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High-performance liquid chromatographic assay for the derivatized enantiomers of propranolol and 4-hydroxypropranolol in human plasma*

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Abstract: A stereospecific method for the analysis of propranolol and 4-hydroxypropranolol in human plasma employing fluorescence detection has been developed using the homochiral derivatizing agent 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT). The use of fluorescence detection provided enhanced sensitivity and cleaner chromatograms for the analysis of plasma samples, when compared to UV detection. Furthermore, parameters such as TAGIT concentration, vortex time and reaction time were examined to optimize conditions for maximum derivatization recoveries. The analyses of S(−)- and R(+)-propranolol from plasma were linear over the concentration range of 2.0–200 ng ml^{-1}, while S(−)- and R(+)-4-hydroxypropranolol were linear from 5.0 to 200 ng ml^{-1}.

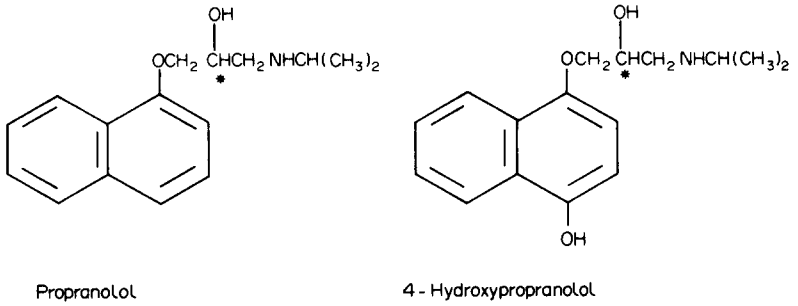
Keywords: *Reversed-phase HPLC; enantiomers; derivatization; propranolol; 4-hydroxypropranolol; fluorescence detection.*

Introduction

The use of β-blocking drugs in the management of cardiovascular disorders is well established. Propranolol [±-1-isopropylamino-3-(1-naphthyloxy)-2-propanol] is a non-selective β-adrenergic receptor blocking agent used in the treatment of hypertension, cardiac arrhythmias and the prophylaxis of angina pectoris [1].

Propranolol (Fig. 1) has a chiral centre and, as such, two enantiomeric forms exist, namely, S(−)- and R(+)-propranolol. The S(−)-enantiomer is statistically significantly more effective than the R(+)-enantiomer in both cats and dogs [2]. The availability of a chiral assay for propranolol and 4-hydroxypropranolol may provide further pharmacokinetic and pharmacodynamic information.

Previously described methods for the analysis of the chiral isomers of propranolol include GC–MS [3], radioreceptor [4], and radioimmunoassays [5]. However, these methods are costly and time consuming. Thus, alternative methods using HPLC have been investigated. The use of HPLC with chiral stationary phases is a potential method of analysis. At the present time, though, many of these chiral columns do not possess the efficiencies that their C18 counterparts do. If selectivity were equivalent this would result in



Propranolol 4-Hydroxypropranolol

Figure 1
Chemical structures of propranolol and 4-hydroxypropranolol. The asterisk denotes the chiral centre.

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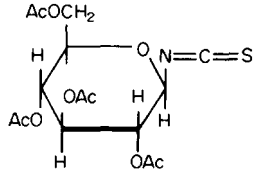
long retention times to achieve adequate resolution and, consequently, lower sensitivity. Therefore, although a few studies adopting this approach have been reported, chiral HPLC still presents difficulties for routine therapeutic analyses. Although the separation of enantiomers is not possible by conventional HPLC, the formation of diastereomers formed through derivatization can enable their separation. The use of chiral derivatization has attracted considerable attention [6–10], with the first being *N*-tri-fluoroacetyl-*S*-(−)-propyl chloride [11]. Unfortunately, this reagent racemized during storage necessitating other reagents to be investigated [11]. The homochiral derivatizing agent TAGIT (Fig. 2) reacts readily with primary or secondary amines to form a corresponding thiourea. TAGIT possesses desirable characteristics, which include being enantiomerically pure, stereochemically stable, and commercially available.

This paper describes the development of a reversed-phase HPLC chiral assay for propranolol and its active metabolite 4-hydroxypropranolol in human plasma employing TAGIT as a derivatization agent and using fluorescence detection.

Experimental

Materials

Racemic propranolol hydrochloride, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate and S(−)- and R(+)-propranolol hydrochloride were purchased from Aldrich (Milwaukee, WI, USA). HPLC grade phosphoric acid 85%, sodium hydroxide, glacial acetic acid, ascorbic acid and ammonium phosphate monobasic were purchased from Fisher Scientific (Montréal, QC, Canada). HPLC grade acetonitrile and hexane were purchased from Caledon (Georgetown, ON, Canada). The water was deionized Type 1, reagent grade (Millipore, Ville St Laurent, QC, Canada).



TAGIT

Figure 2
Chemical structure of TAGIT.

All reagents were used without further purification. 4-Hydroxypropranolol hydrochloride was prepared as previously described by Oatis Jr *et al.* [12].

Instrumentation and chromatographic conditions

The chromatographic system consisted of a Waters Model 590 pump (Waters Associates, Milford, MA, USA), a Varian 9090 autosampler (Varian, Ville St Laurent, QC, Canada) and a Shimadzu RF-535 fluorescence detector (RP Instruments, Montréal, QC, Canada). A stainless-steel column (150 × 4.6 mm i.d.) was packed with 5-μm Hypersil ODS, (prepared in-house). The column was maintained at ambient temperature. Propranolol and 4-hydroxypropranolol were detected by fluorescence at an excitation wavelength of 295 nm and an emission wavelength of 345 nm. The mobile phase consisted of 0.02 M ammonium phosphate monobasic–acetonitrile (2:3, v/v), and was delivered at a flow rate of 1.0 ml min^{-1}. Under these conditions, the retention times of S(−)- and R(+)- 4-hydroxypropranolol and propranolol were 4.1, 4.7, 6.5 and 8.0 min, respectively.

Preparation of standards

A stock solution of racemic propranolol was prepared at 1.0 mg ml^{-1} (free base) in deionized water. Appropriate dilutions of the stock were made with deionized water and used to prepare spiked plasma standards at S(−) and R(+) concentrations of 2.0, 5.0, 25.0, 50.0, 100 and 200 ng ml^{-1}. A stock solution of racemic 4-hydroxypropranolol was prepared at 1.0 mg ml^{-1} (free base) in 0.1 M phosphoric acid containing 50 mg ml^{-1} ascorbic acid. Appropriate dilutions were made with 0.1 M phosphoric acid containing 50 mg ml^{-1} ascorbic acid and used to spike plasma standards at S(−) and R(+) concentrations of 5.0, 25.0, 50.0, 100 and 200 ng ml^{-1}. Individual aliquots of 500 μl were stored in 13 × 100 mm borosilicate tubes at −15°C.

Accuracy and precision

To assess the accuracy and precision of the assay, spiked plasma quality control samples (QCs) were prepared at S(−) and R(+) 4-hydroxypropranolol and propranolol concentrations of 37.5 and 90.0 ng ml^{-1}. To determine accuracy, the concentration of individual enantiomers in the QC samples (n = 8) were

calculated using a standard curve. The per cent difference between the mean measured and the mean added concentrations was considered the accuracy of the assay. Precision was estimated by calculating the inter-assay relative standard deviation (RSD).

Plasma extraction

Aliquots of human plasma (500 μl) were added to 13 × 100 mm borosilicate tubes and alkalinised with 1.0 ml of 0.1 M NaOH and vortexed. Hexane (5 ml) was added and the tubes were shaken for 15 min at low speed (230 ± 30 oscillations per min) on a reciprocating shaker. Following centrifugation for 10 min at *ca* 1500**g**, the organic layer was transferred to a clean 13 × 100 mm disposable borosilicate tube and evaporated to dryness at 37°C under a gentle stream of nitrogen.

Chiral derivatization

After evaporation, the residue was reconstituted in 200 μl of 7.5 mg ml^{-1} TAGIT in acetonitrile and vortexed for 15 s. The tubes were left at room temperature for 40 min and 50 μl was injected onto the liquid chromatograph under the previously stated conditions. Once formed, the diastereomers were stable for at least 36 h at room temperature.

Optimization of derivatization reaction

The extent of derivatisation for both propranolol and 4-hydroxypropranolol was investigated as a function of TAGIT concentration, reaction time at ambient temperature and vortex time. In all cases, a 200 μl aliquot of stock solution containing 200 ng ml^{-1} of each analyte in methanol was evaporated to dryness under nitrogen. The analytes were derivatised with various concentrations of TAGIT ranging from 1.25 to 10.0 mg ml^{-1} in acetonitrile.

The reaction rate was monitored by measuring the formation of the derivative peaks after incubating at room temperature for various times. To the evaporated stock solution, 200 μl of 7.5 mg ml^{-1} TAGIT was added, vortexed 5 s, and chromatographed after incubating between 0.0 and 50 min.

The formation of the diastereomeric derivatives as function of vortex time was also investigated. To the evaporated stock solution 200 μl of 7.5 mg ml^{-1} TAGIT was added, vortexed between 0.0 and 60 s, and incubated for 40 min at room temperature.

Data acquisition

Peak heights for the diastereomeric derivatives of propranolol and 4-hydroxypropranolol were measured using a Chrom-Station (Spectra-Physics Inc., Mountain View, CA, USA). The chromatographic data was automatically processed for absolute peak heights of each enantiomer and fitted to a weighted (1/C) linear regression analysis using the Chrom-Station software.

Derivatization yields

To ascertain the derivatization yields of 4-hydroxypropranolol and propranolol, the absolute peak heights of the underivatized to derivatized analytes were compared. The underivatized analytes were measured using an achiral method with an absolute detection limit of 1.0 ng ml^{-1} for each racemate. The mobile phase was modified to acetonitrile–water–methanol–glacial acetic acid–phosphoric acid (300:225:200:10:1, v/v/v/v/v) and the fluorescence detector was set at 290 and 345 nm for excitation and emission, respectively.

Results and Discussion

The use of TAGIT to resolve the enantiomers of β-blockers in solution with UV detection was first proposed by Sedman and Gal [13]. However, in this study, fluorescence rather than UV detection was utilized to provide enhanced sensitivity and cleaner chromatograms for plasma samples. The derivatization efficiencies for S(−) and R(+) propranolol and 4-hydroxypropranolol were examined with respect to TAGIT concentration, vortex time and reaction time. Based upon the results presented in Tables 1–3, the following operating conditions were used for the analysis of the enantiomeric analytes: 7.5 mg ml^{-1} TAGIT in acetonitrile, vortex 15 s and wait 40 min for complete derivatization. During the incubation period, the S/R-enantiomer peak height ratios were near unity, indicating a similar rate constant for each pair of stereoisomers. Although the rate was lower for the stereoisomers of 4-hydroxypropranolol since an additional 10 min was required to ensure complete derivatization.

Typical chromatograms obtained from extracted plasma blanks, extracted calibration samples at 5.0 ng ml^{-1} and 90.0 ng ml^{-1} are illustrated in Fig. 3(a–c). The retention times of S(−) and R(+) 4-hydroxypropranolol and

Table 1
Normalized peak heights for the diastereomeric derivatives of 4-hydroxypropranolol and propranolol as a function of TAGIT concentration

	TAGIT concentration (mg ml^{-1})					
	1.25	2.50	3.75	5.00	7.50	10.0
S(−)-4-Hydroxypropranolol						
Mean*	97.4	96.1	87.2	85.4	87.4	90.2
SD	8.0	1.9	2.1	1.3	3.3	8.5
RSD (%)	8.2	1.9	2.4	1.5	3.8	9.5
R(+)-4-Hydroxypropranolol						
Mean*	96.2	97.6	86.5	85.4	86.6	90.1
SD	9.5	3.5	1.5	1.4	3.1	8.6
RSD (%)	9.0	3.6	1.7	1.7	3.5	9.5
S(−)-Propranolol						
Mean*	85.4	64.4	92.6	92.9	95.1	93.8
SD	4.8	15.2	1.7	5.9	4.7	5.6
RSD (%)	5.6	23.5	1.8	6.4	5.3	5.9
R(+)-Propranolol						
Mean*	95.7	90.3	92.0	84.1	92.9	91.3
SD	1.8	13.7	0.4	6.4	4.8	3.0
RSD (%)	1.9	15.2	0.5	7.6	5.1	3.3

*n = 3.

Table 2
Normalized peak heights for the diastereomeric derivatives of 4-hydroxypropranolol and propranolol as a function of vortex time

	Vortex time (s)					
	0.0	5.0	15.0	30.0	45.0	60.0
S(−)-4-Hydroxypropranolol						
Mean*	46.7	72.2	96.6	97.4	97.8	97.2
SD	2.6	6.9	5.9	2.4	3.2	2.3
RSD (%)	5.5	9.5	6.1	2.5	3.2	2.4
R(+)-4-Hydroxypropranolol						
Mean*	44.9	69.2	94.5	93.8	95.1	93.1
SD	3.2	7.2	5.0	2.4	3.3	2.7
RSD (%)	7.0	10.4	5.3	2.5	3.5	2.9
S(−)-Propranolol						
Mean*	56.8	81.4	98.6	99.4	97.2	95.7
SD	21.9	15.8	1.6	0.5	1.4	1.0
RSD (%)	38.4	19.4	1.6	0.2	1.4	1.1
R(+)-Propranolol						
Mean*	66.1	89.4	95.5	91.0	94.0	92.5
SD	24.2	2.9	3.9	1.7	2.4	2.3
RSD (%)	36.6	3.3	4.1	1.9	2.6	2.9

*n = 3.

propranolol were 4.1, 4.7, 6.5 and 8.0 min, respectively, with an overall chromatographic run time of 10 min. The resolutions (R_s) between the enantiomers of 4-hydroxypropranolol and propranolol were 1.5 and 2.5, respectively.

Linear response of the absolute peak height of S(−)- and R(+)-4-hydroxypropranolol over the concentration range of 5.0 to 200 ng ml^{-1} was observed, while the linear response of S(−)- and R(+)-propranolol was 2.0–200 ng ml^{-1}. The correlation coefficients for the diastereomeric derivatives were 0.9960 or better (n = 4).

The inter-day accuracy and precision for each stereoisomer was assessed by the analysis of QC samples (Table 4). At spiked plasma concentrations of 37.5 and 90.0 ng ml^{-1} for each analyte, the accuracy and precision were within 6.7 and 7.0%, respectively.

The pure enantiomers of propranolol were chromatographed separately following derivatisation to assess their elution order. Since the pure enantiomers of 4-hydroxypropranolol were not commercially available, a portion of each enantiomerically pure solution of propranolol was left at room temperature for 72 h and then derivatized and chromatographed.

Table 3
Normalized peak heights for the diastereomeric derivatives of 4-hydroxypropranolol and propranolol as a function of reaction time with TAGIT

	Reaction time (min)					
	0	10	20	30	40	50
S(−)-4-Hydroxypropranolol						
Mean*	31.0	55.3	82.0	93.5	94.6	95.3
SD	3.4	14.4	1.4	4.2	3.5	4.5
RSD (%)	11.0	26.0	1.7	4.5	3.7	4.7
R(+)-4-Hydroxypropranolol						
Mean*	32.0	56.8	83.7	95.3	95.0	95.7
SD	3.5	14.9	1.5	4.7	3.0	3.9
RSD (%)	10.8	26.2	1.8	5.0	3.2	4.1
S(−)-Propranolol						
Mean*	91.1	95.8	90.8	94.4	93.5	94.4
SD	4.3	4.4	3.6	2.7	2.0	2.7
RSD (%)	4.7	4.6	4.0	2.8	2.2	2.8
R(+)-Propranolol						
Mean*	76.1	86.2	91.7	83.3	90.4	91.0
SD	7.2	6.4	9.9	0.6	11.6	4.5
RSD (%)	9.5	7.4	10.8	0.7	12.7	5.0

*n = 3.

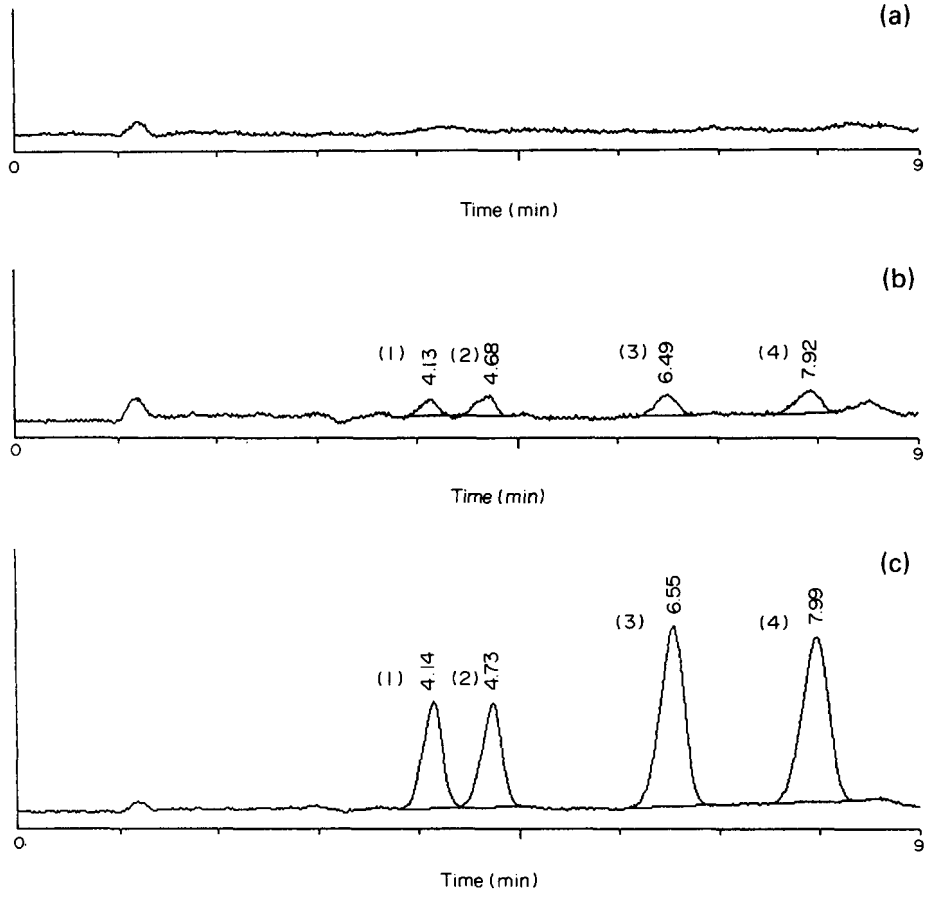


Figure 3
Reversed-phase HPLC chromatograms resulting from TAGIT derivatization of (a) human plasma blank, attenuation = 2; (b) human plasma spiked with 5.0 ng ml^{-1} of each enantiomer, attenuation = 2 and (c) human plasma spiked with 90.0 ng ml^{-1} of each enantiomer, attenuation = 4. Peak assignments were (1) S(−)-4-hydroxypropranolol, (2) R(+)-4-hydroxypropranolol, (3) S(−)-propranolol and (4) R(+)-propranolol.

Table 4
Inter-day accuracy and precision*

Enantiomer	Concentration added (ng ml^{-1})	Concentration measured (mean ± SD) (ng ml^{-1})	Accuracy (error, %)	Precision (RSD, %)
S(−)-4-Hydroxypropranolol	37.5	39.4 ± 1.45	5.1	3.7
	90.0	91.1 ± 5.52	1.2	6.1
R(+)-4-Hydroxypropranolol	37.5	40.0 ± 1.67	6.7	4.2
	90.0	92.2 ± 4.52	2.4	4.9
S(−)-Propranolol	37.5	38.3 ± 1.51	2.1	3.9
	90.0	90.3 ± 2.87	0.3	3.2
R(+)-Propranolol	37.5	38.7 ± 2.72	3.2	7.0
	90.0	90.7 ± 2.79	0.8	3.1

*n = 8.

This yielded two peaks, one corresponding to propranolol and the other to the 4-hydroxy metabolite. Furthermore, this peak assignment corresponds to that of Sedman and Gal [13].

In conclusion, the chiral method described herein is sensitive and rapid for the analysis of propranolol and 4-hydroxypropranolol enantiomers in human plasma. Furthermore, baseline resolution of all stereoisomers is achieved within 10 min making this assay suitable where high sample throughput is necessary.

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