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Determination of chiral purity of ethyl nipecotate using a Chiralcel-OG column

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Abstract

The *R*-(−)- and *S*-(+)-enantiomers of ethyl nipecotate tartaric acid salt were separated by chiral high performance liquid chromatography on a commercially available chiral stationary phase using a non-polar mobile phase. Samples of ethyl nipecotate tartaric acid salt were analysed on a Chiralcel-OG column as the free base of ethyl nipecotate after extraction. The mobile phase was hexane–2-propanol–2-methyl-2-propanol (94:4:2, v/v/v), to which ca. 0.5 ml/l of dimethylamine was added. The method is able to separate the two enantiomers with a resolution factor (R_s) of approximately 1.3 and a selectivity factor (α) of 1.15. The limit of quantification of the *S*-(+)-enantiomer is 0.2% in the *R*-(−)-enantiomer. The method was validated by the standard addition method and determining the recovery of the *S*-(+)-enantiomer in the *R*-(−)-enantiomer. The precision of the method was determined by analysing seven individual sample preparations. The analysis was done by two analysts on different days, using different equipment and reagents.

1. Introduction

In recent years there has been renewed interest in the synthesis of pure enantiomers, specifically because of the increasing awareness of the importance of optical purity in the context of biological activity. The active enantiomer is called the eutomer and the undesirable enantiomer the distomer. Some distomers inhibit the biological activity of the eutomer and sometimes even exhibit severe adverse effects [1–4]. In the last decade, interest in the stereochemical aspects of drug development has intensified because of the more stringent regulations for the marketing of optically active compounds by the US Food and Drug Administration (FDA) and other regulatory agencies [5,6].

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used for the direct and indirect separation and determination of the optical isomers of pharmacologically active compounds [7–14]. The separation of enantiomers by GC or HPLC is one of the fastest growing fields in the area of separation technology. Numerous theoretical and experimental studies have been conducted by the early pioneers of chiral chromatography in order to understand the mechanism of the separation of enantiomers on chiral stationary phases [7,9,15,16]. Indirect separations of enantiomers have also been performed by derivatization of the optically active compounds with pure optically active reagents, forming diastereoisomers [17]. All commercially available chiral stationary phases have been classified by Wainer [18,19] according to the mechanism of separation of

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chiral compounds having different functional groups. Simple, reproducible and sensitive analytical methods are required to determine, and hence control, the chiral purity of the starting materials or intermediates to ensure the desired chiral purity of the optically active drug.

Ethyl nipecotate is a cyclic β-amino acid derivative used as the starting chiral material in the synthesis of tiagabine · HCl. The structures of the free base and tartaric acid salt of ethyl nipecotate are shown in Fig. 1. Tiagabine · HCl is being developed as an antiepileptic/anticonvulsive agent and is currently in phase III clinical trials. The drug is synthesized in pure *R*-(−)-enantiomeric form because it is pharmacologically more potent than the *S*-(+)-enantiomer. However, the toxicological characteristics of the two enantiomers were found to be comparable. As the drug is synthesized and being developed in its *R*-(−)-enantiomeric form, it is critical to control the chiral purity of ethyl nipecotate in order to achieve the desired chiral purity of the final product. Ethyl nipecotate undergoes three steps/substeps in the synthesis of tiagabine · HCl prior to the isolation of the final product, and racemization of ethyl nipecotate (or its derivative) occurs in each of the three steps of synthesis. Therefore, using ethyl nipecotate with a very low *S*-(+)-enantiomer will yield the final product with good chiral purity. From historical data, it has been found that the final compound contains less than 0.5% of the *S*-(+)-enantiomer when the ethyl nipecotate used in the synthesis contained less than 0.2% of the *S*-(+)-enantiomer. Several commercially available columns were investigated during method development using both normal- and reversed-phase modes to achieve optimum resolution and sensitivity for the two enantiomers.

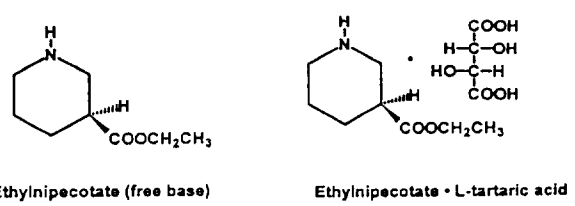

Fig. 1. Structure of ethyl nipecotate free base and ethyl nipecotate tartaric acid salt.

2. Experimental

2.1. Equipment

An HPLC solvent-delivery system (SP 8800) equipped with an injector/autosampler (SP 8780), an integrator (SP4270) and a variable-wavelength UV–visible detector (SP 8450) was used (Spectra-Physics, San Jose, CA, USA). A 25 cm × 4.6 cm I.D., Chiralcel-OG column was used in the method finally developed (Daicel Chemical). The other chiral stationary phase columns investigated were 25 cm × 4.6 mm I.D., 5 μm D-phenylglycine (Regis Chemical, Morton Grove, IL, USA), 25 cm × 4.6 mm I.D., 5 μm Cyclobond-1 β-cyclodextrin (Rainin Instrument, 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 Chemical) and 15 cm × 7.5 mm I.D., 10 μm bovine serum albumin column (manufactured by Machery–Nagel, purchased from Alltech Associates, Deerfield, IL, USA).

2.2. Materials

HPLC-grade hexane, 2-propanol, ethyl acetate and anhydrous sodium carbonate were purchased from Fisher Scientific (Fairlawn, NJ, USA), diethylamine (analytical-reagent grade) and 2-methyl-2-propanol from Aldrich (Milwaukee, WI, USA) and a racemic mixture of *R*-(−)- and *S*-(+)-enantiomers of ethyl nipecotate from Abbott Labs. (North Chicago, IL, USA). Borosilicate scintillation vials and disposable pipettes were obtained from Baxter Scientific (Waukegan, IL, USA).

2.3. Preparation of sample

Approximately 100 mg of the samples were weighed and transferred into a scintillation vial and dissolved in 5 ml of distilled water. About 100 mg of anhydrous sodium carbonate were

added to the aqueous solution of the sample and vortex mixed for about 5 min, then ca. 5 ml of ethyl acetate were added and vortexed for about 2 minutes. The solution was allowed to settle into two layers and the ethyl acetate layer was transferred into a fresh scintillation vial with a disposable pasteur pipette. The ethyl acetate was evaporated to dryness under an air stream. The residue was reconstituted in ca. 10 ml of mobile phase and injected directly into the HPLC system.

2.4. Preparation of mobile phase

To 940 ml of hexane, 40 ml of 2-propanol, 20 ml of 2-methyl-2-propanol and 0.5 ml of diethylamine were added and mixed. This mobile phase mixture was degassed for ca. 5 min and used for analysis.

2.5. Chromatographic conditions

The final chromatographic conditions adopted were as follows: the mobile phase flow-rate was 0.8 ml/min, the samples were monitored with a UV detector at 230 nm and 0.10 AUFS and 10 μl of the sample solution were injected into the HPLC.

2.6. Calculation

Quantification of the *S*-(+)-enantiomer was based on peak-area measurement and the following equation was used for calculation:

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

2.7. Limit of quantification

Samples of *R*-(−)-ethyl nipecotate were analysed to determine the lowest level of the *S*-(+)-enantiomer that can be determined with good reproducibility (R.S.D. less than 10%). The limit of quantification (LOQ) of the method for the *S*-(+)-enantiomer was about 0.2% at a signal-to-noise ratio of 3.

3. Results and discussion

Injection of the blank (mobile phase) into the HPLC system showed no peak eluting with the same retention times as those of the *S*-(+)- and *R*-(−)-enantiomers. Fig. 2 is a typical chromatogram of the racemic mixture of ethyl nipecotate and shows that the peaks of the *S*-(+)- and *R*-(−)-enantiomers are adequately resolved from each other. The selectivity factor (enantiomeric selectivity) of the two optical isomers was 1.15. However, the resolution between the two enantiomers was ca. 1.3.

The amount of the *S*-(+)-enantiomer present in the *R*-(−)-enantiomer was determined on the basis of peak areas. The response of the UV detector at 230 nm was linear from 0.10 to 1.0 mg/ml for 10-μl injections. A typical linear regression equation for the analyte has a correlation coefficient of >0.999 and essentially passed through the origin.

Authentic reference materials of the pure *S*-(+)- and *R*-(−)-enantiomers were used to de-

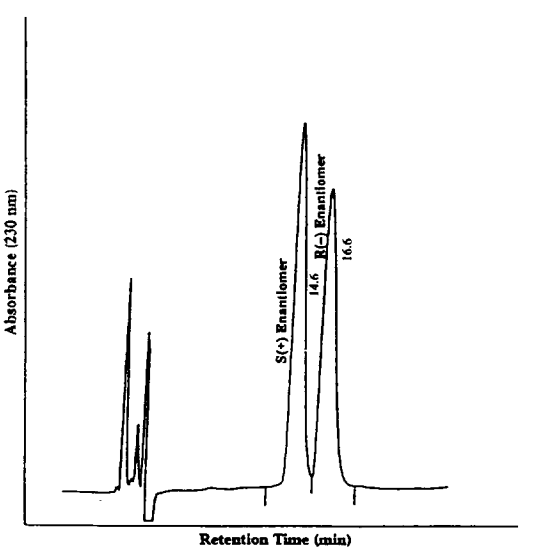

Fig. 2. Typical chromatogram of racemic mixture of ethyl nipecotate using the mobile phase described in the text.

termine the relative retention volumes and the elution order of the two enantiomers. Under the chromatographic conditions described here, the *S*-(+)-enantiomer eluted before the *R*-(−)-enantiomer. Because the *S*-(+)-form is not the enantiomer of interest, the 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 of ethyl nipecotate.

The tartaric acid salt of ethyl nipecotate is used directly in the synthesis of tiagabine · HCl. Ethyl nipecotate tartaric acid salt is not soluble in the mobile phase described here. Therefore, extraction of ethyl nipecotate free base (which is soluble in the mobile phase) was necessary prior to injection of the sample into the HPLC system. The extraction of ethyl nipecotate free base was also necessary because the tartaric acid (if injected onto the chiral column) deteriorates the chiral selectivity of ethyl nipecotate and changes the chiral properties of the column. Occasional deterioration of the column properties such as selectivity factor and efficiency, observed after the injection of certain samples, may also be related to tartaric acid accumulated on the column during sample analysis. Typically, the column performance was regained after cleaning, using the procedure described below.

Experiments were conducted to obtain a mobile phase that will give optimum resolution and selectivity for the two enantiomers on the Chiralcel-OG column. The chiral stationary phase of the Chiralcel-OG column is the methylphenyl carbamate of cellulose, which is agglomerated on silica by a proprietary technique. The types and amounts of solvents that can be used in the mobile phase without damaging the chiral stationary phase of the Chiralcel-OG column are limited. Typical mobile phases are mixtures of hexane or heptane with 2-propanol (typically 10%, v/v). Other solvents such as diethylamine (<0.5%), *tert.*-butanol, 1-octanol and other long-chain alcohols can be used in trace concentrations (<1%) to improve the selectivity, resolution or efficiency of the chromatography. It was also observed that 10% ethanol with 30% 2-propanol in hexane does not deteriorate the chromatographic properties of the stationary phase of the Chiralcel-OG column.

The selectivity for and resolution of the two enantiomers of ethyl nipecotate varies with the type and amount of alcohols present in the mobile phase. Therefore, the ratios of the two alcohols in the mobile phase needs careful adjustment in order to achieve optimum resolution and selectivity. 2-Methyl-2-propanol increases the resolution of the two enantiomers from less than 1 to 1.5. The absence of 2-propanol in the mobile phase has a significant effect on the resolution of the two enantiomers. In the absence of 2-propanol, the resolution factor (R_s) of the two enantiomers remains less than 1 when the concentration of 2-methyl-2-propanol in the mobile phase varies from 0.5 to 15%. On the other hand, the selectivity factor (α) for the two enantiomers remains less than 1.1 when 2-methyl-2-propanol is not present in the mobile phase. Therefore, the optimum resolution and selectivity were obtained by using a combination of the two alcohols in the mobile phase. Experiments were conducted to determine the effect of 2-propanol and 2-methyl-2-propanol in the mobile phase on α and R_s for the two enantiomers of ethyl nipecotate by varying the percentage of one alcohol in the mobile phase and keeping the percentage of the second alcohol constant. The results of these experiments are summarized in Tables 1 and 2.

The presence of diethylamine (in a trace amount) in the mobile phase is critical to obtaining the desired resolution and chromatographic

Table 1
Effect of 2-propanol in the mobile phase on chiral selectivity (α) and resolution (R_s) of ethyl nipecotate

2-Propanol (%)[a]	α	R_s
0.5	1.23	0.45
1.0	1.18	0.89
2.0	1.16	1.10
4.0	1.14	1.28
6.0	1.10	1.37
8.0	1.06	1.58

[a] The content of 2-methyl-2-propanol in the mobile phase was kept constant at 2% (v/v).

Table 2
Effect of 2-methyl-2-propanol in the mobile phase on chiral selectivity (α) and resolution (R_s) of ethyl nipecotate

2-Methyl-2-propanol (%)[a]	α	R_s
0.5	1.31	0.80
1.0	1.27	1.11
2.0	1.21	1.33
5.0	1.16	1.42
7.0	1.08	1.53
9.0	1.01	1.62

[a] The content of 2-propanol in the mobile phase was kept constant at 4% (v/v).

efficiency. Two other amines, *N*,*N*-dimethyloctalamine and triethylamine, were also tested. Both of these amines were found to be less effective than diethylamine in decreasing the band broadening of the two enantiomers and enhancing the chromatography efficiency. It was also found that the chiral selectivity of the Chiralcel-OG column for ethyl nipecotate changed permanently when *N*,*N*-dimethyloctylamine was used in the mobile phase. The resolution of the two enantiomers was less than 1 when a Chiralcel-OC column treated with *N*,*N*-dimethyloctylamine was used for analysis. This finding demonstrates that one has to be careful in trying various trace solvent modifiers in the mobile phase during method development using the Chiralcel-OG or similar types of chiral stationary phases. Typically, 500 ml of the mobile are needed to condition a new column in order to obtain reproducible chromatographic results.

Columns packed with different chiral stationary phases were also investigated for the enantiomeric separation of ethyl nipecotate. Pirkle-type columns, such as phenylglycine and phenylalanine, and also a β-cyclodextrin column were investigated under both normal- and reversed-phase mobile phase conditions for the separation of the two enantiomers of ethyl nipecotate. For normal-phase conditions, hexane, 2-propanol, ethanol, 0.1% trifluoroacetic acid of diethylamine were used as solvents in various proportions in the mobile phase to obtain retention times from 6 to 25 min. For reversed-phase conditions, various ratios of 0.01 *M* phosphate or perchlorate buffers at different pH (2.2–7.5) with different percentages of an organic modifier such as acetonitrile, methanol or 2-propanol were used to obtain retention times ranging from 6 to 20 min. No indication of enantiomeric separation was obtained from all the experiments described above.

Protein columns such as bovine serum albumin (BSA) and α-glycoprotein (AGP-1) were also investigated using different percentages of 2-propanol (BSA column) and methanol–acetonitrile (AGP column) with 0.01 *M* aqueous phosphate buffer (pH $\approx$ 3.5–7.2). The retention time of ethyl nipecotate varied from 7 t o 20 min. These columns also gave no indication of enantiomeric separation under any of the mobile phase conditions used.

Other derivatized cellulose chiral columns such as Chiralcel-OD and Chiralcel-OJ were also investigated for the enantiomeric separation of ethyl nipecotate. Solvents such as hexane, 2-propanol, 2-methyl-2-propanol and ethanol with trace amounts (<0.1%) of trifluoroacetic acid and diethylamine were used in the mobile phase at various solvent strengths. The Chiralcel-OD column showed some indication of enantiomeric separation when a mobile phase of hexane–2-propanol (90:10) containing 0.1% trifluoroacetic acid was used. The Chiralcel-OJ column also showed some indication of chiral separation when a mobile phase of hexane–2-methyl-2-propanol–2-propanol, 94:4:2 containing 0.1% trifluoroacetic acid was used. However, the resolution of and selectivity for the analyte did not improve significantly when the ratios of the solvents (for both the columns) were varied to extreme solvent strengths. The selectivity factors (α) for the Chiralcel-OD and Chiralcel-OJ columns obtained from these experiments were 1.07 and 1.03, respectively.

Chiralcel-OG columns from different lots were tested for column-to-column reproducibility for the enantiomers of ethyl nipecotate. The resolution and chiral selectivity for the two enantiomers of ethyl nipecotate were found to be reproducible on columns having different lot numbers. However, conditioning of the new

column with an appropriate amount of mobile phase (typically 500 ml) was required to achieve satisfactory reproducibility.

Standard addition and recovery experiments were conducted to determine the accuracy of the method for the determination of the *S*-(+)-enantiomer present in the *R*-(−)-enantiomer of ethyl nipecotate. The levels of addition were approximately 2.5–25%. The recovery of the *S*-(+)-enantiomer averaged 100.5% with an R.S.D. of 0.8%. The data for the standard addition and recovery experiments are summarized in Table 3. The mean α value obtained from the chromatograms of the standard addition and recovery experiments was 1.14 ± 0.06 ($n = 6$, R.S.D. $\approx 5\%$).

The precision and short-term ruggedness were also determined by two analysts using a sample of *R*-(−)-ethyl nipecotate containing a small amount of the *S*-(+)-enantiomer. Two samples of *R*-(−)-ethyl nipecotate were prepared by each of the two analysts. The samples were analysed on two different instruments and columns and on different days. The precision of the method was found to be 7.6% (R.S.D.) at an *S*-(+)-enantiomer level of ca. 1% in *R*-(−)-ethyl nipecotate. The data from these experiments are summarized in Table 4. Fig. 3 shows a typical chromatogram for *R*-(−)-ethyl nipecotate containing ca. 1.5% of the *S*-(+)-enantiomer.

The limit of quantification of the *S*-(+)-enantiomer of ethyl nipecotate was determined by injecting samples containing trace levels of the *S*-(+)-enantiomer. The limit of quantification was ca. 0.2% of the peak area of the *R*-(−)-enantiomer at a signal-to-noise ratio of 3.

The Chiralcel-OG column used in the method to separate the two enantiomers of ethyl nipecotate was reasonably stable in terms of selectivity, efficiency, resolution and other chromatographic

Table 3
Standard addition and recovery of the *S*-(+)-enantiomer in the *R*-(−)-enantiomer of ethyl nipecotate tartaric acid salt

(*S*)-(+)-ENP[a] added (%, w/w)	(*S*)-(+)-ENP[a] found (%, w/w)	Recovery (%)
2.4	2.4	100.0
11.3	11.3	100.0
13.6	13.6	100.0
16.0	16.0	100.0
18.1	18.4	101.7
23.8	24.1	101.3
		Mean 100.5
		S.D. 0.8
		R.S.D. 0.8%

[a] ENP = Ethyl nipecotate.

Table 4
Precision data for the determination of the *S*-(+)-enantiomer in the *R*-(−)-enantiomer of ethyl nipecotate by different analysts

Analyst No.	Peak-area percentage of *S*-(+)-enantiomer
1	0.94
1	1.06
1	0.88
1	1.00
2	0.86
2	0.92
2	0.90
	Mean 0.94
	S.D. 0.071
	R.S.D. 7.6%

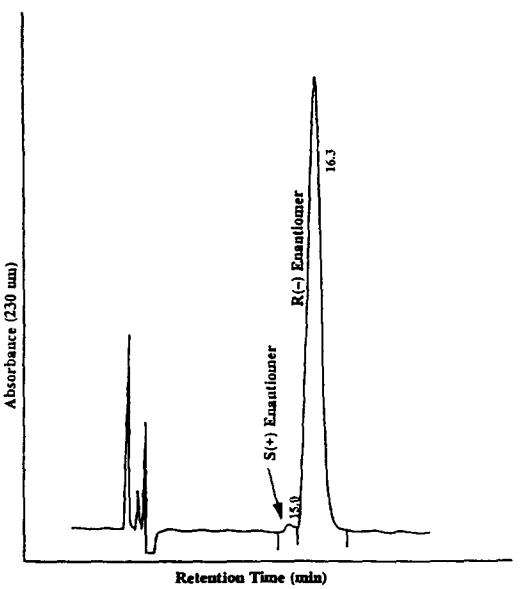

Fig. 3. Typical chromatogram of the *R*-(−)-enantiomer of ethyl nipecotate using the same mobile phase in Fig. 2.

properties. The Chiralcel-OG column did not show any significant change in chromatographic properties after ca. 400 sample injections with the following maintenance procedure applied. On at least two occasions, the selectivity and resolution deteriorated after 60–70 injections of the sample. The column was easily regenerated to its initial chromatographic efficiency, selectivity and resolution simply by washing it with ca. 100 ml of hexane–2-propanol–ethanol (60:30:10, v/v/v) at a flow-rate of 0.3 ml/min. After the cleaning, the column was reconditioned with ca. 100 ml of the mobile phase. The occasional deterioration of the chromatographic properties of the column was probably due to the accumulation of tartaric acid and some unknown impurities from the samples on the column. This column and method were also used to analyse in-process samples that typically contain multiple minor components.

4. Conclusions

Ethyl nipecotate has one chiral centre and is the starting raw material in the synthesis of tiagabine · HCl, which is being developed as an antiepileptic agent. Therefore, it is critical to control the presence of the undesired enantiomer in order to ensure the required chiral purity of the final product. The method described in this paper is rugged, reproducible and capable of providing the chiral purity information necessary to manufacture the final product with the desired quality. The separation of the two enantiomers of ethyl nipecotate was reproducible on five different columns from five different batches.

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