Chemicals Company (Ansan, Korea).

2.2. Instrumentation

The enantiomer standards were collected using a chiral semi-preparative liquid chromatographic system consisting of an LC-9A pump, SPD-6AV UV detector, CR4A integrator from Shimadzu Scientific Corporation (Kyoto, Japan), and a Rheodyne 7725i injector with a 200 μL loop (IDEX Health & Science, Middleborough, MA, USA). The analytical HPLC system used herein consisted of an LC-10A pump, SIL-10A autosampler, CBM-10A integrator (Shimadzu, Kyoto, Japan), and 502T UV detector (GL-Science, Tokyo, Japan).

All ^{1}H-NMR spectra were obtained using a Bruker AMX-400 spectrometer (Bruker, Milton, Ontario, Canada) and were referenced to tetramethylsilane, the signal of which was taken as 0.00 ppm. The instrument was set to acquire at least 128 scans for a proton spectrum. The spectrum was processed using a line broadening of 0.3 Hz.

2.3. Isolation of β-blocker enantiomers

Each β-blocker enantiomer was isolated from racemates using a semi-preparative HPLC system with a Chiralcel OD column (5 µm, 250 × 10 mm I.D., Daicel, Japan) at room temperature by elution at a flow rate of 2.0 mL /min; UV detection was performed at 276 nm. The mobile phase comprised n-hexane/isopropanol/diethylamine. The racemic (rac) β-blockers (10 mg mL^{-1}) were dissolved in the mobile phase before being injected into the chromatographic system. The enantiomeric purity of the collected compounds was checked by chiral HPLC using a Chiralcel OD column (5 µm, 250 × 4.6 mm I.D., Daicel, Japan) with the same mobile phase at a flow rate of 0.5 mL/min.

2.4. Derivatization of β-blockers

(*S*)-TBMB was synthesized by following the procedure proposed by Nishida et al. (see Supporting Information 1).[27] The compounds of interest were derivatized with the synthesized (*S*)-TMBM prior to HPLC and NMR analysis (*Fig.* 2). $SOCl_2$ was added to a solution of (*S*)-TBMB in dry benzene, and the mixture was kept at 60°C. After 10 min, the excess $SOCl_2$ and benzene were removed in vacuo to give (*S*)-TBMB-COCl. A dry pyridine solution of 10 % 4-dimethylaminopyridine (DMAP) and (*S*)-TBMB-COCl was added to the solution of β-blockers in dichloromethane with stirring at room temperature, followed by dilution with acetonitrile and washing with saturated $NaHCO_3$ solution and water. The dichloromethane solution was dried over $MgSO_4$ and evaporated at 40 °C to form di-(*S*)-TBMB-carbonyl-β-blocker. The influence of the reaction time and molar ratio was also investigated to improve the derivatization process. The derivatives were also introduced into a mass spectrometer for determination of the molecular weight.

2.5. ^{1}H-NMR study

The racemate, *R*, and *S* enantiomers of the analytes were derivatized with (*S*)-TBMB-COCl. The diastereomeric derivatives were dissolved in $CDCl_3$ and analyzed using a 400 MHz NMR spectrometer. The chemical shift differences (Δ*δ*) for each enantiomer of the β-blockers were evaluated.

2.6. HPLC conditions

For comparative study, the derivatized enantiomers were also analyzed by using a Phenomenex prodigy silica column (150 × 4.6 mm I.D.; Phenomenex, USA). The mobile phase was hexane-dichloromethane-methanol with various compositions at a flow rate of 1.0 mL/min.

2.7. Analysis of chiral purity of (*S*)-METO

The method was applied for determination of chiral purity of (*S*)-METO, which is relatively more

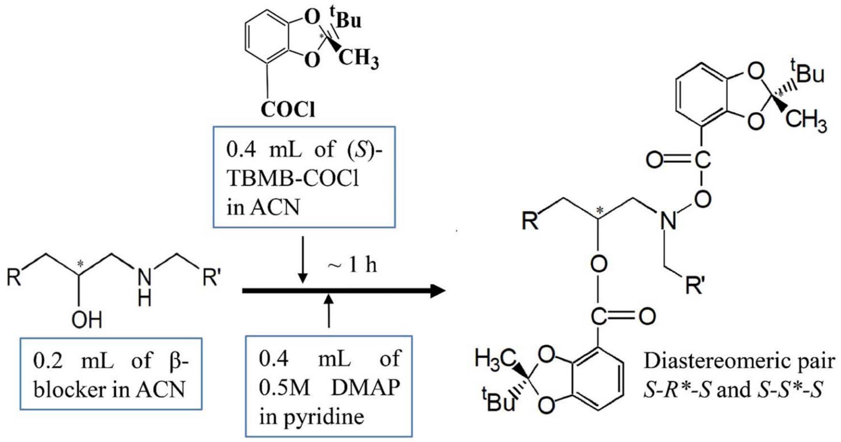

Fig. 2. Process for derivatization of β-blockers with (*S*)-TBMB-COCl. ACN: acetonitrile; DMAP: 4-dimethylaminopyridine.

active among two METO enantiomers. Synthetic mixtures of 2.5 and 5.0 % (*R*)-METO in (*S*)-METO were prepared by spiking the (*R*)-METO derivative into the (*S*)-METO derivative. The mixtures were then analyzed by the proposed HPLC and ^{1}H-NMR methods. The relative content of each enantiomer was calculated based on the peak area.

3. Results and Discussion

3.1. Isolation of β-blocker enantiomers

Each enantiomer was separated and collected by chiral-preparative HPLC within 30 min. All of the (*R*)-enantiomers were eluted before the (*S*)-enantiomers (Supporting Information 2). White amorphous powders with an enantiomeric purity of 100 % were obtained, which could be used as standards in further experiments.

3.2. Derivatization of β-blockers

The isolated β-blocker enantiomers were derivatized, with yields of more than 80 % for all the β-blockers investigated. The reaction time profile was very similar for the four β-blockers. *Fig.* 3 illustrates the influence of the reaction time and concentration of (*S*)-TBMB on the peak area of the derivatized METO enantiomers. The peak area of METO became larger when the reaction time was increased from 0 to 45 min. Further extending the reaction time resulted in no improvement. The reaction time of the other compounds was also within 60 min. An increase of the concentration

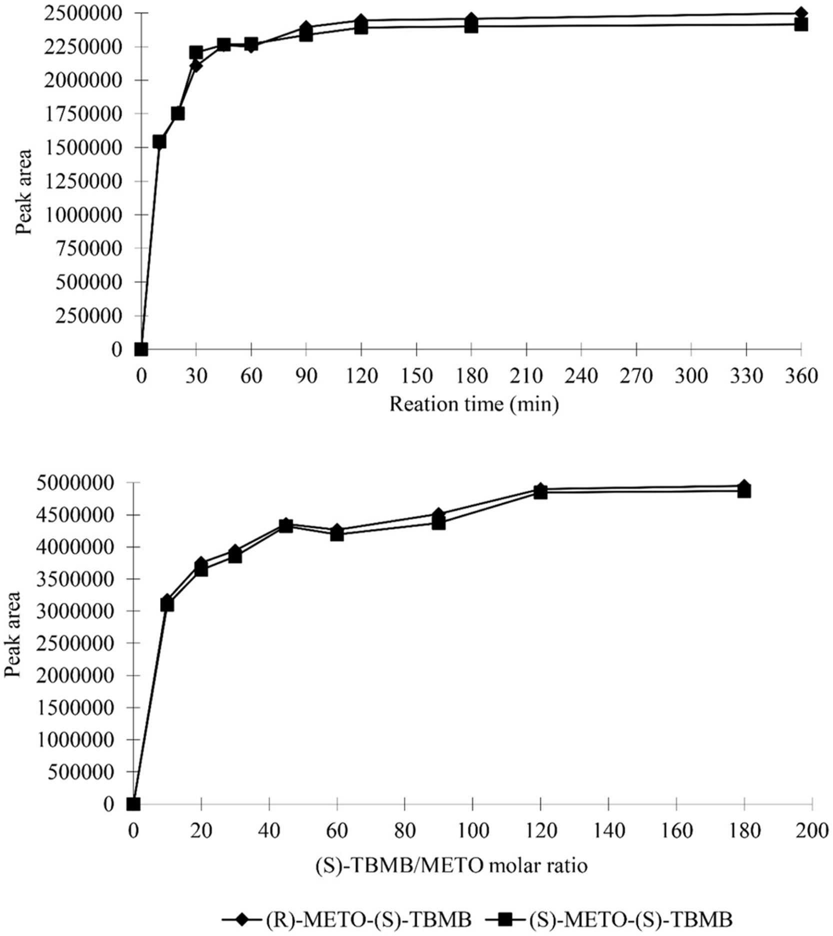

Fig. 3. Influence of reaction time and (*S*)-TBMB concentration on the derivatization of METO.

of (*S*)-TBMB led to a general increase in the formation of the diastereomers. The peak areas of the diastereomers of METO increased when the (*S*)-TBMB/METO molar ratio was 100 and reached a plateau. From the mass spectrometry data, the molecular weight of the derivatives increased by 436 units, which suggests the addition of two (*S*)-TBMB molecules to the β-blocker molecules (Supporting Information 3). The derivatized diastereomeric di-(*S*)-TBMB-β-blockers were stable and no racemization was found during the experiment.

3.3. Chiral discrimination of β-blockers by ^{1}H-NMR

Based on the obtained ^{1}H-NMR spectra of the β-blocker derivatives, the signals arising from the methyl and *tert*-butyl protons of the respective enantiomers

Table 1. Chemical shift difference of protons in β-blocker enantiomers' *t*-butyl group and methyl group

β-blocker	Chemical shift (*S*)-TBMB(δ_H)				$\Delta\delta_H$		ΔHz	
	t-Bu		Me		*t*-Bu	Me	*t*-Bu	Me
	R	S	R	S				
BETX	0.9452	0.9700	1.4831	1.4520	0.025	0.031	9.918	12.436
	1.0307	1.0688	1.5850	1.5265	0.038	0.059	15.262	23.313
BEVA	0.9442	0.9689	1.4819	1.4530	0.026	0.029	10.243	11.545
	1.0308	1.0696	1.5843	1.5278	0.039	0.057	15.497	22.702
BISO	0.9086	0.9122	1.3513	1.3822	0.004	0.031	1.438	12.364
	1.0310	1.0636	1.4739	1.5682	0.033	0.094	13.052	37.755
METO	0.944	0.9718	1.4872	1.4536	0.027	0.034	10.772	14.066
	1.0263	1.0681	1.5863	1.5243	0.042	0.062	16.702	24.810

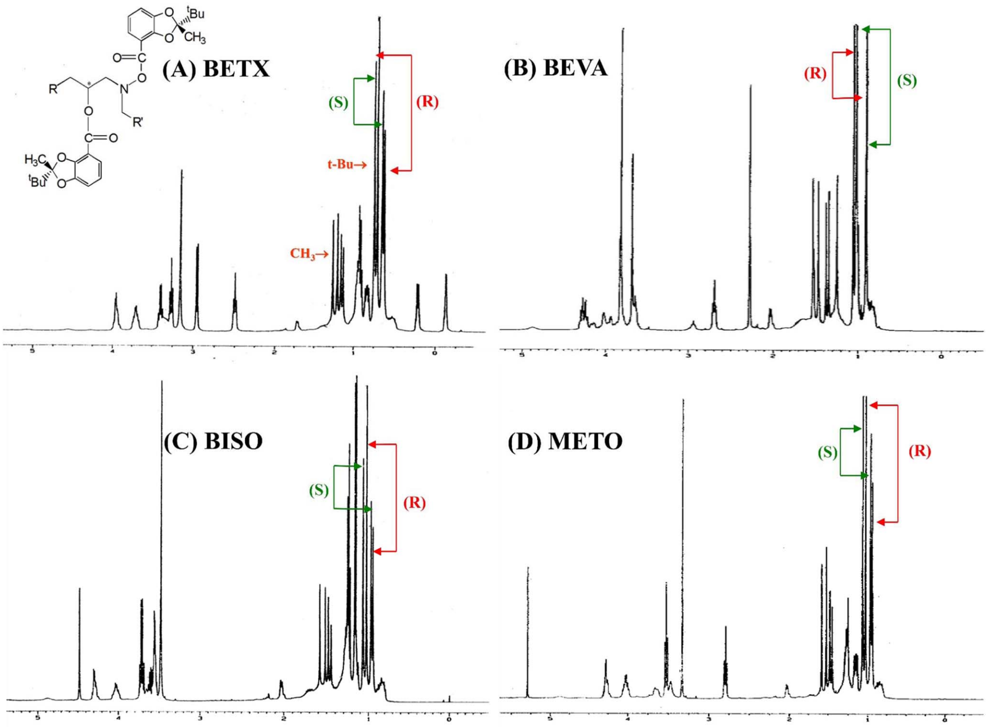

Fig. 4. ^{1}H-NMR spectra of (rac)-β-blockers.

were found to be separated. Based on the NMR peak of *tert*-butyl, down-field chemical shifts were observed for the (*S*)-enantiomers compared with the (*R*)-enantiomers. For the *tert*-butyl and methyl protons of METO, $\Delta\delta$ of the two enantiomers was 0.042 ppm and 0.062 ppm, respectively (*Table* 1). These results allowed the quantification of the (*R*)-enantiomer present in the (*S*)-enantiomer standards and vice versa. The resonance of the *tert*-butyl protons was used for determination of the enantiomers in the samples, as the intensity of these signals was higher than those of the methyl group. *Fig.* 4 shows the expanded ^{1}H-NMR spectra of the (rac) compounds. In comparison with previous published studies on chiral analysis of timolol and propranolol enantiomers using chiral lanthanide shift reagents, cyclosophoraoses, and (-)-Epigallocatechin-3-O-gallate, (*S*)-TBMB provided competent selectivity with similar $\Delta\delta_H$ and ΔHz values.[22-24] However, the other reagents were only investigated with only one compound of interest while (*S*)-TBMB can be applicable for four compounds in β-blocker group.

3.4. Chiral separation of β-blockers by HPLC

The derivatives were injected into the HPLC system. Under normal-phase conditions using a silica column and hexane-dichloromethane-methanol as the mobile phase, complete separation was achieved within 30 min (*Fig.* 5). *Table* 2 lists the chromatographic parameters of the enantiomers under the investigated conditions.

3.5. Determination of chiral purity of (*S*)-METO by developed method

Synthetic mixtures of the (*R*)-METO-di-(*S*)-(+)-TBMB diastereomer and (*S*)-METO-di-(*S*)-(+)-TBMB diastereomer were prepared and analyzed by the proposed methods (*Fig.* 6). The results summarized in *Table* 3 show good agreement between the nominal

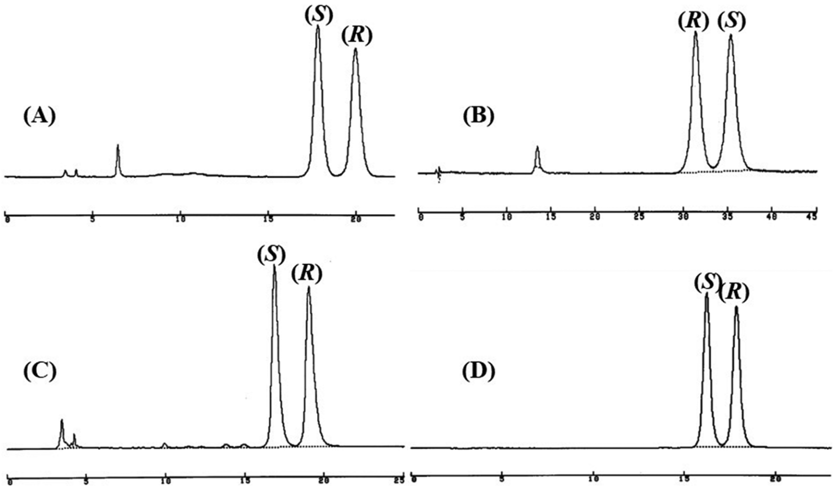

Fig. 5. Chromatograms of diastereomeric derivatives obtained from racemic β-blockers after derivatization with (*S*)-TBMB-COCl. (A) BETX, (B) BEVA, (C) BISO, (D) METO. Conditions are presented in *Table* 2.

Table 2. Chromatographic parameters for β-blocker enantiomers, analyzed by HPLC on Phenomenex Prodigy silica column (5 μm, 250 × 4.6 mm), with UV detection at 276 nm, injection volume: 5 μL

β-blocker	Mobile Phase (Flow rate: 1.0 mL/min)	Peak 1			Peak 2			Rs	α
		t_R (min)	N	As	t_R (min)	N	As		
BETX	n-hexane/dichloromethane/methanol = 100/250/1	17.78	6022	1.31	19.92	5870	1.20	2.20	1.12
BEVA	n-hexane/dichloromethane/methanol = 100/250/1	17.76	6373	1.18	19.82	6333	1.24	2.15	1.10
BISO	n-hexane/dichloromethane/methanol = 100/100/1	16.87	6656	1.76	19.05	6683	1.97	2.50	1.13
METO	n-hexane/dichloromethane/methanol = 200/200/1	16.20	8318	1.16	17.83	9287	1.14	2.23	1.10

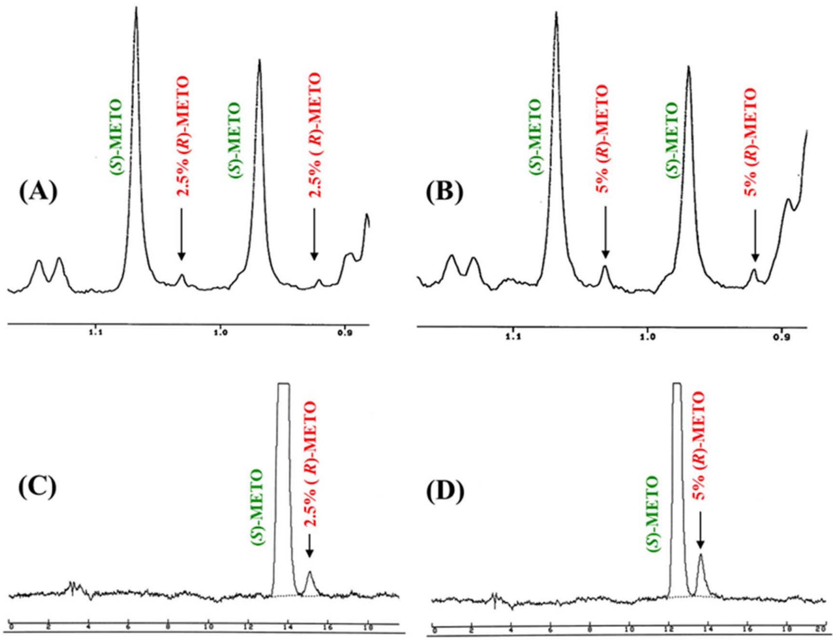

Fig. 6. Typical ^{1}H-NMR spectra (A), (B) and chromatograms (C), (D) of synthesized METO mixtures with 2.5 and 5.0 % isomeric impurity. HPLC condition: Phenomenex Prodigy silica column (5 µm, 250 × 4.6 mm), mobile phase: n-hexane/dichloromethane/methanol = 200/200/1, flow rate: 1.0 mL/min, UV detection at 276 nm, injection volume: 5 µL.

Table 3. Recoveries of (*S*)-(+)-METO-di-(*S*)-TBMB derivatives added to (*R*)-(+)-METO-di-(*S*)-TBMB

Nominal content (%)	Found Content (%)			
	HPLC		NMR	
	Mean (%)	C.V. (%)	Mean (%)	C.V. (%)
2.5	3.19	4.20	3.00	4.31
5.0	5.69	2.15	6.36	3.52

and measured concentrations. The limit of detection and quantitation using ^{1}H-NMR were roughly 1.2 % and 2.0 %, respectively. The sensitivity can be improved by using a higher field instrument and increasing the number of scans acquired or the amount of sample.

4. Conclusions

The chiral discrimination of four commonly used β-blockers by an NMR method was studied for the first time. (*S*)-TBMB was successfully synthesized as a chiral derivative agent for NMR and HPLC analyses. The derivatization was simple and could be completed in less than 1 h at room temperature. The NMR method for chiral discrimination of β-blockers and measuring the chiral purity of (*S*)-METO is convenient and precise. The NMR results were in good agreement with those obtained by the HPLC method.

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