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Stereoselective high-performance liquid chromatography determination of propranolol and 4-hydroxypropranolol in human plasma after pre-column derivatization

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Abstract

A stereoselective reversed-phase HPLC assay to quantify *S*-(−) and *R*-(+) enantiomers of propranolol and 4-hydroxypropranolol in human plasma was developed. The method involved liquid–liquid extraction for sample clean-up and employed 2,3,4,6-tetra-*O*-acetyl-β-glucopyranosyl isothiocyanate as a pre-column chiral derivatization reagent. The internal standard used was 4-methylpropranolol. The derivatized products were separated on an Altex C_{18} column using a mixture of acetonitrile–water–phosphoric acid–triethylamine (58:42:0.1:0.06 and 50:50:0.15:0.06, v/v, for propranolol and 4-hydroxypropranolol, respectively) as mobile phase. The detection of propranolol derivatives was made at $\lambda_{ex}=280$ nm and $\lambda_{em}=325$ nm, and the corresponding 325 and 400 nm were used for 4-hydroxypropranolol derivatives. The assay was linear from 1 to 100 ng/ml and from 2 to 50 ng/ml using 0.5 ml of human plasma for propranolol and 4-hydroxypropranolol enantiomers, respectively. The present assay is used to quantify the enantiomers of propranolol and 4-hydroxypropranolol, respectively, in human plasma for pharmacokinetic studies.

Keywords: Propranolol; 4-Hydroxypropranolol

1. Introduction

Propranolol, a widely used β-adrenergic blocking agent for the treatment of hypertension, was administered as the racemic form. The *S*-(−)-propranolol is approximately 100 times more potent than the *R*-(+)-propranolol, and the *S*-(−) isomer is believed to be largely responsible for the clinical effects of the racemic drug [1]. 4-Hydroxypropranolol, a major metabolite formed in man after oral administration of propranolol [2,3], has the same potency as propranolol, and the *S*-(−)-4-hydroxypropranolol is responsible for the β blocking effect [4]. Numerous investigators have examined the absorption, distribution, metabolism and elimination of propranolol, and the stereoselectivity has been shown to occur in animals and in humans [4–8]. To determine the enantiomers of propranolol, both GC–MS method [8,9] and HPLC analysis have been used. In HPLC assay, the enantiomers were separated either through chiral derivatization to form diastereomers which were then separated on a non-chiral column, or by the addition

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of a chiral counter ion to the mobile phase, in which case separation was achieved through ion pair chromatography [10,11]. Separation of propranolol enantiomers on a chiral column was achieved either after derivatization with achiral reagents [12,13], or without derivatization [14,15]. Among the commercially available pre-column chiral derivatization reagents, *N*-trifluoroacetyl-1-prolyl chloride has been used [4,16], but the reagent is prone to racemize. Another commonly used reagent is 1-phenylethylisocyanate, which is chemically stable, but the resulting method generally does not give adequate detection sensitivity [17–20]. *tert.*-Butoxycarbonyl-L-alanine and *tert.*-butoxycarbonyl-L-leucine can both achieve 1 ng/ml sensitivity from 1 ml human plasma, but the method involves tedious sample cleanup procedures after derivatization [21]. 2,3,4,6-Tetra-*O*-β-D-glucopranosyl isothiocyanate (TAGIT) has been used for the resolution of amino acids, epinephrine and β-blocker, but has not been applied to the biological fluids [22–25]. This paper describes a stereoselective HPLC method for the resolution of propranolol and 4-hydroxypropranolol in human plasma using TAGIT as a pre-column derivatization reagent.

2. Experimental

2.1. Materials

All solvents used were HPLC grade, and all chemicals were reagent grade. Propranolol hydrochloride was obtained from Sigma (St. Louis, MO, USA). 4-Hydroxypropranolol hydrochloride was supplied by Imperial (Macclesfield, UK). 4-Methylpropranolol hydrochloride, which was used as an internal standard (I.S.), was obtained from Ayerst Laboratories (New York, NY, USA). TAGIT was obtained from Polyscience (Warrington, PA, USA). 2,2-Dimethoxypropane and triethylamine were obtained from Aldrich (Milwaukee, WI, USA). Methylene chloride and acetonitrile were dried over molecular sieve before use.

2.2. Instrumentation and chromatography

The liquid chromatographic system consisted of a Beckman 110A pump (Beckman Instruments, Berkeley, CA, USA), an automatic injector (WISP 710B, Waters Associates, Milford, MA, USA) and a spectrofluorometer (Schimadzu RF 530, ISI InstruSpec, Walnut Creek, CA, USA; or Perkin Elmer 204-S, Norwalk, CT, USA). After pre-column derivatization, the diastereomers formed from propranolol, 4-hydroxypropranolol and I.S. were separated on a 250×4.6 mm I.D. Altex Ultrasphere C_{18} column at ambient temperature. The mobile phase consisted of a mixture of acetonitrile–water–phosphoric acid–triethylamine (58:42:0.1:0.06, v/v for propranolol and 50:50:0.15:0.16, v/v for 4-hydroxypropranolol). The detection was made at $\lambda_{ex}=280$ nm and $\lambda_{em}=340$ nm, and $\lambda_{ex}=325$ nm and $\lambda_{em}=400$ nm for propranolol and 4-hydroxypropranolol, respectively. The flow-rate was 1 ml/min in both cases.

2.3. Preparation of standard curve and quality control samples

The stock and working solutions of racemic propranolol and I.S. were prepared in 50% aqueous acetonitrile and stored at 4°C. Ten calibration standards of propranolol, ranging from 2 to 200 ng/ml (i.e., from 1 to 100 ng/ml for each enantiomer) were prepared by spiking drug free human plasma. Drug free human plasma spiked with 6, 20, 50 and 140 ng/ml from a different stock of racemic propranolol, were used as quality control. The stock and working solutions of 4-hydroxypropranolol were prepared in 50% aqueous acetonitrile containing 25% ascorbic acid. Six calibration standards of 4-hydroxypropranolol, ranging from 4 to 100 ng/ml (i.e., from 2 to 50 ng/ml for each enantiomer) were prepared by spiking drug free human plasma. Drug free plasma spiked with 8, 16, 40 and 80 ng/ml from a different stock of racemic 4-hydroxypropranolol, were used as quality control. All QC samples were stored at −70°C until analysis.

2.4. Extraction and derivatization

To 0.5 ml of plasma, 60 μl of I.S. working solution (1 ng/μl) and 0.5 ml of 1 *M* potassium phosphate (pH 10) were added. The mixture was vortexed for 1 min. Six ml of methylene chloride was added. The mixture was rotated for 15 min and then centrifuged at 1500 *g* for 10 min. The organic layer was transferred to a clean tube and evaporated

to dryness under a nitrogen stream. The residue was further dried sequentially with 1 ml of dry methylene chloride and 0.2 ml of dimethoxypropane. The residue was subjected to pre-column derivatization. To the dried residue, 150 μl of TAGIT solution (100 μg/ml in acetonitrile) was added. The tube was capped and the mixture was vortexed for 1 min and left to stand at room temperature overnight. Finally, the reaction mixture was evaporated to dryness under a nitrogen stream. The residue was reconstituted with 200 μl of 50% acetonitrile and an aliquot of 70 μl of the resulting solution was injected onto the HPLC system.

2.5. Quantitation and assay validation

The intra- and inter-day precision and accuracy were obtained by analyzing the calibration curve standards, and four spiked controls in six replicates within one day and on six consecutive days, respectively. The best fit lines were determined by least-squares linear regression analysis. The concentration of each enantiomer was determined by assaying plasma samples in conjunction with frozen controls and calibration curve standards. Calibration curves were constructed by plotting, for each enantiomer, peak height ratios of the enantiomer to the internal standard versus the enantiomer concentration. Peak height ratios were calculated using the second peak of I.S. which eluted later. Concentrations were calculated backward using regression parameters.

2.6. Application of the assay

The present assay has been used to quantify the enantiomers of propranolol and 4-hydroxypropranolol in plasma after an oral dose of 80 mg propranolol in human. Plasma samples were taken at predose and at various time periods after drug administration, and stored at −70°C until analysis.

3. Results and discussion

3.1. Extraction and derivatization

For plasma sample cleanup, 1 *M* potassium phosphate (500 μl, pH 10) was used to achieve the cleanest chromatogram while maintaining high recovery. To enhance the detection sensitivity, TAGIT was used as a pre-column chiral derivatization reagent in the present assay. TAGIT is not naturally fluorescent. The reaction of TAGIT with propranolol generated thiourea derivative as the major product (Fig. 1). The reaction yield was not measured but was reproducible under the specified reaction conditions of this assay. The other minor product eluted at shorter retention times than the thiourea derivative and did not interfer the assay. The reaction between 4-hydroxypropranolol and TAGIT presumably generated mono-derivatized thiourea, since a weaker mobile phase was needed for elution in comparison to the propranolol derivative. TAGIT reacts readily with amines, alcohols and water, therefore the reaction medium should be free of amines, alcohols and water. To achieve a water free reaction environment, besides using dried solvent for the reaction, the extracted sample should also be dried. To dry the methylene chloride extract, all solid drying reagents such as anhydrous sodium sulfate, potassium carbonate, calcium chloride, magnesium sulfate and molecular sieves were found to adsorb analytes in different amount. In this assay, after evaporating methylene chloride, a liquid drying reagent was used to further remove residual moisture in the sample before adding the derivatization reagent. This assay used methylene chloride to form an azeotropic mixture with water and further employed dimethoxypropane as a moisture scavenger which effectively removed traces of moisture and resulted in a high reaction yield, therefore, achieved a very high detection sensitivity. The optimal amount of TAGIT used in the reaction was examined. In order to avoid a kinetic resolution problem (i.e., TAGIT preferentially reacted with one enantiomer), a large excess of TAGIT was used. It was found that approximately 15–20 μg of TAGIT per sample was the optimal amount in the present assay. Additional amounts of TAGIT generated side products which caused interference, whereas lesser amounts of TAGIT resulted in a low yield which accordingly decreased the detectability. The present derivatization reaction was carried out at room temperature. It was found that after leaving the reaction mixture overnight (~12 h), the reaction yield reached to a maximum. Further elevating the reaction temperature could accelerate the reaction, but this was not examined.

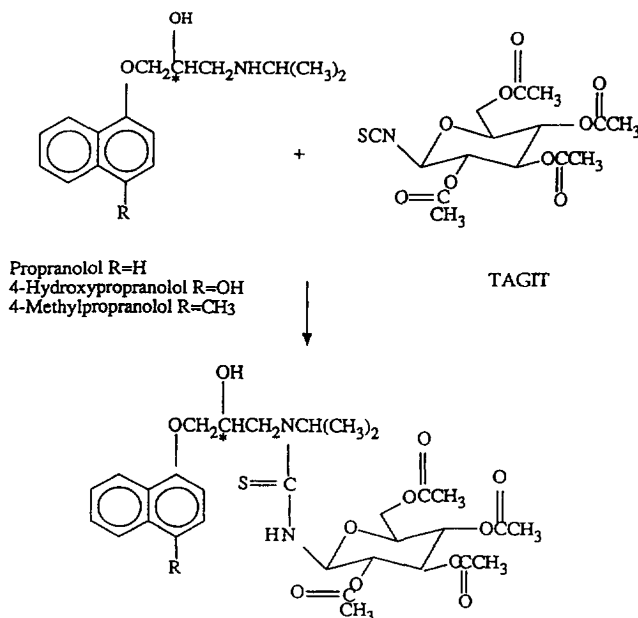

Fig. 1. Pre-column derivatization of propranolol, 4-hydroxypropranolol and 4-methylpropranolol (I.S.) with TAGIT.

3.2. Chromatography

The initial mobile phase used was a mixture of 58% acetonitrile and 42% ammonium phosphate, monobasic (pH 4), but there were interfering peaks with the analytes. However, ion pair chromatographic technique, for example, addition of heptane sulfonate to a mixture of 60% acetonitrile, 40% water and 0.15% phosphoric acid, was found able to move the interfering peaks away from the analytes, whereas the analytes, as they were neutral (forming thiourea after derivatization), remained unaffected. The results suggested that the interfering peaks probably were ionic compounds. In fact, triethylamine was found successfully fine-tuned the retention times of analytes. In the present assay, the retention times of the *S*-(−)- and the *R*-(+)-propranolol derivatives were approximately 9.0 and 10.5 min, respectively. The retention times of the *S*-(−)- and the *R*-(+)-4-hydroxypropranolol were approximately 13.8 and 16.9 min, respectively. The representative chromatograms are shown in Figs. 2 and 3. No interferences were observed at the retention times of the analytes.

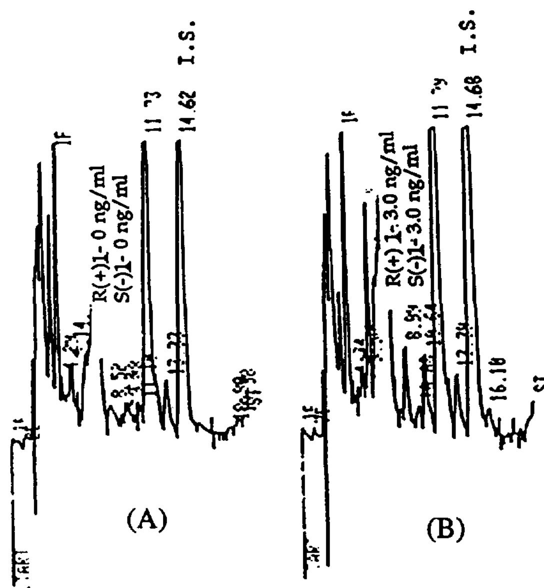

Fig. 2. Representative chromatograms of human blank plasma spiked with I.S. (A), and blank plasma spiked with I.S. and 3 ng/ml of propranolol (1) (B).

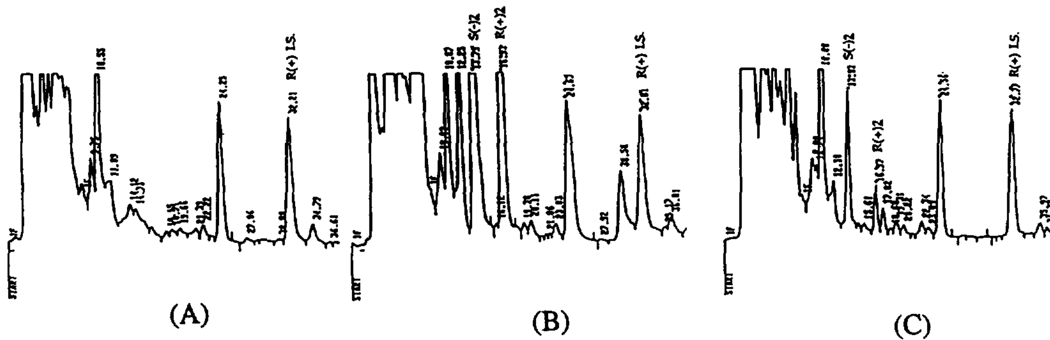

Fig. 3. Representative chromatograms of blank plasma spiked with I.S. (A), blank plasma spiked with I.S. and 40 ng/ml of 4-hydroxypropranolol (2) (B), and 1-h plasma sample spiked with I.S. following an 80 mg oral dose of propranolol (C).

3.3. Linearity, precision and accuracy

The present method for the resolution of propranolol was validated by analyzing ten plasma standards with concentrations ranging from 1 to 100 ng/ml of propranolol enantiomers. The calibration curves of *S*-(−) and *R*-(+) isomers were linear over the concentration ranges studied [*R*-(+) isomer: $y=0.0334x+0.0010$, $r^2=0.9991$ over range 1–15 ng/ml; $y=0.0347x-0.0399$, $r^2=0.9994$ over range 8–100 ng/ml. *S*-(−) isomer: $y=0.0359x-0.0011$, $r^2=0.9999$ over range from 1–15 ng/ml; $y=0.0380x-0.0429$, $r^2=0.9997$ over range 8–100 ng/ml]. The intra-day coefficient of variation and accuracy ranged from 2.7 to 13.1% and 95.6 to 104.3%, respectively. The results are shown in Table 1. For the same concentration range, the inter-day coefficient of variation and accuracy ranged from 2.45 to 6.69% and 94.0 to 103.0%, respectively. The results are shown in Table 2. The method for 4-hydroxypropranolol was validated by analyzing six plasma standards ranging from 4–100 ng/ml of racemic 4-hydroxypropranolol. The calibration curves for *S*-(−) and *R*-(+) isomers were linear over the concentration ranges studied [*R*-(+) isomer: $y=0.0085x-0.0049$, $r^2=0.9982$ over range 2–50 ng/ml. *S*-(−) isomer: $y=0.0109x-0.0017$, $r^2=0.9992$ over range 2–50 ng/ml]. The intra-day coefficient of variation and accuracy ranged from 4.01 to 10.4% and 88.8 to 108.5%, respectively, for concentrations ranging between 4.0 and 40.0 ng/ml of enantiomer. The results are shown in Table 3. For the same concentration range, the inter-day coefficient of variation and accuracy ranged from 2.91 to 13.0% and 95.0 to 100.3%, respectively. The results are

Table 1
Intra-day precision and accuracy data for propranolol enantiomers in plasma ($n=6$)

Enantiomer	Spiked concentration (ng/ml)	Found concentration (mean±S.D.) (ng/ml)	Precision (% C.V.)	Accuracy (% Error)
R-(+)	70.0	66.9±2.98	4.45	4.40
	25.0	24.8±0.67	0.70	0.80
	10.0	10.4±0.49	4.71	4.00
	3.0	3.1±0.41	13.10	4.30
S-(−)	70.0	68.5±2.48	3.62	2.10
	25.0	25.8±1.20	4.69	2.40
	10.0	10.2±0.38	3.73	2.00
	3.0	2.9±0.34	11.80	4.30

Table 2
Inter-day precision and accuracy data for propranolol enantiomers in plasma ($n=6$)

Enantiomer	Spiked concentration (ng/ml)	Found concentration (mean±S.D.) (ng/ml)	Precision (% C.V.)	Accuracy (% Error)
R-(+)	70.0	70.3±2.32	3.30	0.40
	25.0	25.6±1.09	4.26	2.40
	10.0	10.0±0.50	5.00	0.00
	3.0	2.99±0.20	6.69	0.30
S-(−)	70.0	69.7±3.36	4.82	0.40
	25.0	24.9±0.61	2.45	0.80
	10.0	10.3±0.50	4.85	3.00
	3.0	2.8±0.16	5.07	6.00

Table 3
Intra-day precision and accuracy data for 4-hydroxypropranolol enantiomers in plasma ($n=6$)

Enantiomer	Spiked concnetration (ng/ml)	Found concnetration (mean±S.D.) (ng/ml)	Precision (% C.V.)	Accuracy (% Error)
R-(+)	40.0	39.4±2.94	7.46	1.50
	20.0	20.8±1.26	6.09	4.00
	8.0	7.1±0.74	10.40	11.20
	4.0	4.2±0.36	8.58	4.00
S-(−)	40.0	41.0±1.83	4.45	2.50
	20.0	20.1±0.81	4.03	0.50
	8.0	7.8±0.31	4.01	2.10
	4.0	4.3±0.43	9.79	8.50

shown in Table 4. Excellent linearity and reproducibility were obtained for both assays.

3.4. Extraction recovery

The relative extraction recovery of propranolol and 4-hydroxypropranolol was determined from three extracted plasma samples and three water standards. The results are shown in Tables 5 and 6. The recovery ranged from 95.8 to 105.0% for propranolol and 72.4 to 85.3% for 4-hydroxypropranolol.

3.5. Application of the assay

The present assay has been used to analyze *R*-(+) and *S*-(−) isomers of propranolol in plasma samples from 12 human subjects following oral administra-

Table 4
Inter-day precision and accuracy data for 4-hydroxypropranolol enantiomers in plasma ($n=6$)

Enantiomer	Spiked concentration (ng/ml)	Found concentration (mean±S.D.) (ng/ml)	Precision (% C.V.)	Accuracy (% Error)
R-(+)	40.0	40.1±1.42	3.55	0.30
	20.0	19.0±2.48	13.00	5.00
	8.0	8.0±0.29	3.62	0.10
	4.0	3.8±0.26	6.87	4.00
S-(−)	40.0	39.7±1.16	2.91	0.70
	20.0	19.5±2.35	12.10	2.50
	8.0	8.0±0.48	6.03	0.10
	4.0	3.9±0.39	9.99	3.70

Table 5
Extraction recovery of propranolol enantiomer from plasma

Concentration (ng/ml)	Peak-height ratio (mean±S.D.) ($n=3$)		Recovery (%)
	Water sample	Plasma sample	
70.0	2.190±0.011	2.100±0.088	95.8
25.0	0.780±0.008	0.802±0.007	103.0
10.0	0.321±0.002	0.333±0.005	104.0
3.0	0.097±0.000	0.102±0.004	105.0

Table 6
Extraction recovery of 4-hydroxypropranolol enantiomer from plasma

Concentration (ng/ml)	Peak-height ratio (mean±S.D.) ($n=3$)		Recovery (%)
	Water sample	Plasma sample	
40.0	0.896±0.060	0.674±0.041	75.2
20.0	0.440±0.004	0.318±0.017	80.8
10.0	0.236±0.020	0.191±0.005	72.4
4.0	0.116±0.007	0.099±0.013	85.3

tion of 80 mg of propranolol in drug interaction studies. The mean pharmacokinetic variables are shown in Table 7. The mean plasma peak concentration of the S-(−) enantiomer is approximately 40% higher than the R-(+) isomer. The half life ($t_{1/2}$) of the S-(−) isomer is approximately 25% shorter than the R-(+) isomer. The area under the curve (AUC) of the plasma concentration-time profile of the S-(−) isomer is approximately 25% higher than the R-(+) isomer, indicating the stereoselective metabolism of propranolol in human. The assay with modified mobile phase also has been used to analyze the enantiomers of 4-hydroxypropranolol in plasma samples from one human subject following oral administration of 80 mg of propranolol. The plasma concentration-time profile is shown in Fig. 4. Since the plasma concentrations of R-(+) enantiomer were below the assay range, the values obtained were intended for reference only. The plasma concen-

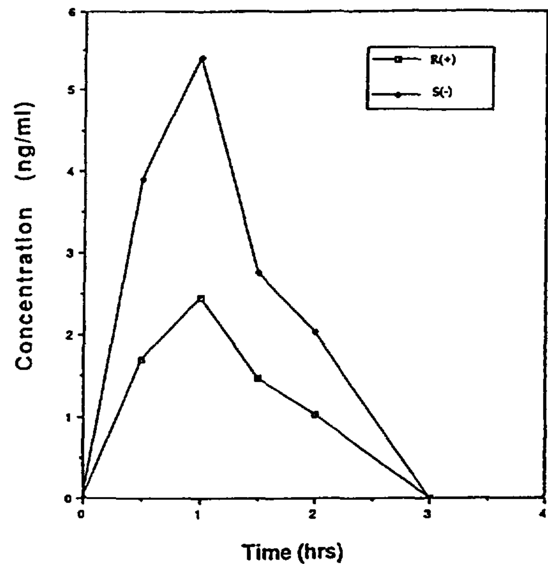

Fig. 4. Plasma concentration–time plot of R-(+)- and S-(−)-4-hydroxypropranolol following an 80-mg oral dose of propranolol.

Table 7
Pharmocokinetic variables following an oral administration of 80 mg of propranolol in human ($n=12$)

Variable	R-(+) Isomer	S-(−) Isomer	Ratio of S-(−)/R-(+)
C_{peak} (ng/ml)	29.79±19.81	41.62±24.57	1.40
T_{peak} (h)	2.58±1.06	2.38±1.17	
$T_{1/2}$ (h)	11.84±3.68	8.65±1.93	0.73
AUC, unextrapolated (ng·h/ml)	210.9±146.3	264.8±183.2	1.26
AUC, extrapolated (ng·h/ml)	241.1±161.1	308.2±189.9	1.28

tration of the S-(−) isomer was higher than the R-(+) isomer, demonstrating the stereoselective ring oxidation of propranolol in human.

4. Conclusions

The present assay, using liquid–liquid extraction for plasma sample cleanup and employing TAGIT as a pre-column derivatization reagent, has successfully resolved the stereoisomers of propranolol and 4-hydroxypropranolol in human plasma. The assay is sensitive and reproducible, as demonstrated in the analysis of plasma samples from human subjects. TAGIT is stereo chemically pure and not fluorescent which are the desired properties for use as a pre-column derivatization reagent, however, the reagent is moisture sensitive, therefore achieving a moisture free reaction condition is the key for this reaction. The present method, with slight modifications, might also be applicable for the analysis of other chiral amines and alcohols.

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