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Separation and quantitation of the *S*-(+)-enantiomer in the bulk drug tiagabine·HCl by chiral high-performance-liquid chromatography using a Chiralcel-OD column

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Abstract

Tiagabine·HCl is being developed as an anti-convulsant/anti-epileptic agent for seizure disorders. The pharmacological activity of the *R*-(−)-enantiomer is higher than that of the *S*-(+)-enantiomer. Therefore, the drug is synthesized in the pure *R*-(−)-enantiomeric form. The enantiomers of tiagabine·HCl were separated on a modified cellulose stationary phase (Chiralcel-OD) with a mobile phase of hexane–isopropanol–ethanol (80:14:06, v/v/v). Approximately 5 ml of trifluoroacetic acid was added for each liter of the mobile phase mixture. The method is capable of separating the two enantiomers with a selectivity factor of 1.55 and a resolution factor of 3.4. The samples of tiagabine·HCl were monitored by a UV detector at 260 nm. The method was validated by conducting standard addition and recovery of the *S*-(+)-enantiomer in tiagabine·HCl. The R.S.D. of the method is 3.2%. The limit of quantification (LOQ) of the *S*-(+)-enantiomer present in tiagabine·HCl is about 0.03%. © 1998 Elsevier Science B.V.

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1. Introduction

The biological activity of different enantiomers of a pharmaceutical agent can differ significantly [1]. In recent years, stringent regulations for marketing enantiomeric drugs have been implemented by the regulatory agencies of all the major countries (Pharmaceutical Consumers) of the world [2–4]. Therefore, in recent years, all of the major pharmaceutical companies have paid special attention to the development of pure enantiomers (instead of racemic mixtures) as potential drug candidates.

Various analytical techniques, including high-performance liquid chromatography (HPLC) and gas chromatography, have been extensively used for the separation and quantification of the enantiomers of pharmacologically active chemical entities [5–12]. Numerous theoretical and experimental studies have been conducted by the early pioneers of this field to gain an understanding of the mechanisms of chiral separation on chiral stationary phases [11–16]. Derivatization of chiral drugs with optically pure compounds to form a diastereoisomer was a common practice in the early days of this field [17]. Wainer [18] has classified all of the commercially available

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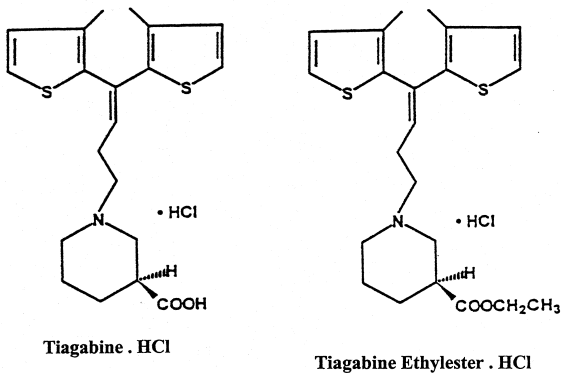

Fig. 1. Chemical structures of tiagabine·HCl and it ethyl ester.

chiral stationary phases according to their mode of separation with respect to different functional groups of the optically active analytes. A simple, sensitive, and reproducible analytical method is required to determine the optical purity of a compound and also to determine the pharmacokinetic profiles and pharmacodynamic effects of individual enantiomers of a chirally active drug.

In this report, a commercially available chiral stationary phase (Chiralcel-OD) was used to separate and estimate the *R*-(−) and *S*-(+)-enantiomers of tiagabine·HCl without derivatization. The chemical structures of tiagabine·HCl and its ethyl ester derivative are shown in Fig. 1. Various commercially available chiral stationary phases 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 Chiralcel-OD column was used in the method that was finally developed (Daicel, Exton, PA, USA). The other chiral stationary phase columns investigated in this experiment were a 25 cm×4.6 mm, 5 μm, D-phenylglycine (Regis, Morton Grove, IL, USA) column, a 25 cm×4.6 mm, 5 μm, β-cyclodextrin column, a Cyclobond-1 column (Rainin, Woburn, MA, USA), a 25 cm×4.6 mm, 5 μm, phenylalanine column (Jones Chromatography, Mid Glamorgan, UK), 25 cm×4.6 mm, 10 μm, Chiralcel-OJ, OG, AD and ODR columns (Daicel) and a 15 cm×7.5 mm, 10 μm, bovine serum albumin column (manufactured by Macherey Nagle, bought from Alltech Associates, Deerfield, IL, USA).

2.2. Materials

HPLC-grade hexane, isopropanol and ethanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). Trifluoroacetic acid (reagent grade) was purchased from Aldrich (Milwaukee, WI, USA). The racemic mixture, *R*-(−)- and *S*-(+)-enantiomers, of tiagabine·HCl was from Abbott Laboratories (North Chicago, IL, USA).

2.3. Preparation of the sample

Approximately 50 mg of tiagabine·HCl (racemic or pure enantiomers) were weighed and transferred to a 25-ml volumetric flask. Five to ten drops of methanol were added to the sample flask to dissolve the sample. The sample was then diluted with isopropanol to the 25-ml volumetric mark. The standard solutions of *R*-(−)- and *S*-(+)-enantiomers and the racemic (±) mixture were similarly prepared. The mobile phase was injected as a blank. The analytical concentration of the sample solution was approximately 25 mg/ml.

2.4. Preparation of the mobile phase

To 800 ml of hexane, 140 ml of isopropyl alcohol, 60 ml of ethanol and 5 ml of trifluoroacetic acid were added and the solution was mixed. The mobile phase was degassed for approximately 5 min prior to use.

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 260 nm and 0.20 A.U.F.S.; a 10-μl volume of the sample was injected into the HPLC system.

2.6. Calculation

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

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

2.7. Limit of quantification (LOQ)

Samples of the *R*-(−)-enantiomer of tiagabine·HCl were analyzed to determine the lowest level of *S*-(+)-enantiomer that can be quantitated with good reproducibility (R.S.D. value of less than 10%). The limit of quantification (LOQ) of the final method for the *S*-(+)-enantiomer present in the *R*-(−)-enantiomer was approximately 0.03% (peak area) at a signal-to-noise ratio of three.

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. 2a is a typical chromatogram of the racemic (±) mixture of tiagabine·HCl and shows that the peaks of *S*-(+)- and *R*-(−)-enantiomers are adequately resolved from each other. Fig. 2b is a typical chromatogram of the *R*-(−)-enantiomer (tiagabine sample) of tiagabine·HCl. The selectivity factor (α) for the *R*-(−)- and *S*-(+)-enantiomers of tiagabine·HCl was 1.55. The resolution factor between the two enantiomers was 3.4.

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 ~0.0007 to 4.3 mg/ml for 10 μl injections. A typical regression line equation of the analyte has a correlation coefficient >0.9999, with a negligible *y*-intercept, and essentially passes through the origin.

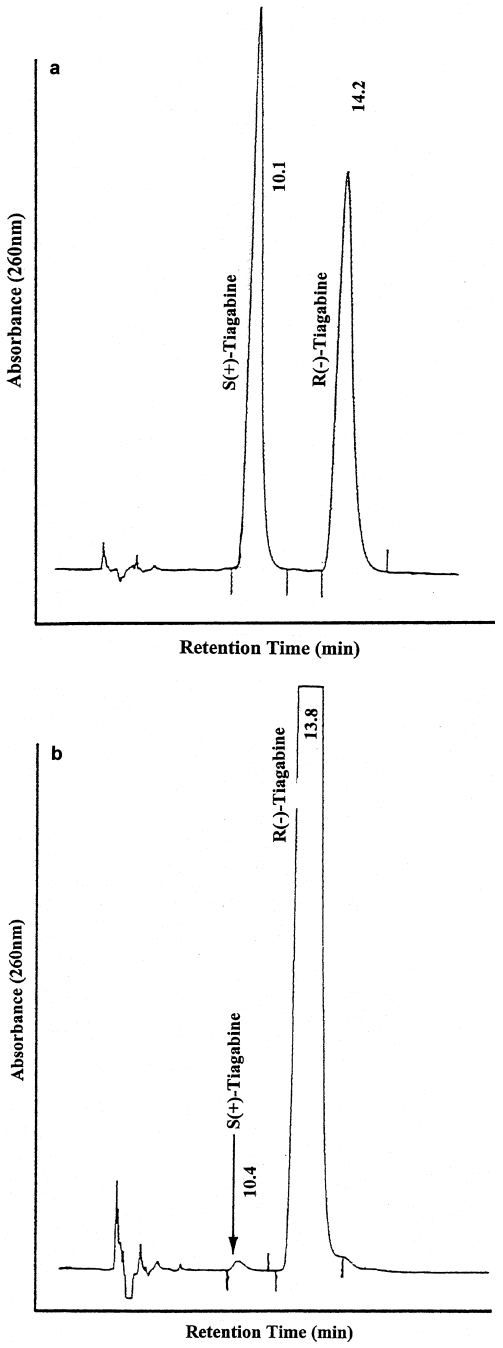

Fig. 2. (a) Typical chromatogram of a racemic (±) mixture of tiagabine·HCl using the column and mobile phase conditions of the final method described in this report. (b) Typical chromatogram of the *R*-(−)-enantiomer of tiagabine·HCl. Chromatographic conditions were the same as for (a).

Authentic reference materials of 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.

Various experiments were conducted to select stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. After the selection of the best stationary phase (Chiralcel-OD), experiments were conducted to obtain a mobile phase that would give optimum separation and sensitivity on the Chiralcel-OD column. The stationary phase of the Chiralcel-OD column is modified cellulose, agglomerated on silica (as the backbone). Therefore, the type (and amount) of solvent that can be used in the mobile phase on Chiralcel-OD column is limited. If solvents other than the solvents suggested by Diacel are used in the mobile phase, the column may lose selectivity and the back pressure may also increase (due to swelling of the stationary phase), which may lead to destruction of the column.

The selectivity and resolution of the two enantiomers varies with the choice of alcohol. For example, if methanol, 2-methyl-2-propanol or butanol is used in the mobile phase instead of ethanol and 2-propanol, the resolution between the two enantiomers of tiagabine·HCl is severely reduced. The presence of 2-propanol in the mobile phase greatly enhanced the resolution between the two enantiomers. The resolution between the *R*-(−)- and *S*-(+)-enantiomers of tiagabine·HCl is approximately two when 2-propanol is not present in the mobile phase. The resolution between the two enantiomers increases to ~3.4 when 2-propanol is added to the mobile phase. For tiagabine·HCl, in addition to the enhancement of resolution, the addition of 2-propanol to the mobile phase also increased the sharpness of both peaks and, hence, increased the chromatographic efficiency of the system.

The presence of trifluoroacetic acid in the mobile phase plays an important role in the retention time of the two enantiomers and also in improving the chromatographic efficiency and the separation. If trifluoroacetic acid is not present in the mobile phase, the two enantiomers would not elute from the column for at least 1 h. The addition of ~1 ml of trifluoroacetic acid to the mobile phase is adequate for the enantiomers to elute with normal (expected) retention times. However, the addition of more trifluoroacetic acid to the mobile phase improves the shape of the peaks, with little or no significant change in the retention times. This phenomena levels off after adding 5 ml of trifluoroacetic acid to the mobile phase. Therefore, the sharper peaks of the analyte in the presence of trifluoroacetic acid are most likely due to suppression of the secondary interactions between the active sites of the analyte and the stationary phase. Two other acids, acetic acid and formic acid, were also tested. The presence of formic acid and acetic acid in the mobile phase did not yield any separation between the two enantiomers of tiagabine·HCl. Both of these acids were also found to be less effective than trifluoroacetic acid in terms of improving the peak shape and decreasing the peak widths of the two enantiomers. This experiment was conducted using pure *R*-(−)- and *S*-(+) standards of tiagabine·HCl, because the retention times of both enantiomers were identical on a Chiralcel-OD column when trifluoroacetic acid (in the mobile phase) was replaced by acetic acid or formic acid.

The separation of two enantiomers on a chiral stationary phase can only take place if one enantiomer, on a time-average basis, has a stronger interaction than that of 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. 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. The retention time of the *S*-(+)-enantiomer (undesired isomer) was less than the retention time of the *R*-(−)-enantiomer under the chromatographic conditions used in this method. Therefore, this method is ideal for trace analysis of the *S*-(+)-enantiomer in tiagabine·HCl samples.

Columns packed with phenylglycine, phenylalanine (Pirkle type) and β-cyclodextrin were also investigated (both under normal- and reversed-phase conditions) for the enantiomeric separation of

tiagabine·HCl. Solvents such as hexane, methylene chloride, chloroform, ethylacetate, isopropanol, ethanol and trifluoroacetic acid were used in various combinations to obtain retention times from 6 to 80 min. For reversed-phase conditions, various ratios of 0.01 *M* phosphate and acetate buffers at different pH values (2.2 to 7.5) with different percentages of organic modifier (such as methanol, acetonitrile and isopropanol) were used to obtain retention times ranging from 7 to 60 min. There was no indication of enantiomeric separation from any of the above experiments when tiagabine·HCl was injected directly into the HPLC system without any derivatization.

Bovine serum albumin (BSA) and α-glycoprotein (AGP) columns were also investigated using mobile phases that had different percentages of isopropanol (1 to 10%) in 0.01 *M* aqueous phosphate and acetate buffers. The pH of the mobile phase was varied from three to seven and the retention time of the analyte ranged from 12 to 25 min. Both of these columns were able to separate the two enantiomers. However, reproducibility of the resolution and selectivity (of the two enantiomers) from column-to-column (different lots) and day-to-day varied widely. The *S*-(+)-enantiomer (unwanted isomer) of tiagabine·HCl eluted after the *R*-(−)-enantiomer on both of the protein columns under the various mobile phase conditions investigated during method development. Therefore, these columns (protein) were not suitable for trace analysis of the undesired enantiomer in the samples of tiagabine·HCl, given that the resolution and chromatographic efficiency were poor to start with.

Chiralcel-OJ and OG columns were also investigated for use in the enantiomeric separation of tiagabine·HCl. 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 8 to 45 min. The Chiralcel-OJ column showed some indication of enantiomeric separation (resolution ~0.4) when a mobile phase of hexane–isopropanol–ethanol–trifluoroacetic acid (~90:6:4, v/v/v) and 0.1% trifluoroacetic acid was used. However, the resolution and selectivity of the analyte did not improve to any significant extent when the ratios of the solvents were varied widely. The Chiralcel-OG column did not show resolution under any of the mobile-phase conditions used during this investigation.

The column-to-column reproducibility of the separation between the two enantiomers of the analyte was tested by using five Chiralcel-OD columns from different lots. The separation was found to be extremely reproducible on columns with different lot numbers. However, conditioning of the new column with the mobile phase (for two of the five columns) was required to achieve reproducibility. Typically, 1 l of the mobile phase, at a flow-rate of approximately 0.5 ml/min, was required to condition a new column.

Standard addition and recovery experiments were conducted 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 0.05 to 1.0%. Recovery of the *S*-(+)-enantiomer averaged 99.2, with an R.S.D. value of 1.1%. The data for the standard addition and recovery experiments are summarized in Table 1.

Table 1
Standard addition and recovery data for the *S*-(+)-enantiomer of tiagabine·HCl

Added (%)	Found (%)	Net found[a] (%)	Recovery (%)
0.0	0.143	–	–
0.046	0.190	0.047	101.1
0.093	0.235	0.092	98.9
0.303	0.442	0.299	98.7
0.606	0.744	0.601	98.2
1.01	1.136	0.993	98.3
Mean	99.2		
Standard deviation	±1.1		
Relative standard deviation	1.1		

[a]Net = % found !0.143%

The precision and short-term ruggedness of the method were also determined by analysing seven independent sample preparations, by two analysts on the same day on two columns from different lots. The samples were analyzed using two different instruments, and different mobile phases and reagents. The precision (R.S.D.) of the method is 3.2%. The results of this experiment are presented in Table 2. Fig. 2b is a typical sample chromatogram of tiagabine·HCl containing approximately 0.05% of the *S*-(+)-enantiomer.

The Chiralcel-OD column that was used to separate the two enantiomers was found to be very stable under the mobile-phase conditions used in the final method. The efficiency, selectivity, resolution and other chromatographic properties of the Chiralcel-OD column did not show any significant change after approximately 1000 sample injections when the following maintenance procedure was used. On several occasions, the resolution and efficiency of the column deteriorated after 50 to 100 injections of the sample. The column was easily regenerated to its original activity simply by washing it with approximately 200 ml of 2-propanol–ethanol–hexane (70:20:10, v/v/v) at a flow-rate of 0.2 ml/min. After washing, the column was reconditioned with approximately 200 ml of the mobile phase. The occasional deterioration of the column's performance was probably due to the presence of some unknown impurities in the samples, which had accumulated on the top of the column with time. This method was also used to analyze the in-process samples. The in-process samples at the R&D stage of any project usually contain high level of impurities of unknown character and vary 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 column (performance) deterioration.

Table 3
Chiral stability of tiagabine·HCl in sample preparations (solutions)

Sample no.	% of *S*-(+)-enantiomer (peak area)	
	Day 0	Day 4
1	0.147	0.149
2	0.143	0.142
3	0.142	0.141
4	0.154	0.140
Mean	0.147	0.143
Standard deviation	±0.0054	±0.0036
Relative standard deviation (%)	3.7	2.5

The possibility of potential chiral-inversion of tiagabine·HCl in the sample preparation was also investigated. Four independent samples of tiagabine·HCl were prepared, at approximately 2 mg/ml each. The samples were analyzed on Day zero, then stored at room temperature and darkness for approximately four days before being reanalyzed. The results of this study are summarized in Table 3. Inspection of the data in Table 3 reveal that tiagabine·HCl is chirally stable in the sample solution for at least four days, when stored in darkness and at room temperature. The results obtained for the samples between zero and four days have overlapping ranges, and their means agree within the normal variability of the procedure.

Table 2
Precision data for the analysis of the *S*-(+)-enantiomer present in the *R*-(−)-enantiomer of tiagabine·HCl

Analyst	Sample preparation no.	% *S*-(+)-enantiomer (peak area)
1	1	0.118
1	2	0.125
1	3	0.127
1	4	0.125
2	1	0.126
2	2	0.123
2	3	0.131
Mean		0.125
Standard deviation		±0.004
Relative standard deviation		3.2

Chromatographic interference of the ethyl ester (penultimate product) of tiagabine·HCl with the analyte was also investigated by using the mobile-phase conditions described in Section 2. Fig. 3 shows a chromatogram of the ethyl ester of tiagabine·HCl. Under the mobile phase and stationary phase conditions of this method, the ethyl ester of tiagabine·HCl has a retention time that is much shorter than the retention times of the two enantiomers of tiagabine·HCl. The *S*-(+)- and *R*-(−)-enantiomers of the ethyl ester of tiagabine·HCl eluted with the same retention times under the mobile phase and stationary phase conditions used in this method. No further investigation was conducted to determine if the two enantiomers would separate on the Chiralcel-OD column under different mobile phase (solvent ratios) conditions, which would give longer retention times for the two enantiomers.

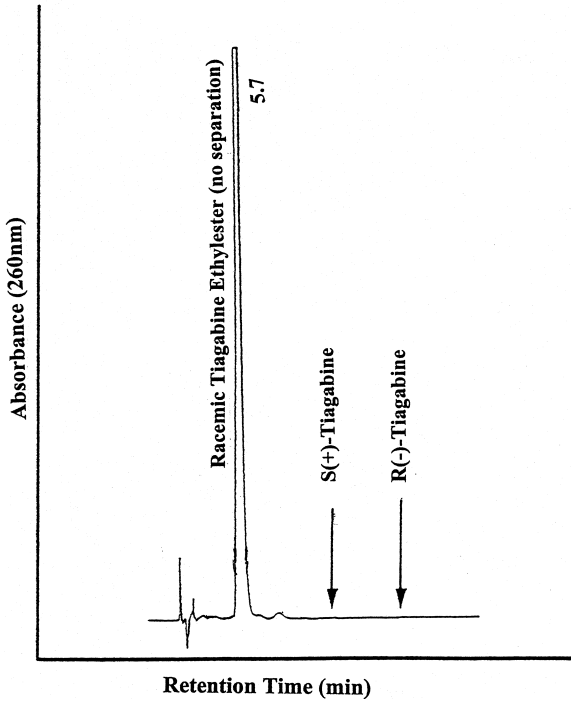

Fig. 3. Typical chromatogram of a sample of tiagabine ethyl ester·HCl. Chromatographic conditions were the same as those of Fig. 2a.

4. Conclusion

An analytical method was required to determine the presence of the *S*-(+)-enantiomer in the bulk drug of tiagabine·HCl in order to assure the chiral purity of the final product. The normal-phase HPLC method described in this paper for the separation of the two enantiomers of the analyte is simple, reproducible and relatively sensitive. The separation of the two enantiomers was reproducible on different columns obtained from different lots. This method is also ideal for the trace analysis of the *S*-(+)-enantiomer because the elution time of the *R*-(−)-enantiomer elution is greater than that of the unwanted enantiomer.

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