A High-Performance Liquid Chromatographic Assay for the Enantiomers of Bevantolol in Human Plasma

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A method was developed and validated for the simultaneous analysis of (+)- and (−)-bevantolol in human plasma. The assay involves plasma protein precipitation, derivatization of racemic bevantolol to its diastereomeric thioureas with 2,3,4,5-tetra-*o*-acetyl-α-D-glucopyranosyl isothiocyanate, and solid-phase extraction of the diastereomers from 0.5 ml human plasma. Chromatographic separation was accomplished under isocratic conditions using a reversed-phase C-18 analytical column and mobile phase consisting of equal parts of 75 m*M* dibasic ammonium phosphate buffer (adjusted to pH 3.5 with phosphoric acid) and acetonitrile, with a detection wavelength of 220 nm. The absolute peak-height method was employed for quantitation. Retention times for the diastereomers of (+)- and (−)-bevantolol were 7.4 and 6.4 min, respectively. The method is suitable for the quantification of the enantiomers over a concentration range of 40 to 800 ng/ml per enantiomer.

KEY WORDS: bevantolol; stereoselective assay; high-performance liquid chromatography; diastereomeric derivatives.

INTRODUCTION

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 (denoted by * in Fig. 1a) 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.

There are considerable pharmacologic, pharmacokinetic, and metabolic differences between the enantiomers of several β-blockers (1–4). Generally, the (−) forms elicit the greater pharmacologic effects for this class of compounds. The (−)-enantiomer of propranolol, for example, is responsible for most of the drug's antihypertensive and other cardiovascular actions (1). Similarly, bevantolol's β-blocking activity in dogs has been shown to reside in the (−)-isomer (5). The pharmacokinetic profile and metabolism of the two propranolol enantiomers differ as well. For example, in man the apparent oral clearances of (+)- and (−)-propranolol are 19.4 and 15.0 ml/min/kg, respectively (6).

The possibility that the pharmacokinetic profile of bevantolol's enantiomers are different and the fact that enantioselective pharmacokinetic profiles of chiral compounds are of interest to regulatory agencies, prompted the development of a high-performance liquid chromatographic assay for (+)- and (−)-bevantolol in human plasma. The method was adapted from a technique described by Kinoshita *et al.* (7) involving derivatization of the enantiomers to their respective diastereomers. This paper describes the assay and its validation and demonstrates its use in the assay of plasma samples of volunteers following administration of racemic bevantolol.

MATERIALS AND METHODS

Materials

Racemic bevantolol HCl and the separate enantiomers (as free bases) were prepared at Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI (8). All reagents were of analytical reagent grade. Ammonium phosphate buffer, 75 m*M*, was prepared from dibasic ammonium phosphate, adjusting to pH 3.5 with phosphoric acid. Derivatization was with 2,3,4,5-tetra-*o*-acetyl-α-D-glucopyranosyl isothiocyanate (GITC), obtained from Polysciences, Inc., Warrington, PA (Fig. 1b). Derivatization solution was prepared fresh daily by dissolving 25 mg GITC in 100 ml acetonitrile.

Instrumentation

The chromatographic system consisted of an Altex 110A high-pressure pump (Altex, Berkeley, CA), a Model 481 Lambda Max UV multiwavelength detector (220 nm; Waters, Milford, MA), a C-R1A integrator (Shimadzu, Kyoto, Japan), a Partisil 5 RAC II column (10 cm × 4.6-mm i.d.; particle size, 5 μm; Whatman, Clifton, NJ), and a WISP 712 automated sample processor (Waters, Milford, MA).

Derivatization, Extraction, and Separation

For calibration standards 0, 20, 40, or 80 μl of 1 μg/ml racemic bevantolol HCl stock solution or 20, 40, or 80 μl of 10 μg/ml racemic bevantolol HCl stock solution was added to 0.5 ml blank human plasma to provide the equivalent of 0, 20, 40, 80, 200, 400, or 800 ng/ml, respectively, of each enantiomer. Total volume adjustment to 580 μl was accomplished with water. Similarly, for clinical samples, 80 μl of water was added to a 0.5-ml aliquot of each plasma sample. Protein precipitation required the addition of 1 ml acetonitrile to each standard and sample, followed by vortexing (10 sec) and centrifuging (10 min, 2000 rpm). Supernatant was combined with 1 ml of 25 m*M* ammonium hydroxide (pH 10.5) with vortexing. GITC reagent mixture (100 μl) was then added with vortexing, and the reaction allowed to proceed at room temperature for 10 min prior to extraction. The proposed reaction scheme for a secondary amine with GITC to form the thiourea derivative is depicted in Fig. 1c.

Solid-phase extraction cartridges (Bond-Elut, C-18, 3-ml volume; Analytichem International, Harbor City, CA) were preconditioned by drawing through 1 cartridge volume each of acetonitrile and 25 m*M* ammonium hydroxide under vacuum (5 to 10 in. Hg). The sorbent bed was not allowed to dry prior to the addition of sample. Each sample was poured into its respective conditioned cartridge and drawn through

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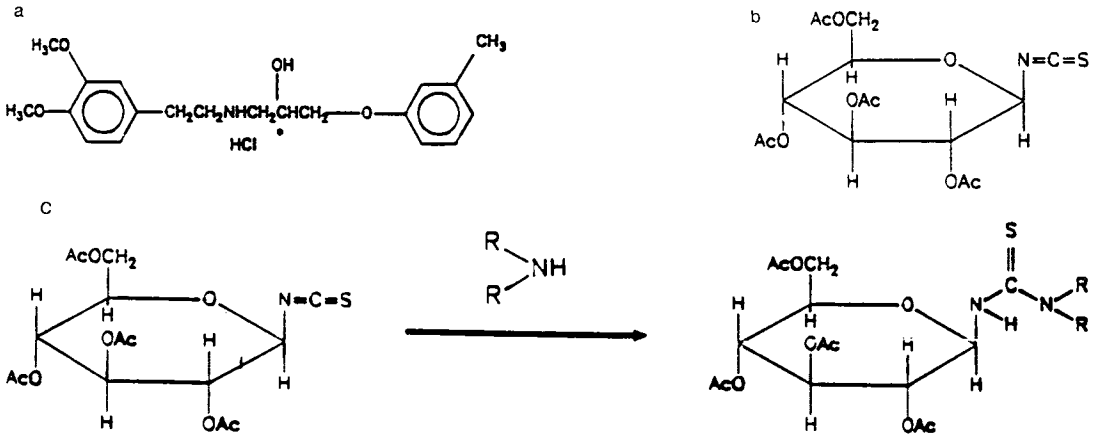

Fig. 1. Chemical structures: (a) bevantolol; (b) GITC; (c) proposed reaction of GITC with a secondary amine to form the thiourea product.

under the same vacuum. Cartridges were rinsed with 1 cartridge volume each of 25 m*M* ammonium hydroxide and water. Elution was with 1.25 ml methanol. Eluent was evaporated to dryness under a current of nitrogen in a dry bath at 45°C. Residue was reconstituted with 0.5 ml mobile phase [equal parts of 75 m*M* dibasic ammonium phosphate buffer (adjusted to pH 3.5 with phosphoric acid) and acetonitrile], after which 75 µl was injected onto the column. The analytical column was eluted at 45°C with mobile phase at a flow rate of 2.0 ml/min. Detection was at 220 nm.

Calculations

The absolute peak height in millimeters corresponding to each derivatized enantiomer was recorded for each sample. Six concentrations ranging from 40 to 1600 ng/ml of racemate, corresponding to the equivalent of 20 to 800 ng/ml of each enantiomer, were used to construct calibration curves. Calibration curves were fitted by least-squares linear regression with a weighting factor of unity. Extrapolated enantiomer concentrations and percentage deviation were determined for each calibration standard.

Specificity

Specificity of the assay for the derivatized enantiomers of bevantolol was based on retention characteristics of these compounds under the chromatographic conditions. Blank human plasma samples were subjected to the derivatization procedure to check for interferences in the quantitation of either enantiomer. Specificity was further evaluated by rapid-scan photodiode array detection.

Isolation/Extraction Efficiency

Recovery of the diastereomeric derivatives of bevantolol was determined by comparing peak heights of derivatized/extracted samples to peak heights of derivatized/unextracted controls. The latter were prepared by evaporating aliquots of racemic bevantolol HCl aqueous stock solution to dryness and derivatizing the residue according to the method described above. Extraction/isolation efficiency at 140 and 1400 ng/ml (equivalent to 70 and 700 ng/ml of each enantiomer) was determined.

Amount of Derivatization Reagent

Aliquots of aqueous standard solution providing 350 ng of either (+)- or (−)-bevantolol were evaporated to dryness. To each were added 500 µl of 25 m*M* ammonium hydroxide (pH 10.5) and either 2.5, 5, 10, 25, 50, or 100 µl of 0.025% (w/v) GITC in acetonitrile. The derivatization reaction was stopped at about 10 min by evaporating the reaction mixture to dryness, after which residue was reconstituted in 0.5 ml mobile phase. A 75-µl aliquot was injected onto the column. The effect of amount of reagent used was monitored for each enantiomer by comparing peak height of derivatized species to amount of reagent.

Derivatization Time Course

Aliquots of aqueous standard solution providing 350 ng of either (+)- or (−)-bevantolol were evaporated to dryness. To each tube were added 500 µl 25 m*M* ammonium hydroxide (pH 10.5) and 100 µl 0.025% (w/v) GITC in acetonitrile. The derivatization reaction was stopped at either 10, 20, 30, 45, or 60 min by evaporating the reaction mixture to dryness. Residue was reconstituted in 0.5 ml mobile phase and a 75-µl aliquot was injected onto the column. Reaction time course was monitored for each enantiomer by monitoring peak height of derivatized species as a function of reaction time.

RESULTS AND DISCUSSION

System suitability, specificity, isolation/extraction efficiency, reproducibility, linearity, precision, and accuracy were evaluated for the assay validation of the enantiomers of bevantolol in human plasma.

System Suitability

Suitability of the system for the enantiomers of bevantolol was based on the following chromatographic parameters and the performance of the method throughout validation. The retention times of derivatized (+)- and (−)-bevantolol were 7.4 and 6.4 min, respectively. Capacity factors (κ') for the derivatized (+)- and (−)-enantiomers were 11.0 and 9.3, respectively. The respective numbers of theoretical plates for derivatized (+)- and (−)-bevantolol

were 23,000 and 17,000. Relative retention of the derivatized (−)- to (+)-enantiomer was expressed by the separation α, determined to be 1.18 from the ratio of the capacity factors. These data indicate that the method is suitable for analyzing the two derivatized enantiomers of bevantolol in human plasma.

Specificity

No interferences were observed in derivatized blank human plasma at the retention times of (+)- and (−)-bevantolol. Figure 2 depicts typical chromatograms for blank human plasma, a 400-ng/ml (each enantiomer) calibration standard, and a subject plasma sample from a pharmacokinetic study. System specificity was evaluated by multiple-wavelength, rapid-scan photodiode array detection of the upslope, apex, and downslope of derivatized (+)- and (−)-bevantolol chromatographic peaks. Spectra of the two derivatized enantiomers in water, extracted from human plasma calibration standard, and extracted from human plasma sample, were identical. Small interferences detectable at wavelengths greater than 250 nm did not interfere with quantitation of the enantiomers at 220 nm.

Isolation/Extraction Efficiency

The recovery of (+)-bevantolol by the derivatization/extraction method for 70 ng/ml was 81.3% (6.7% RSD), with a range of 75.7 to 89.9%, and for 700 ng/ml the recovery was 84.2% (4.2% RSD), with a range of 81.0 to 90.9%. At the

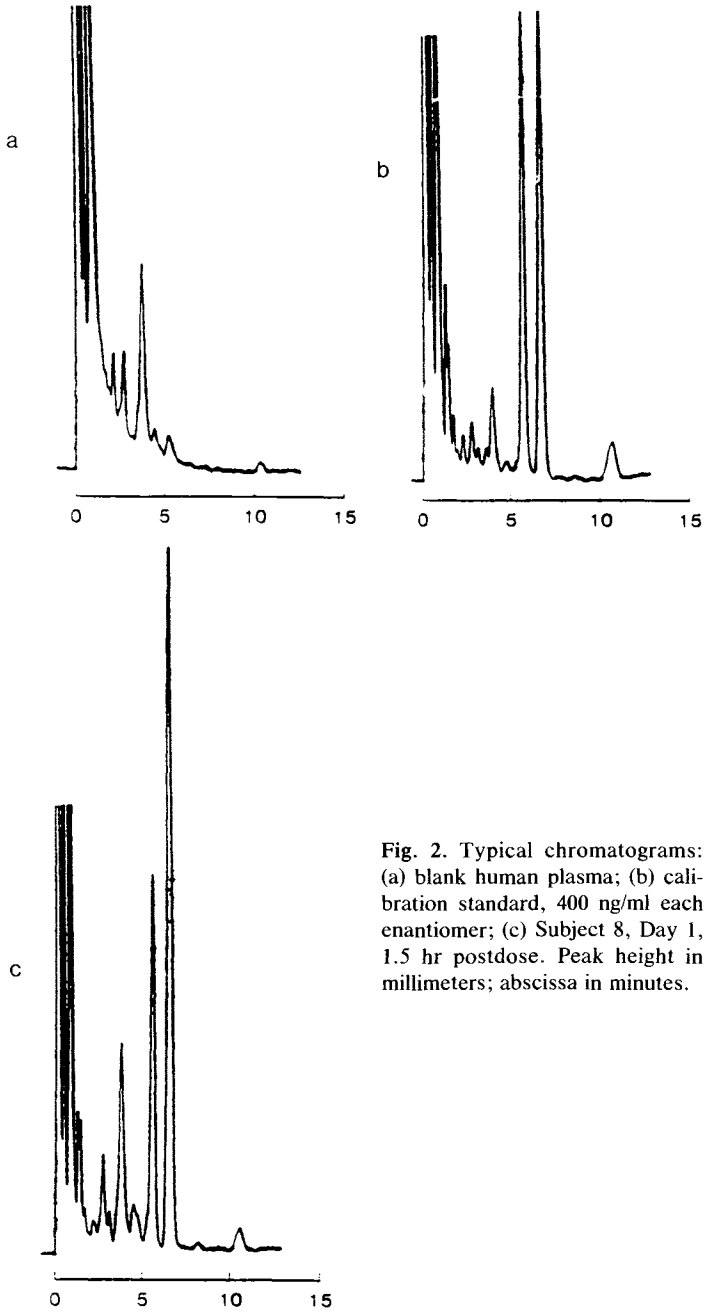

Fig. 2. Typical chromatograms: (a) blank human plasma; (b) calibration standard, 400 ng/ml each enantiomer; (c) Subject 8, Day 1, 1.5 hr postdose. Peak height in millimeters; abscissa in minutes.

same two concentrations, the recovery of (−)-bevantolol was 86.2% (5.0% RSD), with a range of 81.6 to 92.3%, and 89.8% (2.2% RSD), with a range of 87.3 to 92.3%, respectively.

Amount of Derivatization Reagent

The addition of derivatizing reagent solution in volumes greater than 50 μl resulted in no further increase in the peak heights of the derivatized species. As a precaution, 100-μl aliquots of the reagent were used routinely in conducting the assay.

Effect of Reaction Time on Extent of Reaction

Within the practical reaction-time range of 10 to 60 min, no further increase in peak height of either enantiomer was observed. Consequently, the reaction is complete within 10 min. A 10-min incubation time was therefore used for routine analysis.

Reproducibility

System reproducibility was determined by six replicate injections of processed sample at both 80 and 800 ng/ml (equivalent of each enantiomer). Relative standard deviations for repeat injections at these concentrations were 4.3 and 0.8%, respectively, for the (+)-enantiomer and 4.0 and 0.4%, respectively, for the (−)-enantiomer.

Linearity, Precision, and Accuracy

Standard curves were fitted to a first degree polynomial, $y = ax + b$, where y is the peak-height ratio, a and b are constant coefficients, and x is concentration of drug. A weighting factor of unity gave the best description of the data within the working concentration range of 40 to 800 ng/ml of each enantiomer. Triplicates of each calibration standard were assayed on three separate occasions. The peak heights for the derivatives of (+)- and (−)-bevantolol are listed in Table I, and extrapolated concentrations are listed in Table II. Precision and accuracy of calibration curves were based on the relative standard deviations and the relative errors of calibration standards, which ranged from 1.8 to 14.9% and 0 to 15.0%, respectively, over the three trials (Table II). Linear correlation coefficient (r^2) for standard curves ranged from 0.9948 to 0.9964 for (+)-bevantolol and from 0.9924 to 0.9982 for (−)-bevantolol.

Limit of Quantitation

A level of 80 ng/ml racemate (40 ng/ml equivalent of each enantiomer) was determined to be the lowest concentration of bevantolol which could be quantified with an acceptable relative standard deviation and relative error. The minimum detectable amount was determined to be 40 ng/ml racemate (20 ng/ml equivalent of each enantiomer).

The method was applied in determining plasma concentration–time curves of subjects given 400-mg racemic bevantolol. Figure 3 illustrates the resultant mean ($n = 6$) disposition of bevantolol enantiomers. (+)-Bevantolol had a slightly higher mean maximum plasma concentration and a faster elimination rate relative to that of (−)-bevantolol fol-

Table I. Peak Heights (mm) of (+)- and (−)-Bevantolol Calibration Standards in Human Plasma

Concentration (ng/ml)											
20		40		80		200		400		800	
(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
					Trial 1						
3.00	4.03	4.75	6.25	8.33	9.98	17.8	20.8	32.9	38.9	73.6	81.7
2.75	3.81	4.50	6.50	8.08	9.66	16.5	20.5	37.8	43.5	70.7	77.6
2.54	4.12	4.18	7.27	8.41	10.1	17.7	22.5	—[a]	—[a]	70.6	76.5
					Trial 2						
2.80	4.80	4.50	6.80	7.30	10.1	17.9	22.4	41.2	46.9	77.8	87.1
2.40	4.20	4.70	6.60	7.30	11.5	17.4	24.3	40.9	46.8	77.1	87.4
2.70	4.60	4.60	6.70	7.50	11.6	17.8	21.8	40.8	47.0	77.8	87.5
					Trial 3						
2.90	4.80	5.25	6.90	8.60	11.2	19.5	23.4	40.9	46.5	80.1	89.0
3.10	5.00	5.00	7.30	7.90	10.8	19.8	22.3	41.5	46.8	80.3	89.1
3.00	4.60	4.40	7.30	8.50	11.2	18.0	26.0	—[a]	—[a]	76.7	86.7
					Mean						
2.80	4.44	4.65	6.85	7.99	10.7	18.0	22.7	39.4	45.2	76.1	84.7
					% RSD[b]						
8.2	9.2	6.9	5.5	6.5	6.8	5.6	7.6	7.9	6.2	4.8	5.7

[a] Sample lost.
[b] Percentage relative standard deviation.

Table II. Extrapolated (+)- and (−)-Bevantolol Concentrations of Calibration Standards in Human Plasma

Concentration (ng/ml)											
20		40		80		200		400		800	
(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
					Trial 1						
15	24	38	44	77	85	190	193	379	364	825	828
13	21	41	41	74	82	187	178	427	420	782	795
16	19	49	38	79	86	208	191	447	485	771	794
					Trial 2						
20	23	39	41	70	70	185	180	415	421	793	800
14	19	37	43	83	70	203	174	415	418	796	792
18	22	38	42	84	72	180	179	416	417	797	800
					Trial 3						
19	19	38	43	78	78	192	189	408	409	805	811
20	21	42	41	75	70	182	193	411	415	806	813
17	20	42	35	78	77	217	174	345	329	783	776
					Mean						
17	21	40	41	78	77	194	183	407	409	795	801
					% RSD[a]						
14.9	9.0	9.2	7.4	5.6	8.4	6.5	4.3	7.2	10.5	2.0	1.8
					% RE[b]						
−15.0	5.0	0.0	2.5	−2.5	−3.8	−3.0	−8.5	1.8	2.3	−0.6	0.1

[a] Percentage relative standard deviation.
[b] Percentage relative error.

lowing oral dosing with racemic bevantolol. In contrast, the elimination rates of propranolol enantiomers were similar after oral dosing in man and dog (9,10).

In summary, a method was developed and validated for the analysis of the enantiomers of bevantolol in human plasma. The method is suitable for the separation and quantification of the enantiomers over a concentration range of 40 to 800 ng/ml per enantiomer.

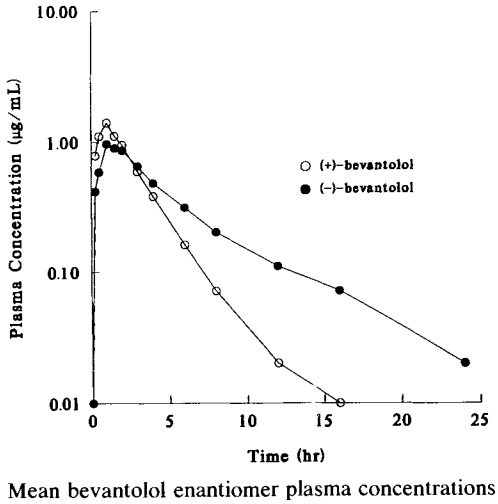

Fig. 3. Mean bevantolol enantiomer plasma concentrations following single 400-mg oral racemic doses in healthy subjects (n = 6).

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