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Enantiomeric Purity Test of Bevantolol by Reversed-Phase High Performance Liquid Chromatography after Derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl Isothiocyanate

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A reversed-phase high-performance liquid chromatographic method was developed to determine the optical purity of bevantolol enantiomers. (S)-(-)-Menthyl chloroformate((-)-MCF), (S)-(-)-α-methylbenzyl isocyanate((-)-MBIC) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate(GITC), which can react with the secondary amine group of bevantolol were investigated as chiral derivatization reagents. Among them indirect chiral HPLC method using GITC gave the best result. The derivatization proceeded quantitatively within 20 min at room temperature. Separation of the enantiomers as diastereomers was achieved by reversed-phase HPLC within 20min using ODS column. Different ratios of (S)-(-)-bevantolol and (R)-(+)-bevantolol were prepared. Enantiomeric separation of these mixtures took place on a chiralcel OD column or, after derivatization with GITC, on a ODS column. No racemization was found during the experiment. This method allowed determination of 0.05% of either enantiomer in the presence of its stereoisomer and method validation showed adequete linearity over the required range.

Key words: Chiral separation, 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl Isothiocyanate, GITC, Bevantolol, Optical purity

INTRODUCTION

Chirality is one of the words in the pharmaceutical language which comes from the Greek. The Greek used the word 'cheiros' to refer to handedness, i.e., being left-handed or right-handed. The existence of optical isomerism has been known since the first half of the 19th century. About 40% of the drugs in use at this time are chiral and only 12% are administered as enantiomers. Chemical production of molecules with a chiral centre most often results in racemic mixture because synthetic processes do not favour the production of one of the enantiomers and are administered as a racemic mixture. However due to enantiomeric purity in pharmacokinetics, administration of racemate will not lead to a racemic mixture in the body. It is established that in most of the cases only one of the enantiomers exhibits pharmacological effects, whereas the other one may also show side effects. An important technique for analytical quantification of single enantiomers in mixtures of enantiomers is chromatography. Chromatographic separations of enantiomers can either be carried out by an indirect method, which involves the formation of diastereomeric pairs by using chiral derivatization agents or directly by using a chiral stationary phase or chiral additives to the mobile phase. The advantage of using the indirect method of separation include improved peak symmetry and resolution since the separation occurs on achiral columns. Disadvantage may include: (1) the need for a derivatizable functional group. which allows for diastereomer formation; (2) knowledge of the chiral purity and stability of the derivatizing agents; (3) mild and reproducible reaction conditions; (4) quantitative derivatization conditions; (5) sample preparation complexity. The advantage of using the direct method is that no derivatization of the analyte is required. Disadvantage of the direct mode include: (1) higher limit of detection due to poor peak symmetry; (2) poor selectivity between achiral impurities and the enantiomers; (3) sample solubility problems due

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to limited mobile phase choices and limited organic modifier concentrations; (4) limited ability to control elution order which can result in loss of the enantiomeric impurity peak if it elutes on the tailing edge of the desired enantiomeric peak(Walhagen *et al.*, 1991).

In recent years, progress in the synthesis of chiral compounds has promoted the development of new analytical methods for the determination of the enantiomeric composition of racemic mixtures. There are many chiral derivatization reagents used in indirect method (ex. R-1-(2-Napthyl)ethyl isothiocyanate, (R)-(+)-(1-Napthyl) ethylamine, R-1-(1-Napthyl)ethyl isocyanate, etc). And (S)-(-)-Menthyl chloroformate((-)-MCF), (S)-(-)-α-methylbenzyl isocyanate ((-)-MBIC) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate(GITC), which can react with the secondary amine group of bevantolol has been used as chiral derivatization reagents. (-)-MBIC has been used as a chiral derivative reagent for β-blocking drugs (Pflugman *et al.*, 1987). GITC was introduced by Kinoshita and co-workers (Nimura *et al.*, 1980 and Kinoshita *et al.*, 1981) as a chiral reagent for the derivatization of amino acid enantiomers followed by reversed-phase HPLC separation of the resulting diastereomers. Bevantolol is an experimental drug belonging to the therapeutic category of adrenergic β-receptor blockers. The compound has a chiral center at the 2-carbon which is responsible for the existence of bevantolol as two enantiomers, denoted (+) and (-) by convention. Physicochemically, the enantiomers differ only in their rotation of plane-polarized light. Bevantolol is synthesized manufactured and dispensed as the racemate. So Analytical chirotechnology is needed for the development of enantiomeric pure β-blockers which avoid the side effect, for example, lowered heart rate. There are considerable pharmacologic, pharmacokinetic, and metabolic differences between the enantiomers of several β-blockers (Barrett *et al.*, 1968, Lennard *et al.*, 1983, Hsyu *et al.*, 1985 and Walle *et al.*, 1983). Generally, the (-)-forms elicit the greater pharmacologic effects for this class of compounds. The (-)-enantiomer of propranolol, for example, is mainly responsible for antihypertensive and other cardiovascular actions (Barrett *et al.*, 1968) of the drugs. Similarly, bevantolol's β-blocking activity has been shown to reside in the (-)-isomer. This paper describes the derivatization of bevantolol used as mixture of (R)-(+)-bevantolol and (S)-(-)-bevantolol into each diastereomer by three chiral derivatization reagents, (-)-MCF, (-)-MBIC and GITC, which can react with the secondary amine group of bevantolol followed by analysis by the HPLC system. Indirect HPLC method using GITC gave the best result. Separation of the enantiomers of bevantolol as diastereomers was successfully achieved by reversed-phase HPLC using ODS column.

MATERIALS AND METHODS

Materials and equipment

Bevantolol hydrochloride, 1-[[2-(3,4-dimethoxyphenyl) ethyl]amino]-3-(3-methyl-phenoxy)-2-propanol, was provided by LG Chemical Corporation(Chung-ju, Korea). 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) was purchased from Sigma(St. Louis, MO, USA). (S)-(-)-menthyl chloroformate((-)-MCF) was purchased from Tokyo Kasei Organic chemicals(Tokyo, Japan). Trans-4-hydroxy-L-proline(proline) was purchased from Sigma(St. Louis, MO, USA). (S)-(-)-α-methylbenzyl isocyanate((-)-MBIC), triethylamine and diethylamine were purchased from Aldrich (Milkwaukee, WI, USA). Distilled water were prepared with Milli-RO 15 water purification system (Nihon Millipore, Japan) and was filtered through 0.22 μm membrane filter. Acetonitrile, n-hexane, isopropanol as a HPLC grade and all other reagents as a analytical grade were obtained from Duksan Pure Chemicals Co. (Ansan, Korea). The chiral semi-preparative high performance liquid chromatographic system consisted of LC-9A pump, C-R4A intergrator, SPD-6AV detector(Shimadzu, Kyoto, Japan) with a semi-preparative flow cell and Rheodyne 7725*i* injector with a 200 μL loop. The achiral analytical high performance liquid chromatographic system consisted of LC-10A pump, SIL-10A autoinjector, CLASS-10LC with CBM-10A computerized intergrator (Shimadzu, Kyoto, Japan), and 502T UV detector(GL-Science, Tokyo, Japan) with a analytical flow cell.

Chromatography

The chromatographic columns were Chiralcel OD (5 μm, 250 × 10 mm I.D., Daicel, Japan) for chiral semi-preparative HPLC, Chiralcel OD (5 μm, 250 × 4.6 mm I.D., Daicel, Japan) for chiral analytical enantiomeric purity test and Inertsil ODS-3 (5 μm, 150 × 4.6 mm I.D., GL Science, Japan) for achiral reversed-phase HPLC of derivatized diastereomers. The mobile phases were n-hexane-isopropanol-diethylamine(10/90/0.1) at a flow rate 2.0 mL/min for chiral semi-preparative HPLC and n-hexane-isopropanol-diethylamine(10/90/0.1) at a flow rate 0.45 mL/min for chiral analytical enantiomeric purity test. 77% methanol in water, 65% methanol in water and 65% acetonitrile in 25 mM ammonium acetate buffer (pH 5.0 adjusted with acetic acid) were used as mobile phases for achiral reversed-phase HPLC of derivatized diastereomers of bevantolol with (-)-MCF, (-)-MBIC and GITC respectively.

Preparation of bevantolol enantiomers

Bevantolol hydrochloride 100 mg was dissolved in 10 ml of mobile phase. This solution was injected into the chiral semi-preparative HPLC system and resolved into each enantiomer on the Chiralcel OD chiral column at room temperature at a flow rate of 4.0 ml/min monitored

at UV 276 nm. Fractions containing single enantiomers were collected and evaporated to dryness under nitrogen stream. Optical purity was determined by the chiral HPLC using Chiralcel OD analytical column.

Derivatization of bevantolol with (-)-MCF, (-)-MBIC and GITC

Aliquots of a 0.4 mg/ml solution of bevantolol in acetonitrile(100 μl) were pipetted into a 4 ml vial, evaporated to dryness under a stream of nitrogen, and the residues were dissolved in 400 μl of (-)-MCF solution (2.55 M, in acetonitrile). The solution was throughtly vortex mixed and kept at room temperature for 30 min. After incubation, 200 μl of *trans*-4-hydroxy-L-proline solution (5.10 M, in saturated Na_2CO_3 solution) was added immediatly to quench the reaction and then the reaction tube was centrifuged for 5 min at 3000 rpm. A 20 μl of acetonitrile layer was injected into the achiral reversed-phase HPLC system at UV 278 nm.

Aliquots of a 0.4 mg/ml solution of bevantolol in acetonitrile (100 μl) and 100 μL of (-)-MBIC solution (4.6 μL/mL in acetonitrile) were placed in 4 mL vial. After stirring at room temperature for 30 min, 20 μL of this solution was injected into achiral reversed-phase HPLC system at UV 278 nm.

Aliquots of a 0.4 mg/ml solution of bevantolol in acetonitrile(100 μl) and 1000L of GITC solution(2.76 mg/mL in acetonitrile) were placed in 4mL vial. And 500 μL of triethylamine solution (4.7 μL/mL in acetonitrile) was added. The resulting mixture was vigorously shaken and allowed to stand at room temperature for 20 min, and 20 μL of this solution was injected into achiral reversed-phase HPLC system at UV 276 nm.

Enantiomeric purity test of bevantolol using GITC

To check the molar response of GITC derivatized bevantolol at UV 276 nm, 200 μL of bevantolol solution (0.2 mg/mL in acetonitrile) and 1000 μL of GITC solution (0.276 mg/mL in acetonitrile) were placed in 4 mL vial. And 200 μL of triethylamine solution (4.7 μL/mL in acetonitrile) followed by nitrogen purging. The mixture was vortexed for 1 min and stirred at room temperature for 20 min. 20 μL of this solution was injected into achiral reversed-phase HPLC system. For enantiomeric purity test of bevantolol, 1 mg of (S)-(-)-and (R)-(+)-bevantolol enantiomer were dissolved in acetonitrile. These solutions was transferred to 4 mL vial according to various ratio of (S)-(-)-bevantolol and (R)-(+)-bevantolol. 200 μL of GITC solution (2.76 mg/mL in acetonitrile) was added and 200 μL of triethylamine solution (4.7 μL/mL in acetonitrile). The mixture was vortex mixed for 1 min and allowed to stand at room temperature for 20 min. 20 μL of this solution was injected into achiral reversed-phase HPLC system.

RESULTS AND DISCUSSION

Preparation of (R)-(+)-bevantolol and (S)-(-)-bevantolol

Bevantolol racemate was separated to each enantiomer by chiral semi-preparative HPLC within 45 min(Fig. 1). Resolution(Rs) was 4.3 and R-(+)-bevantolol was eluted first. White-colored amorphous powder of each enantiomer was obtained. Enantiomeric purity was 100.0% for each enantiomer. These bevantolol enantiomers could be used as standards for purity test of bevantolol after derivatization with chiral derivative reagent by achiral HPLC system.

Separation of bevantolol enantiomers by chiral derivatization method

(-)-MCF, (-)-MBIC and GITC reacted selectively with bevantolol enantiomers to form the corresponding diastereomers (Fig. 2) which were resolved by achiral HPLC system within 45, 40 and 20 min, respectively (Fig. 3, 4, 5). No racemization was found during or after derivatization reaction in all the chiral dervatization reagents used. When (-)-MCF was used as a chial derivatization reagent, the resolution and selectivity factor were 1.12 and 1.08, respectively and (S)-(-)-bevantolol was eluted first. When (-)-MBIC was used, the resolution and selectivity factor were 0.79 and 1.06, respectively and (R)-(+)-bevantolol was eluted first. When GITC was used, the resolution and selectivity factor were 1.22 and 1.15, respectively and (S)-(-)-bevantolol was eluted first. GITC was found as the most effective chiral derivatization reagent for the chiral purity test of bevantolol judged from resolution and analytical time (Table I).

Chiral purity of bevantolol after derivatization with GITC

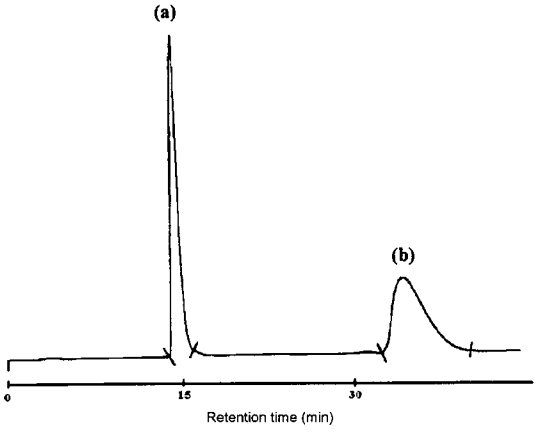

Fig. 1. Chiral semi-preparative HPLC chromatogram of bevantolol enantiomers. [Column, Chiralcel OD, 250×10 mm I.D.; mobile phase, n-hexane-isopropanol-diethylamine (10/90/0.1, v/v/v); detector, UV 276 nm]. Peak (a), (R)-(+)-bevantolol; peak (b), (S)-(-)-bevantolol.

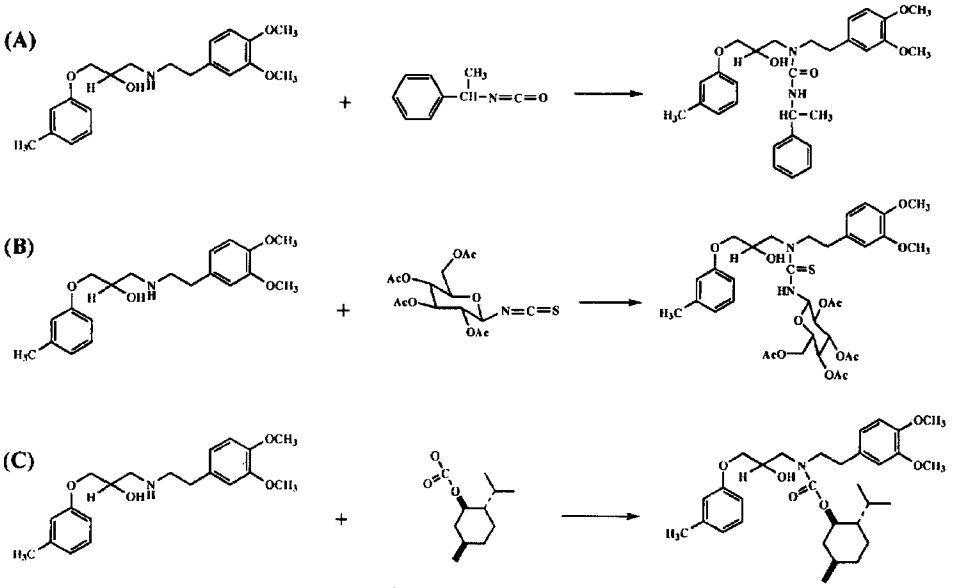

Fig. 2. Proposed derivatization reaction of bevantolol with (A) S-(-)-α-methylbenzyl isocyanate, (B) 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, and (C) (-)-menthyl chlorformate.

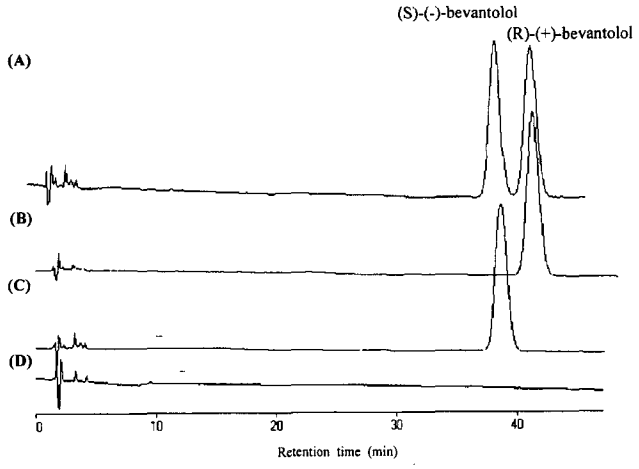

Fig. 3. Achiral HPLC chromatogram of (A) bevantolol racemate, (B) (R)-(+)-bevantolol, (C) (S)-(-)-bevantolol and (D) blank after derivatization with (-)-MCF. [Column, Inertsil ODS-3, 150 × 4.6 mm I.D.; mobile phase, 77% methanol in water; detector, UV 278 nm]

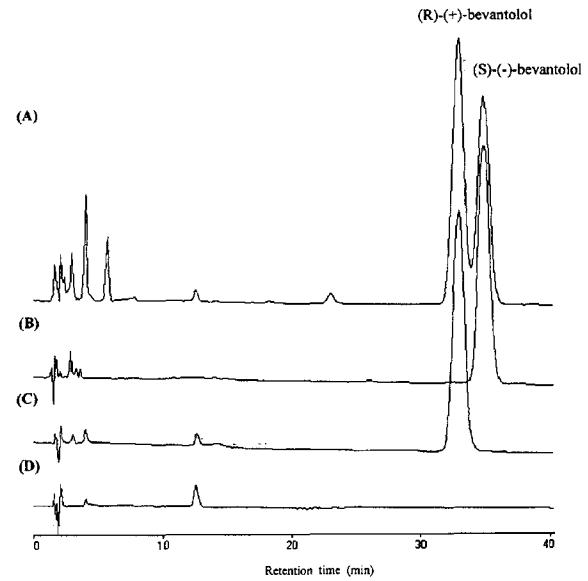

Fig. 4. Achiral HPLC chromatogram of (A) bevantolol racemate, (B) (S)-(-)-bevantolol, (C) (R)-(+)-bevantolol and (D) blank after derivatization with (-)-MBIC. [Column, Inertsil ODS-3, 150 × 4.6 mm I.D.; mobile phase, 65% methanol in water; detector, UV 278 nm]

Table I. Comparison of capacity factor (k), selectivity factor (α) and resolution (Rs) of bevantolol racemate using (-)-MCF, (-)-MBIC and GITC

CDR	k_A	k_B	α	Rs	N_A	N_B
(-)-MCF	24.36	26.25	1.08	1.12	4333	3498
(-)-MBIC	19.49	20.67	1.06	0.79	3102	3269
GITC	3.40	3.92	1.15	1.22	1688	2199

k_A : capacity factor of the peak eluted first
k_B : capacity factor of the peak eluted last
N_A: the theoretical number of plate of the peak eluted first
N_B: the theoretical number of plate of the peak eluted first

The derivatization procedure for bevantolol was optimized by varying reaction temperature, time and the amount of a GITC reagent. The derivatization conditions were selected to result in the greatest degree of the peak area of the GITC derivatized bevantolol. Under the exess amount a GITC reagent (more than 2 times of a sample amount), the reaction proceeded rapidly and quantitatively without warming. The peak areas GITC derivatized

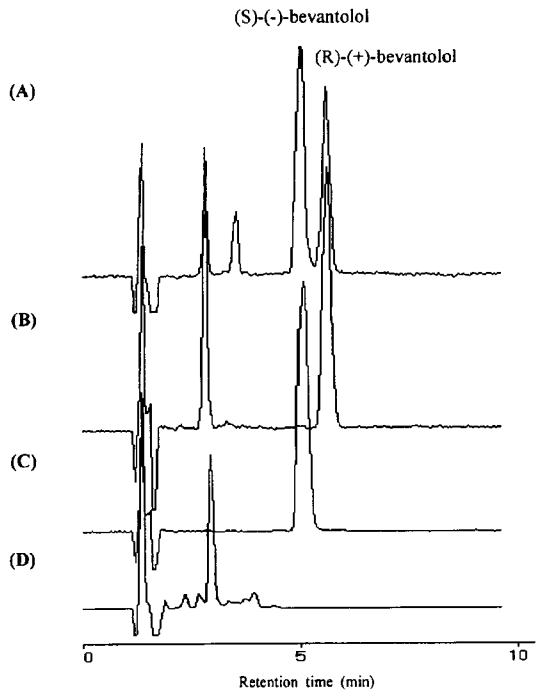

Fig. 5. Achiral HPLC chromatogram of (A) bevantolol racemate, (B) (R)-(+)-bevantolol, (C) (S)-(-)-bevantolol and (D) blank after derivatization with GITC. [Column, Inertsil ODS-3, 150 x 4.6 mm I.D.; mobile phase, 65% methanol in 25 mM ammonium acetate buffer (pH 5.0); detector, UV 276 nm]

Table II. The linearity and recovery of (R)-(+)-bevantolol and (S)-(-)-bevantolol in the reversed-phase HPLC method after derivatization with GITC

n	(S)-(-)-bevantolol -GITC	(R)-(+)-bevantolol -GITC	(-)-form/(+) -form
1	50.12	49.88	1.005
2	49.23	50.77	0.970
3	49.71	50.29	0.988
4	50.10	49.90	1.004
5	49.55	50.45	0.982
6	49.94	50.06	0.988
7	50.88	49.12	1.036
8	50.05	49.95	1.002
9	50.06	49.94	1.002
10	49.83	50.17	0.993
average	49.95	50.05	0.998
C.V. (%)	0.87	0.86	1.736

bevantolol at room temperature became constant over the reaction time 10 min~120 min. The solutions reacted in the water bath at 40°C for 20 min gave the same peak areas as those reacted in room temperature for 20 min. When monitored at UV 276 nm, the ratio of the molar response of GITC derivtized (S)-(-)-bevantolol to that of (R)-(+)-bevantolol was 0.998 (Table II).

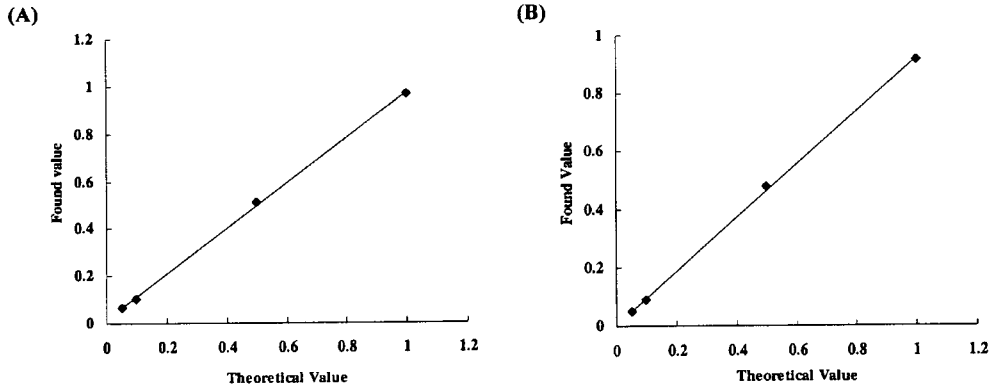

Fig. 6. Linearity of response of (A) (R)-(+)-bevantolol in (S)-(-)-bevantolol and (B) (S)-(-)-bevantolol in (R)-(+)-bevantolol

Table III. The linearity and recovery of (R)-(+)-bevantolol and (S)-(-)-bevantolol in the reversed-phase HPLC method after derivatization with GITC

(R)-(+)-bevantolol				(S)-(-)-bevantolol			
Added (%)	Found (%)	Recovery (%)	C. V. (%)	Added (%)	Found (%)	Recovery (%)	C. V. (%)
0.05	0.07	130.35	12.39	0.05	0.05	98.30	13.16
0.10	0.10	103.00	7.19	0.10	0.09	90.25	7.44
0.50	0.51	101.73	8.67	0.50	0.48	95.66	2.02
1.00	0.97	96.72	3.48	1.00	0.91	91.42	2.69

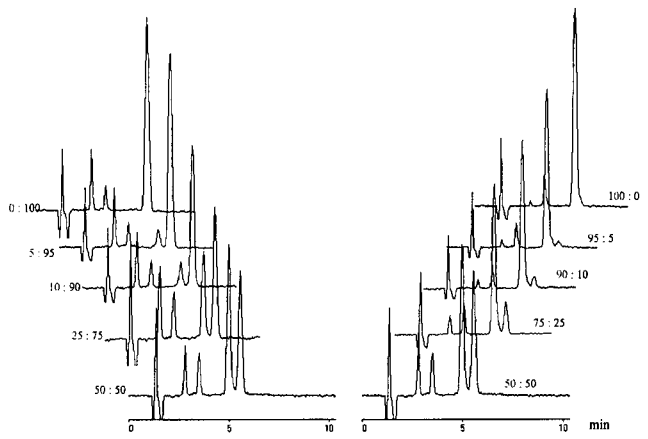

Fig. 7. Chiral purity test of bevantolol after derivatization with GITC by achiral HPLC. [Column, Inertsil ODS-3, 150×4.6 mm I.D.; mobile phase, 65% methanol in 25 mM ammonium acetate buffer (pH 5.0); detector, UV 276 nm]. First order peak, (S)-(-)-bevantolol; the last order peak, (R)-(+)-bevantolol.

The linearity and the recovery of peak area response of the (R)-(+)-bevantolol added to (S)-(-)-bevantolol and the (S)-(-)-bevantolol added to (R)-(+)-bevantolol was examined over the range 0.05%~1.00%(w/w). The linearity and the recovery testing results are summarized in Table III and Fig. 6, indicating that this method is suitable and applicable for use as the optical purity testing method. The correlation coefficient(r) of 0.999 for the (R)-(+)-bevantolol and (S)-(-)-bevantolol, respectively. The recovery of the antipode was 90.25%~130.35% and the RSD ranged from 2.69% to 13.16%. The detection limit of both enantiomers at a signal to noise ratio of 3 was ca. 0.03%. Chromatograms of the mixtues combing different ratios of (S)-(-)-bevantolol and (R)-(+)-bevantolol are shown in Fig. 7.

CONCLUSION

It was found that the derivatization of bevantolol with GITC is an useful technique for the separation of the enantiomers by reversed-phase HPLC. The GITC reagent is stable and commercially available. The derivatization procedure is simple and fast. And derivatization reaction of bevantolol with GITC is independent of the amount of each enantiomer presented in the reaction mixtue. The detection limit of the antipode in their stereoisomers was down the 0.03% level. This method could be applied to optical purity testing of drugs containing metoprolol enantiomer.

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