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Separation of the $R(-)$- and $S(+)$-enantiomers of the ethyl ester of tiagabine · HCl using a Chiralcel-OG column

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Abstract

The $R(-)$- and $S(+)$-enantiomers of ethyl-N-[4,4-di(3-methyl-thien-2-yl)-but-3-enyl]-nipecotate–hydrochloride are separated by a normal-phase chiral HPLC on a commercially available chiral stationary phase. Samples of $R(-)$- and $S(+)$-enantiomers were first dissolved in a few drops of 2-methyl-2-propanol, diluted with the mobile phase, and then injected directly into the HPLC system. The HPLC system was equipped with a Daicel, Chiralcel-OG column. The mobile phase consisted of hexane–2-methyl-2-propanol–1-octanol, (990:8:2, v/v/v) to which approximately 0.5 ml of diethylamine was added. The method is able to separate the $R(-)$- and $S(+)$-enantiomers with a resolution factor of 1.2 and a selectivity factor of 1.16. The limit of quantification of the $S(+)$-enantiomer is 0.2% in the $R(-)$-enantiomer. The analytical procedure was validated by conducting standard addition and recovery of the $S(+)$-enantiomer in the $R(-)$-enantiomer. The precision of the method was determined by multiple analysis of each of the two samples of $R(-)$-enantiomer containing approximately 1% of the $S(+)$-enantiomer.

1. Introduction

The differences in biological activity between the enantiomers of pharmaceutical agents have been reported in the literature [1]. In recent years, interest in the stereochemical aspects of drug action has been intensified [2–4] because of the more stringent regulations for the marketing of optically active drugs by the United States Food and Drug Administration (U.S.F.D.A.) and other regulatory agencies of the world.

High-performance liquid chromatography (HPLC) and gas chromatography (GC) have been used to separate and quantitate the enantiomers of pharmacologically active compounds [5–12]. To understand the separation mechanism of the enantiomers on a chiral stationary phase, numerous theoretical studies have been conducted by the early pioneers of this field [13–16]. Gal [17] conducted studies on derivatization of optically active compounds with pure optically active reagents, forming diastereoisomers. The diastereoisomers were separated using various chromatographic conditions. Wainer [18,19] has classified all the commercially available chiral stationary phases according to their mode of separation mechanism of the optically active compounds having different functional groups. To ensure the optical purity of a chiral drug, and to determine the pharmacokinetic profiles and pharmacodynamic effects of individual enantiomers of a chirally active drug, a simple reproducible and sensitive analytical method is required.

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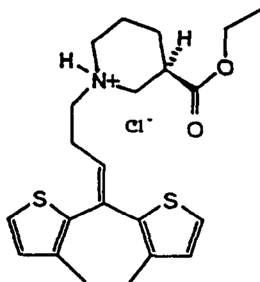

Fig. 1. Chemical structure of the ethylester of Tiagabine · HCl.

In this report, a commercially available chiral stationary phase (Chiralcel-OG) was used to separate and quantitate the *R*(−)- and *S*(+)-enantiomers of ethyl-N-[4,4-di(3-methyl-thien-2-yl)-but-3-enyl] nipecotate–hydrochloride (ethyl ester of Tiagabine · HCl) without derivatization. The chemical structure of the ethyl ester of Tiagabine · HCl is shown in Fig. 1. This compound is a pro-drug and is the penultimate intermediate in the synthesis of Tiagabine · HCl, which is under development as an anti-convulsive agent. The *R*(−)-enantiomer of the drug has been found to be pharmacologically more active than the *S*(+)-enantiomer. Several commercially available columns were investigated using normal-phase and reversed-phase modes to obtain the maximum resolution and sensitivity of the two enantiomers.

2. Experimental

2.1. Equipment

A HPLC solvent-delivery system (SP 8800) equipped with an injector/autosampler (SP 8780), an integrator (SP 4270), and a variable-wavelength UV–visible detector (SP 8450) was used in the experiment (Spectra-Physics, San Jose, CA, USA). A 25 cm × 4.6 mm I.D., Chiralcel-OG column was used in the method finally developed (Daicel, USA). The other chiral stationary phase columns investigated in this experiment were 25 cm × 4.6 mm I.D., 5 μm, D-phenylglycine (Regis, Morton Grove, IL, USA), 25 cm × 4.6 mm I.D., 5 μm, β-cyclodextrin column, cyclobond-1 (Rainin Instruments, Woburn, MA, USA), 25 cm × 4.6 mm I.D., 5 μm, phenylalanine (Jones Chromatography, Mid Glamorgan, UK), 25 cm × 4.6 mm I.D., 10 μm, Chiralcel-OJ (Daicel, USA), and 15 cm × 7.5 mm, 10 μm, bovine serum albumin column (manufactured by Machery–Nagel, bought from Alltech Assoc., Deerfield, IL, USA).

2.2. Materials

A HPLC-grade hexane and 1-octanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). Diethylamine (reagent grade) and 2-methyl-2-propanol were purchased from Aldrich (Milwaukee, WI, USA). The racemic mixture, *R*(−)- and *S*(+)-enantiomers of the ethyl ester of Tiagabine · HCl, was from Abbott Laboratories (North Chicago, IL, USA).

2.3. Preparation of the sample

Approximately 10 mg of the samples were weighed and transferred into a 10-ml volumetric flask. Aliquots of 5–10 drops of 2-methyl-2-propanol were added into the sample flask to dissolve the sample. The sample was then diluted with the mobile phase. The standard solutions of *R*(−)- and *S*(+)-enantiomers and the racemic (±) mixture were similarly prepared. The mobile phase was injected as a blank.

2.4. Preparation of the mobile phase

To 990 ml of hexane, 8.0 ml of 2-methyl-2-propanol, 2.0 ml of 1-octanol, and 0.50 ml of diethylamine were added and mixed. The mobile phase mixture was degassed for approximately 5 min and used for analysis.

2.5. Chromatographic conditions

The typical chromatographic conditions used in this experiment are as follows: the flow-rate of the mobile phase was 0.8 ml/min; the samples were monitored with a UV detector at 280 nm

and 0.10 to 0.20 AUFS; 10 μl of the sample were injected into the HPLC system.

2.6. Calculation

Quantification of the $S(+)$-enantiomer present in the $R(-)$-enantiomer was done using the following equation:

$$\% \text{ of } S(+)\text{-enantiomer} = 100 \cdot \frac{\text{Peak area of the } S(+)\text{-enantiomer}}{\text{Sum of the peak areas of the } S(+)\text{- and } R(-)\text{-enantiomers}}$$

2.7. Limit of quantification (LOQ)

Samples of $R(-)$-enantiomer of tiagabine-ethyl ester were analyzed to determine the lowest level of $S(+)$-enantiomer which can be quantitated with good reproducibility (% R.S.D. less than 10). The limit of quantification (LOQ) of the method for $S(+)$-enantiomer present in the $R(-)$-enantiomer was about 0.2% at a signal-to-noise ratio of about 3.

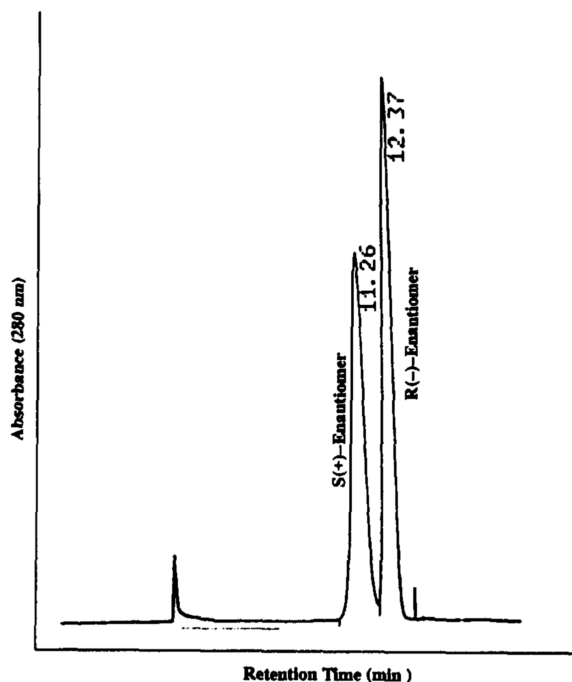

Fig. 2. Typical chromatogram of a racemic (±) mixture of the ethyl ester of Tiagabine · HCl using the mobile phase (with 1-octanol) described in this report.

3. Results and discussion

The blank (mobile phase) was injected and no peak eluted with the same retention times as those of the $S(+)$- and $R(-)$-enantiomers. Fig. 2 is a typical chromatogram of the racemic (±) mixture of the chiral compound which shows that the peaks of $S(+)$- and $R(-)$-enantiomers are adequately resolved from each other. Figs. 3 and 4 are typical chromatograms of the racemic (±) and $R(-)$-enantiomers of the ethyl ester of Tiagabine · HCl when 1-octanol was not present in the mobile phase.

The capacity factors (k') of the $S(+)$- and $R(-)$-enantiomers were approximately 2.80 and 3.25 under the chromatographic conditions used in this experiment. The resolution between the two enantiomers was approximately 1.2 with a selectivity factor (α) of 1.16.

The amount of the $S(+)$-enantiomer present in the $R(-)$-enantiomer was quantified by peak area percent. The response of the UV detector was linear from 0.10 to 1.0 mg/ml for 10-μl injections. Typical regression line equation of the analyte has a correlation coefficient of >0.999 with a negligible y-intercept, and essentially passes through the origin. Authentic reference materials of the pure $S(+)$- and $R(-)$-enantiomers were available for the determination of relative retention volumes, *i.e.* elution order. Under the chromatographic conditions of these experiments, the $S(+)$-enantiomer elutes before the $R(-)$-enantiomer. Elution of the $S(+)$-enantiomer prior to the $R(-)$-enantiomer makes this method ideal for trace analysis of the $S(+)$-enantiomer present in the $R(-)$-enantiomer.

Experiments were conducted to obtain a mobile phase which will give optimum separation and sensitivity on the Chiralcel-OG column. The stationary phase of the Chiralcel-OG column is the methylphenyl carbamate derivative of cellulose, agglomerated on silica. Therefore, the type and amount of solvents which can be used in the mobile phase on Chiralcel-OG column are lim-

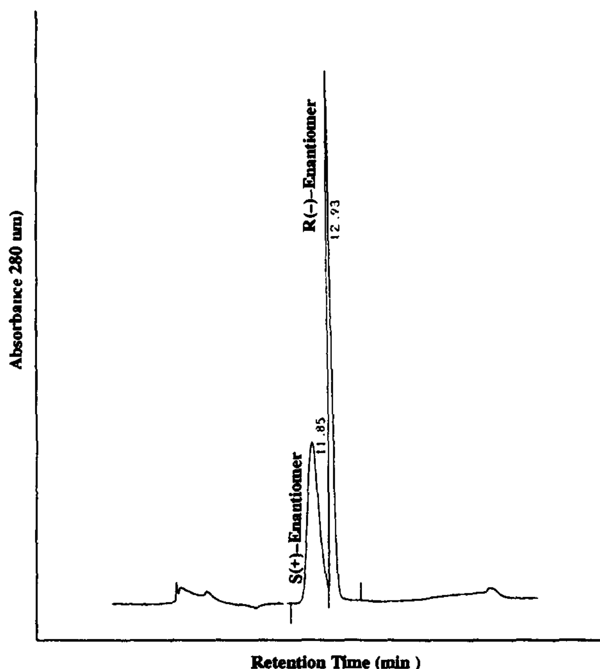

Fig. 3. Typical chromatogram of a racemic (±) mixture of the ethyl ester of Tiagabine · HCl using the same mobile phase as of Fig. 2, except 1-octanol was not added in the mobile phase.

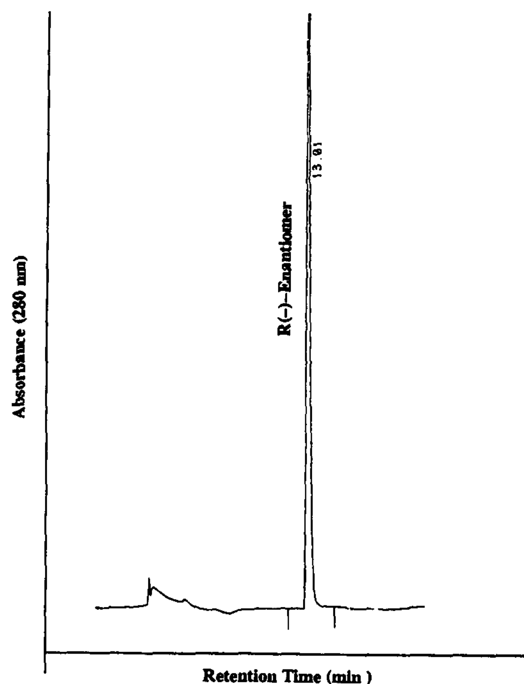

Fig. 4. Typical chromatogram of the $R(-)$-enantiomer of the ethyl ester of Tiagabine · HCl. Mobile phase is same as that of Fig. 2, except 1-octanol was not added in the mobile phase.

ited. Typically, isopropanol is used in the range of 5 to 10%, mixed with hexane or heptane as the mobile phase. Other solvents which can be used in the mobile phase in small quantities are *tert.*-butanol, ethanol and other long chain alcohols. If solvents other than the above are used in the mobile phase, the column may lose selectivity, and the back pressure may also increase (due to the swelling of the stationary phase) and may destroy the column. Trace amounts of diethylamine (less than 0.5%) can be used in the mobile phase when the analyte molecule contains an amine functionality.

The selectivity and resolution of the two enantiomers varies with the choice of alcohol. For example, if 2-propanol is used in the mobile phase instead of 2-methyl-2-propanol, the resolution between the two enantiomers of the ethyl ester of Tiagabine · HCl is totally lost. The presence of a second alcohol in the mobile phase in trace amounts can also enhance the resolution between the two enantiomers. The resolution between the $R(-)$ and $S(+)$-enantiomers of the ethyl ester of Tiagabine · HCl is approximately 1.1 without 1-octanol in the mobile phase. The resolution between the two enantiomers increases to 1.5, when 0.2% of 1-octanol is added in the mobile phase. For the ethyl ester of Tiagabine · HCl, in addition to resolution enhancement, addition of 0.2% 1-octanol in the mobile phase has also broadened the peak width of the unusual sharp peak of the $R(-)$-enantiomer which elutes after the $S(+)$-enantiomer (Figs. 3 and 4).

The unusual chromatographic behavior of the $R(-)$-enantiomer (later eluting peak) is difficult to explain satisfactorily with the established phenomena of chromatography. Typically, in isocratic chromatography, the later eluting peak is wider than the early eluting peak when the core structures (active sites) of both the molecules are similar, except for the number of carbon atoms. The broadening of the $R(-)$-enantiomer peak, which elutes later, cannot be

satisfactorily explained with the known theories and phenomena of chromatography, such as eddy diffusion, mass transfer of the analyte from mobile phase to stationary phase and vice versa, viscosity of the mobile phase and other various interactions (hydrophobic, ionic, charge dispersion, etc.) between the analyte and the stationary phase. Addition of 1-octanol in the mobile phase improved the resolution and also broadened the peaks of the two enantiomers. However, the relative peak-broadening for the *R*(−)-enantiomer was found to be greater than the *S*(+)-enantiomer. This observation suggests that the interaction of the two enantiomers with the active sites of the chiral stationary phase or with the active sites of the silica backbone of the stationary phase increases in the presence of 1-octanol in the mobile phase, hence resulting in a broader peak. However, because of different spatial configurations of the two enantiomers, the chiral interaction of the *R*(−)-enantiomer with the chiral stationary phase (when 1-octanol is added in the mobile phase) appears to be greater than the *S*(+)-enantiomer, hence resulting in a higher percentage of peak-broadening than the *S*(+)-enantiomer.

Inspection of Figs. 3 and 4 reveals that the peak shape and width of the pure *R*(−)-enantiomer in the standard solution is the same as the peak shape and width of *R*(−)-enantiomer in the standard solution of the racemic (±) mixture. It is not possible to explain satisfactorily by the known theories and phenomena of chromatography the sharper peak shape of the *R*(−)-enantiomer (later eluting is sharper) than the *S*(+)-enantiomer; however, a possible explanation could be that the secondary interactions of the *S*(+)-enantiomer directly with the chiral stationary phase or with the silica backbone of the stationary phase are stronger than the interactions of the *R*(−)-enantiomer with the stationary phase. In chiral chromatography, the retention of the enantiomers is primarily dictated by the chiral interaction between the chiral sites of the analyte and stationary phase. Therefore, due to different spatial configurations of the two enantiomers, one enantiomer can have stronger secondary interactions with the stationary phase (or its backbone) but weak chiral interaction with the chiral stationary phase, resulting into a broader peak but a shorter retention time than the other enantiomer. In general, the mass transfer of the analyte from stationary phase to mobile phase is poor when the analyte experiences strong secondary interactions with the active sites of the stationary phase or with the active sites of the backbone of the stationary phase. It is difficult to prove the above hypothesis regarding the band widths of the two enantiomers with the data available at this time. However, the explanation given above seems logical and may quite possibly be true.

The presence of diethylamine in the mobile phase plays an important role in the chromatographic efficiency and the separation. If diethylamine is not present in the mobile phase, the two enantiomers elute as overlapping wide peaks with a resolution factor of less than one. The extra broadening of the peaks of the two enantiomers in the absence of diethylamine in the mobile phase can be explained in terms of strong interaction of the nitrogen atom of the nipecotic acid moiety of the analyte with the silanols of the silica backbone of the stationary phase, or with the carbamate or hydroxyl functionality of the chiral stationary phase. Therefore, the sharper peaks of the analyte in presence of diethylamine is due to the suppression of the secondary interactions between the basic sites of the analyte and the stationary phase. Two other amines such as N,N-dimethyloctylamine and triethylamine were also tested. Both of these amines were found to be less effective than diethylamine in terms of decreasing the overlapping of the two peaks, and increasing the resolution.

The stationary phase of the Chiralcel-OG column is very susceptible to trace amounts of mobile phase modifiers; the effects on the retention and selectivity behavior of the original stationary phase by different mobile phase modifier may be either temporary or permanent. Therefore, columns should be used for dedicated analyses. When a new column is obtained, it should be conditioned with at least 1 l of mobile phase at a flow-rate of 0.5 ml/min to 0.8 ml/min to achieve a reproducible separation.

The separation of two enantiomers on a chiral stationary phase can only take place if one enantiomer, on a time average basis, has stronger interaction than the other enantiomer. The elution order of the $S(+)$- and $R(-)$-enantiomers of the analyte is dictated by the absolute configuration of the chiral stationary phase. The elution of the undesired enantiomer prior to the desired enantiomer is always preferred. Otherwise, a large resolution factor and good chromatographic efficiency would be required between the two enantiomers for accurate quantitation of the undesired enantiomer.

Columns packed with phenylglycine, phenylalanine (Pirkle type) and β-cyclodextrin were also investigated (both under normal- and reversed-phase conditions) for enantiomeric separation of the ethyl ester of Tiagabine · HCl. Solvents, such as hexane, isopropanol, ethanol and 0.1% trifluoroacetic acid were used at various combination, to obtain retention times from 10 to 60 min. For reversed-phase conditions, various ratios of 0.01 *M* phosphate buffer at different pH (2.2 to 7.5) with different percentages of organic modifiers such as methanol, acetonitrile and isopropanol were used to obtain retention time ranged from 10 to 50 min. There was no indication of enantiomeric separation from any of the above experiments.

A bovine serum albumin (BSA) column was also investigated using mobile phases having different percentages of isopropanol (1 to 10%) in a 0.01 *M* aqueous phosphate buffer. The pH of the mobile phase was varied from 3 to 7 and the retention time of the analyte ranged from 12 to 35 min. Again, no indication of enantiomeric separation was obtained from any experiments conducted by using the BSA column.

A Chiralcel-OJ column was also investigated for enantiomeric separation of the ethyl ester of Tiagabine. Solvents such as hexane, isopropanol, ethanol, trace amounts of diethylamine and trifluoroacetic acid (0.1%, v/v) were used at different solvent strengths. The retention time of the analyte varied from 10 to 30 min. This column (Chiralcel-OJ), showed some indication of enantiomeric separation when a mobile phase of hexane–isopropanol–ethanol–trifluoroacetic acid at a ratio of 93:5:2:0.1 (v/v) was used. However, the resolution and selectivity of the analyte did not improve to any significant magnitude when the ratios of the solvents were varied from 100% hexane to hexane–isopropanol (90:10), and hexane–ethanol (90:10).

The column-to-column reproducibility of the separation between the two enantiomers of the analyte was tested by using three Chiralcel-OG columns of different lots. The separation was found to be reproducible on columns having different lot numbers, but conditioning of the new column with the mobile phase was required to achieve reproducibility. Typically, 1 l of the mobile phase at a flow-rate of approximately 0.5 ml/min was needed to condition a new column.

Standard addition and recovery experiments were conducted by two analysts to determine the accuracy of the method for the quantification of trace amounts of $S(+)$-enantiomer present in the $R(-)$-enantiomer. The range of addition levels used in this study was approximately 0.5% to 2.0%. The recovery of the $S(+)$-enantiomer averaged 100.5% with 2.1% R.S.D. The data for the standard addition and recovery experiments are summarized in Table 1.

The precision and short-term ruggedness of the method were also determined by two analysts using one lot of Tiagabine ethyl ester

Table 1
Standard addition and recovery data for $S(+)$-enantiomer

Baseline signal (%)[a] (*A*)	Addition level (%) (*B*)	Percent theory (*A*+*B*)	Percent found (*C*)	Percent recovery $\left(\frac{C}{A+B}\cdot 100\right)$
1.00	0.55	1.55	1.56	100.6
1.00	1.00	2.00	1.99	99.5
1.00	2.00	3.00	3.00	100.0
1.05	0.55	1.60	1.67	104.4
1.05	1.00	2.05	2.07	101.0
1.05	2.00	3.05	2.97	97.4
Mean				100.5
S.D.				±2.1
R.S.D. (%)				±2.1

[a] Baseline signal is the percent of $S(+)$-enantiomer already present in the sample used in this experiment.

which contained trace amount of $S(+)$-enantiomer. Two samples of $R(-)$-enantiomer of Tiagabine ethyl ester were prepared by each of the two analysts. The samples were analyzed using two different instruments, columns, and on two different days. The precision (R.S.D.) of the method is 6.4%. The results of this experiment are presented in Table 2. Fig. 5 is a typical sample chromatogram of the ethyl ester of Tiagabine · HCl containing approximately 0.3% of the $S(+)$-enantiomer.

The Chiralcel-OG column used in this method to separate the two enantiomers was found to be very stable under mobile phase conditions used in the final method. The efficiency, selectivity, resolution, and other chromatographic properties of the Chiralcel-OG column did not show any significant change after approximately 500 sample injections with the following maintenance procedure applied. On several occasions (three times), the resolution and efficiency of the column deteriorated after 20 to 30 injections of the sample. The column was easily regenerated to its original activity simply by washing it with approximately 200 ml of hexane–isopropanol–ethanol (70:20:10, v/v/v) at a flow-rate of 0.2 ml/min. After the washing, the column was reconditioned with approximately 200 ml of the mobile phase. The occasional deterioration of the column performance was probably due to the presence of some unknown impurities in the samples. This method is also used to analyze the in-process samples. The in-process samples at R&D stage of any project usually contain impurities of unknown characteristics and varies widely from lot to lot. Some of these unknown impurities may not elute from the column with the mobile phase used, and therefore could be the potential cause of deterioration of the efficiency, selectivity and resolution.

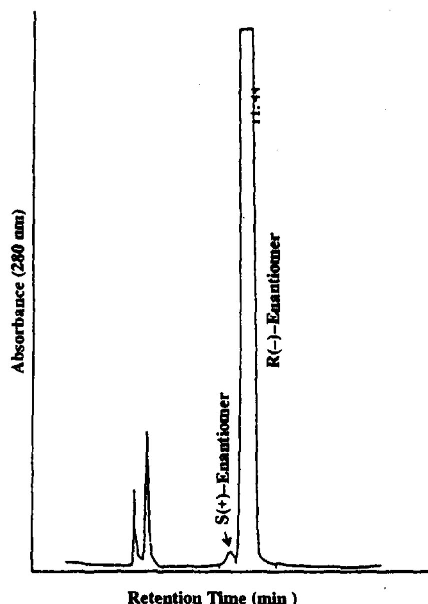

Fig. 5. Typical chromatogram of a sample of the ethyl ester of Tiagabine · HCl containing trace amount of the $S(+)$-enantiomer. Mobile phase is same as that of Fig. 2.

Table 2
Precision data for analysis of $S(+)$-enantiomer present in the $R(-)$-enantiomer

Analyst identification	Peak area (%) of $S(+)$-enantiomer
1	0.481
1	0.463
1	0.474
1	0.459
1	0.489
1	0.472
2	0.429
2	0.414
2	0.402
Mean	0.454
S.D.	±0.029
R.S.D. (%)	±6.4

4. Conclusions

An analytical method was required to determine the presence of $S(+)$-enantiomer in the ethyl ester of Tiagabine · HCl in order to assure the chiral purity of the final product. If the content of the $S(+)$-enantiomer at this stage of synthesis is higher than the desired level, then it can be reduced, to some extent, by recrystallization. Once this synthetic step is completed and the final product is obtained, the $S(+)$-enantiomer cannot be further reduced to any significant

amount by recrystallization. Therefore, it was important to control the final enantiomeric purity at this stage of synthesis. The normal-phase HPLC method described in this paper to separate the two enantiomers of the analyte is simple, reproducible, and relatively sensitive. The separation of the two enantiomers was reproducible on three different columns obtained from three different lots.

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