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Reversed-phase high-performance liquid chromatographic separation of the enantiomers of N-[4,4-di(3-methylthien-2-yl)-but-3-enyl] nipecotic acid on a Pirkle-type phenylglycine stationary phase

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SUMMARY

The enantiomers of N-[4,4-di(3-methylthien-2-yl)-but-3-enyl] nipecotic acid hydrochloride are separated by reversed-phase high-performance liquid chromatography on a commercially available chiral stationary phase. Prior to chromatography, the chiral compound was derivatized into an amide by reacting it with an achiral amine. The amide derivative of the *S*- and *R*-enantiomers were baseline resolved on a commercially available Pirkle-type L- or D-phenylglycine chiral stationary phase with a selectivity factor (α) of 1.2. The limit of detection of the *S*-enantiomer is less than 0.03% in the *R*-enantiomer. The derivatized enantiomers were monitored by UV at 260 nm and 0.20–0.01 a.u.f.s. Several other commercially available chiral stationary phases were also investigated to achieve optimum sensitivity and separation of the enantiomers of the chiral compound.

INTRODUCTION

The enantiomers of different drugs which have one or multiple asymmetric center(s), may differ widely in their pharmacodynamic and toxicological properties. To establish the enantiomeric purity of chiral drugs and to determine the chiral selectivity of the synthesis, simple, sensitive, and reproducible analytical methods are required.

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used to separate and quantify the enantiomers in a mixture[1–9]. Numerous theoretical studies on the chiral stationary phases of HPLC and chiral derivatizing reagents have been conducted to understand the separation mechanisms of the enantiomers[10–15]. The results of these studies aided scientists for better understanding and optimization of the separation parameters. Wainer[16,17] has classified chiral stationary phases according to their mode of separation mechanism of chiral compounds which have different functional groups. Gal[18] has conducted some useful

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studies on the formation of diastereoisomers with optically pure reagents and subsequent chromatographic separations. Because of the availability of the technology, the United States Food and Drug Administration (U.S. FDA) has more stringent regulations for the marketing of drugs which have (an) optically active center(s)[19]. Therefore, an analytical method is required to study the pharmacokinetic behavior and other pharmacological activities of each of the pure enantiomers of a racemic drug to satisfy the requirements of the U.S. FDA.

In this work, D- and L-phenylglycine chiral stationary phases (CSPs) were used to separate and quantify the enantiomers of N-[4,4-di(3-methylthien-2-yl)-but-3-enyl] nipecotic acid hydrochloride. The chemical structure of the chiral compound is shown in Fig. 1. This compound has potential therapeutic use as an antiepileptic agent. The *R*-enantiomer has been found to be therapeutically more active than the *S*-enantiomer. An analytical method is required to determine the purity of the pharmacologically active enantiomer in order to obtain pharmacological, toxicological, and metabolic fate data of this potential drug candidate.

In this study, several chiral stationary phases were investigated in normal-phase and reversed-phase modes to obtain maximum resolution and sensitivity of the enantiomers.

EXPERIMENTAL

Equipment

An HPLC solvent delivery system (SP8800) equipped with an injector/autosampler (SP8780), an integrator (SP4270), and a variable-wavelength UV–visible detector (SP8450) was used in the experiment (Spectra-Physics, San Jose, CA, U.S.A.). A 25 cm × 4.6 mm, Pirkle Covalent L- or D-phenylglycine Hi-Chrom 5-μm HPLC column was used in the method finally developed (Regis, Morton Grove, IL, U.S.A.). The other CSP columns investigated in this experiment were 5-μm, 25 cm × 4.6 mm D-phenylalanine and 25 cm × 4.6 mm D-phenylglycine (Jones, Littleton, CO, U.S.A.), and a 25 cm × 4.6 mm, 5-μm Cyclobond-I (Rainin, Woburn, MA, U.S.A.). Deionized water was collected from a Milli-Q System (Millipore, Bedford, MA, U.S.A.). All quantitative (volume transfer) work was performed with an Eppendorf digital pipette (VWR, Chicago, IL, U.S.A.). A filter apparatus, 4.7 cm (Millipore, Milford, MA, U.S.A.) with nylon-66 membrane filter, 4.7 cm × 0.45 μm was used to filter the mobile phase (Alltech, Deerfield, IL, U.S.A.). Liquid chromatography–mass spectrometry (LC–MS) was performed on a Nermag Model R30-10 with Vestec thermospray interface (Nermag, France).

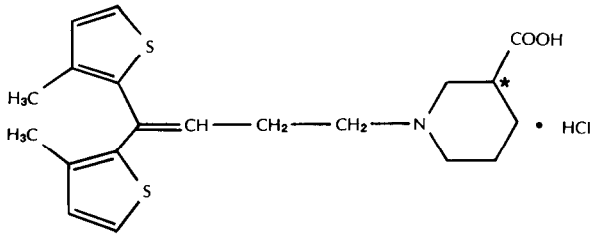

Fig. 1. Chemical structure of N-[4,4-di(3-methylthien-2-yl)-but-3-enyl] nipecotic acid hydrochloride; ★ indicates the chiral center.

Materials

HPLC-grade methanol, acetonitrile, glacial acetic acid (reagent grade), monobasic potassium phosphate and phosphoric acid were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Ammonium acetate, ACS grade, was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). The reagents 1-naphthalenemethylamine (NMA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl (EDC) and 1-hydroxybenzotriazole hydrate (HOBT) were purchased from Aldrich (Milwaukee, WI, U.S.A.). The trifluoroacetic acid was purchased from Mallinckrodt (Paris, KY, U.S.A.). The glass scintillation vials were purchased from American Scientific Products (McGraw Park, IL, U.S.A.). The chiral compound (drug candidate) in its *S*- and *R*-enantiomeric forms was synthesized at Abbott Labs. (North Chicago, IL, U.S.A.).

Derivatization of the chiral compound

Ca. 25 mg of *S*- or *R*-enantiomer or racemic mixture of the chiral compound were transferred into a 20-ml scintillation vial. *Ca.* 10 mg of HOBT, 20 mg of EDC and 8 ml of methylene chloride were taken in a scintillation vial containing the chiral compound. The mixture was sonicated until all the solid materials dissolved in the methylene chloride solution. *Ca.* 15 μl of NMA were added to the solution and sonicated again for another 10 min. The amide derivative of the parent compound is formed in this step which has the structural formula as shown in Fig. 2. At the completion of the reaction, some precipitate appeared and was removed by filtration through a filter paper. The precipitate was discarded and the filtrate was dried by evaporation with nitrogen. The dry residue was reconstituted into solution with 10 ml of the mobile phase. An aliquot (20–100 μl) of this solution was injected into the HPLC system.

Preparation of blank

A blank for the derivatization of the chiral compound was prepared by using the same procedure as described, except that no chiral compound was added in the reaction.

Chromatographic conditions of the method finally developed

The mobile phase consisted of 0.10 *M* ammonium acetate (pH 3.70±0.05)–acetonitrile (61:39, v/v). The pH of the ammonium acetate solution was adjusted with glacial acetic acid. The rate of mobile phase delivery through the HPLC system was 1.5 ml/min. The derivatized chiral compound was monitored by a UV–visible detec-

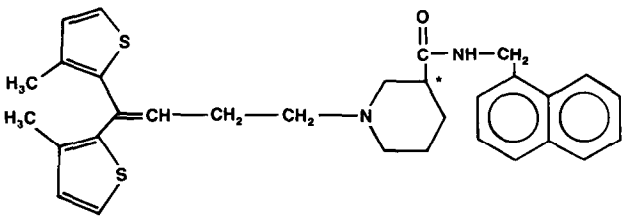

Fig. 2. Chemical structure of the amide derivative of N-[4,4-di(3-methylthien-2-yl)-but-3-enyl] nipecotic acid; ★ indicates the chiral center.

tor at 260 nm at a sensitivity of 0.20–0.01 a.u.f.s. The analytical column and the mobile phase were maintained at ambient temperature during analysis.

Calculation

Quantification of the *S*-enantiomer was done using the following equation:

$$\text{percent of } S\text{-enantiomer} = \frac{\text{peak area of derivatized } S\text{-enantiomer}}{\left(\begin{array}{c}\text{sum of the peak areas of derivatized} \\ R\text{- and } S\text{-enantiomers}\end{array}\right)} \times 100$$

Limit of quantification

The limit of quantification was determined by injecting the derivatized *R*-enantiomer with added amounts of the *S*-enantiomer. The peak of the derivatized *S*-enantiomer was clearly distinguished from the baseline noise when peak area percent of the *S*-enantiomer was 0.03% compared to the peak area of the *R*-enantiomer.

Test for interconversion of the enantiomers during derivatization reaction

The test for the interconversion of the enantiomers during the derivatization reaction was determined by taking the ratio of the weight of the *R*- and *S*-enantiomers, dividing it by the ratio of the peak areas of the *R*- and *S*-enantiomers and multiplying by 100.

RESULTS AND DISCUSSION

Fig. 3 is a typical chromatogram of the blank for the derivatization reaction of the chiral compound which shows that no chromatographic peak from the reagents elutes with the same retention time as those of the derivatized enantiomers. Fig. 4 is a typical chromatogram of the derivatized racemate of the chiral compound which shows that the peaks of derivatized *S*- and *R*-enantiomers are baseline resolved from each other.

The above chromatograms were obtained by using an L-phenylglycine column (Regis). The capacity factors (k') of derivatized *S*- and *R*-enantiomers were *ca.* 7.1 and 8.8 under the chromatographic conditions used in this experiment. The selectivity factor (α) for the derivatized enantiomers was 1.24. A D-phenylglycine column was also tested under identical conditions to those used for the L-phenylglycine column. The α values of the derivatized enantiomers (on D-phenylglycine) remained unchanged, except that the elution profiles of the enantiomers were reversed. This information can be used in trace analysis when the small peak of the trace enantiomer is overlapped by the huge peak of the bulk enantiomer which elutes before the trace enantiomer.

The quantification of the derivatized enantiomers was conducted by peak area percent. The response of the UV detector was linear to at least 30 μg of the derivatized *S*- or *R*-enantiomers. The retention times of the *S*- and *R*-enantiomers were determined by the individual injection of each pure enantiomer (derivatized) into the HPLC system. Complete evaporation of the methylene chloride after the derivatization reaction is very critical. If trace amounts of methylene chloride are present in the injected samples, the retention times of the enantiomers change abruptly. The abrupt

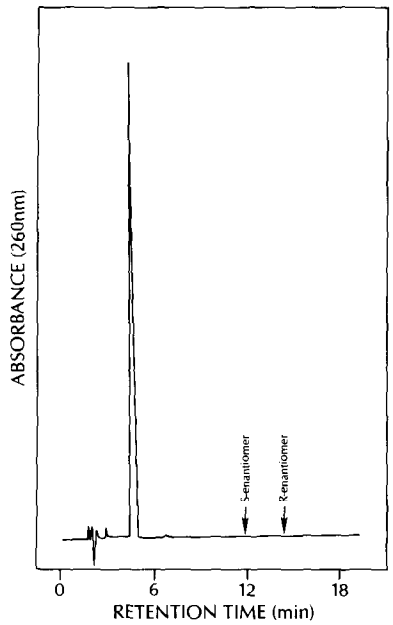

Fig. 3. Chromatogram of a blank for the derivatization reaction of the chiral compound. Column, 25 cm x 4.6 mm, 5 μm, L-phenylglycine. Detector, 260 nm, 0.10 a.u.f.s.

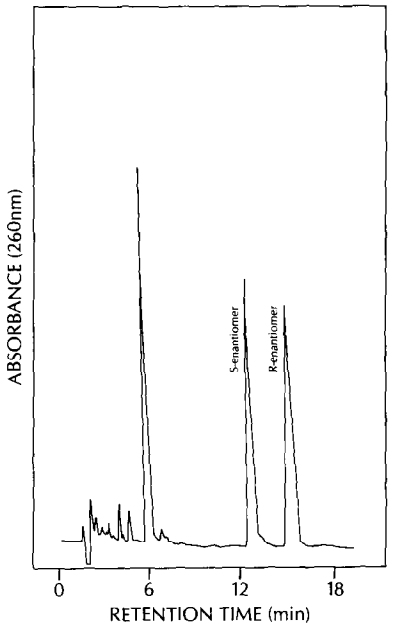

Fig. 4. Chromatogram of derivatized racemate of the chiral compound. Column, 25 cm x 4.6 mm, 5 μm L-phenylglycine. Detector, 260 nm, 0.10 a.u.f.s.

change in the retention times of the enantiomers due to the presence of methylene chloride, was confirmed by adding one or two drops of methylene chloride in the sample solution prior to injection into the HPLC.

An experiment was conducted to determine if the derivatization reaction of the enantiomers (to form an amide) is dependent on the percent of the *S*- or *R*-enantiomer present in the reaction mixture. Different ratios of the *S*- and *R*-enantiomers were taken into a container and derivatized with NMA. This experiment showed that the peak area ratio of the derivatized product is equal to the weight ratios of the starting (underivatized) *S*- and *R*-enantiomers. The results of this experiment have been summarized in Table I. The data indicate that the interconversion of the enantiomers during the derivatization reaction is practically nil. The results of this experiment also indicate that the extent of derivatization of the *S*- and *R*-enantiomers with NMA is independent of the amount of each enantiomer present in the reaction mixture.

The completion of the derivatization reaction was studied by injecting the derivatized *S*- or *R*-enantiomer into the HPLC system and monitoring the underivatized peak of the enantiomers in the chromatogram under the chromatographic conditions used in this experiment. The retention time of the underivatized peak of the *S*- and *R*-enantiomers were identical and *ca.* 8 min. No peak eluted with the same retention time as the underivatized enantiomers when the derivatized samples of the *S*- and *R*-enantiomers were injected into the HPLC system. This indicates that all the material (*S*- and *R*-enantiomers) used for derivatization formed the amide derivative by reacting with NMA. Therefore, it can be concluded that the derivatization reaction went into completion even when the ratios of the enantiomers in the reaction mixtures were different.

Identification of the derivatized *S*- and *R*-enantiomers (Fig. 4) of the chiral compound was conducted by LC–MS detection. Fig. 5 shows a total ion chromatogram of m/z 515. The peaks with retention times of *ca.* 14.3 and 17.5 min have $(M + 1)^+$ of 515. This corresponds to the molecular weight of the derivatized chiral compound which is 514, indicating that the two peaks are enantiomers having different retention times due to the different extents of chiral interaction with the CSP of the column.

To obtain further evidence for identification of the derivatized peaks in Fig. 4,

TABLE I

DATA ON THE INTERCONVERSION OF THE ENANTIOMERS DURING THE DERIVATIZATION REACTION

Weight ratio of S- and R-enantiomer (A)	*Peak area ratio of S- and R-enantiomer (B)*	*Ratio (A/B × 100)*
1.200	1.226	102.2
0.333	0.335	100.6
0.786	0.785	99.8
0.754	0.748	99.2
0.491	0.512	104.2
0.952	0.924	94.1

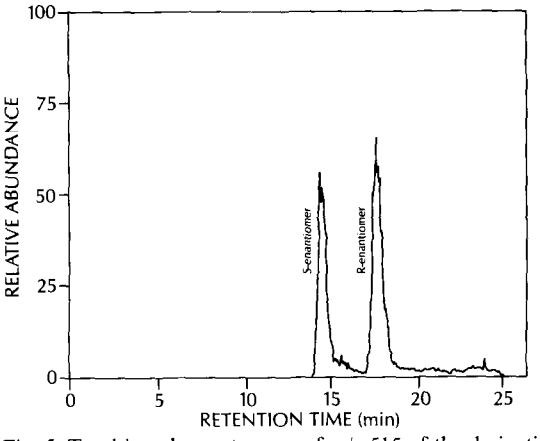

Fig. 5. Total ion chromatogram of m/z 515 of the derivatized chiral compound.

LC–MS analysis of the pure *S*-enantiomer and the *R*-enantiomer was conducted. Fig. 6A and B show the mass spectra (chemical ionization scan) of the derivatized *S*- and *R*-enantiomers. In both spectra, the main peak appeared with an m/z of 515 indicating that they have the same molecular weight, but are resolved on a CSP because of different spatial configurations (Figs. 4 and 5).

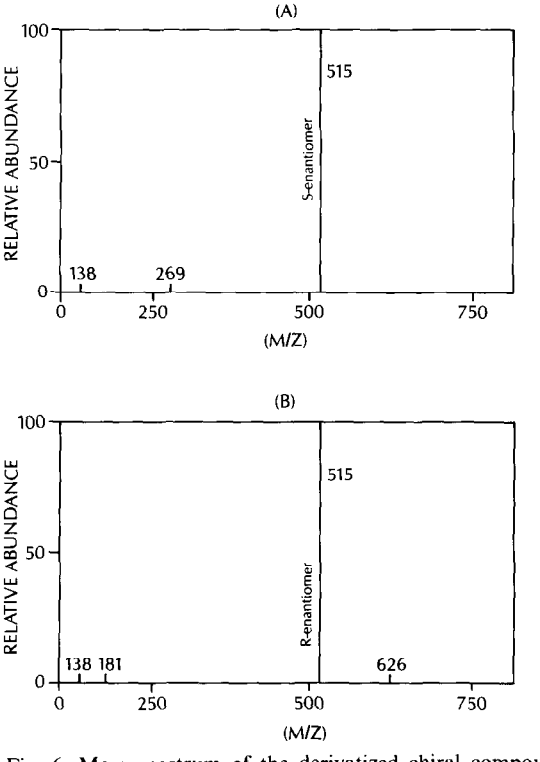

Fig. 6. Mass spectrum of the derivatized chiral compound, (A) m/z of *S*-enantiomer; (B) m/z of *R*-enantiomer.

Standard addition–recovery studies were conducted by two analysts to determine the accuracy of the method for the quantification of 0.05% (w/w) quantities of the *S*-enantiomer in the *R*-enantiomer. The recoveries of the derivatized *S*-enantiomer averaged 94.8% with 7.2% relative standard deviation (R.S.D.) over a range of addition levels from *ca.* 0.5% to 2.0%. The data from these experiments are summarized in Table II.

The precision of the method was also determined by two analysts using a 2–5 mg/ml sample of the derivatized *R*-enantiomer containing trace amounts of the *S*-enantiomer. The precision of the method is 9.0% R.S.D. The results are presented in Table III.

Derivatization of the acid functional group of the chiral compound into an amide (with NMA), was necessary to obtain the resolution of the enantiomers in both the reversed- and normal-phase modes for phenylglycine (D and L) columns. The underivatized chiral compound gave identical retention times (in the reversed- and normal-phase modes) for both the *S*- and *R*-enantiomers on all CSPs [except for bovine serum albumin (BSA)] investigated in this experiment. The identical retention times of the underivatized enantiomers indicate that no significant chiral interaction occurs during equilibration of the chiral compound with the CSP.

The separation of the enantiomers after derivatization may occur due to the deactivation of the strong acid group and also by the attachment of a naphthyl group in the chiral carbon of the molecule, which is rich in π-electrons. According to Pirkle and Hamper[20], as the ability of π-electron donation of a chiral molecule increases, the interaction of the molecule with the Pirkle type CSP also increases. The separation of the two enantiomers can only take place if one enantiomer on a time-average basis, has more interaction with the CSP than the other. The spatial configuration of the stationary phase determines which enantiomer should have more chiral interaction than the other, on a certain CSP.

The interesting part of this method is that the separation was achieved in reversed-phase mobile phase conditions. Most of the published literature shows that the separation of chiral compounds on phenylglycine-type of stationary phase takes place with solvents (mobile phase) having little or no hydrogen-bonding ability, because the

TABLE II

STANDARD ADDITION–RECOVERY DATA FOR *S*-ENANTIOMER

Analyst	*Baseline signal (A) (%)*	*Addition level (B) (%)*	*Theory (A+B) (%)*	*Found (C) (%)*	*Recovery [C/(A + B)] (%)*
1	0.046	0.500	0.546	0.513	94.0
1	0.046	1.000	1.046	1.100	105.2
1	0.046	2.000	2.046	1.988	97.2
2	0.046	0.570	0.616	0.542	88.0
2	0.046	2.240	2.286	2.050	89.7
				Mean recovery	94.8%
				S.D.	±6.8
				R.S.D.	7.2%

TABLE III

PRECISION DATA FOR ANALYSIS OF *S*-ENANTIOMER PRESENT IN *R*-ENANTIOMER

Analyst	*Peak area % of S-enantiomer*
1	0.052
1	0.042
1	0.049
2	0.044
2	0.044
Mean recovery	0.046%
S.D.	±0.004
R.S.D.	9.0%

solvent molecules compete with the chiral molecule for the hydrogen-bonding sites of the stationary phase. Since the mobile phase used in this experiment is highly polar, the separation of the derivatized enantiomers cannot be explained satisfactory by the so-called "three point interaction theory of chiral separation"[20] when hydrogen bonding is considered to be an important factor in controlling the separation of the enantiomers. The separation of the enantiomers under the chromatographic conditions used in this experiment may have taken place predominantly due to steric interaction rather than hydrogen bonding with the stationary phase.

The separation of the derivatized enantiomers was also achieved on phenylglycine stationary phases in normal-phase conditions. The resolution mechanism of the enantiomers under normal-phase experimental conditions can be explained quite well by the "three point chiral interaction theory". The mobile phase used was hexane–acetonitrile–isopropanol–trifluoroacetic acid (69:8:19:4, v/v). All other chromatographic conditions were maintained identical to those of reversed-phase mode. The selectivity factor (α) under normal-phase conditions was greater than 1.40, which is higher than the reversed-phase mode. Baseline resolution of two peaks is obtained when α is 1.1. Therefore, in the normal-phase mode, one can obtain baseline separation of the derivatized *S*- and *R*-enantiomers by using a short column.

The moisture content in the solvents of normal-phase chromatography (mobile phase) is sensitive on α and on resolution. Therefore, irreproducible chromatographic parameters may be obtained due to the slight variation of the moisture content in the mobile-phase solvents. Reversed-phase chromatography is more resistant to this effect, and hence, is more reproducible. Also, the solvents in the normal-phase mode are not compatible with LC–MS for conducting the identification work. Therefore, the reversed-phase mode was chosen for separation and identification of the derivatized enantiomers.

An HPLC method to separate the underivatized enantiomers was developed by NOVO, Denmark[21]. A CSP of BSA attached to 7-μm silica particles was used for the separation. The advantage of this method over the method developed in this report, is that the enantiomers can be separated without derivatization. This advantage was overshadowed by several disadvantages. It is quite difficult to reproduce the separation of the enantiomers (under the same mobile phase conditions) when columns from different batches from the same manufacturer were used. The BSA stationary

phase can be easily damaged if propanol is used in excess of 5% (with water) in the mobile phase. Chromatographic efficiency of the BSA column was lower than the phenylglycine column, probably due to the sluggish mass transfer of the enantiomers on the stationary phase, the mobile phase, or both. The elution order of the enantiomers cannot be reversed (for trace analysis) by using a BSA column, and the limit of detection is only 1% of the *S*-enantiomer in a mixture with the *R*-enantiomer.

A D-phenylalanine CSP (Apex Chiral AP) also separated the derivatized enantiomers in the normal-phase mode when a mobile phase of hexane–acetonitrile–isopropanol–trifluoroacetic acid (73:5:19:3, v/v) was used. The α value for this column was also greater than 1.40. This stationary phase did not give any separation of the derivatized enantiomers when used in the reversed-phase mode. The trifluoroacetic acid (TFA) in the mobile phase (under normal-phase conditions) was necessary in order to elute the derivatized enantiomers from the column. When TFA was not used, the derivatized and underivatized enantiomers did not elute from the column.

A D-phenylglycine stationary phase column from Jones (Apex Chiral PK) was also tested in the reversed-phase and normal-phase modes. The derivatized *S*- and *R*-enantiomers eluted with the same retention time in both modes. A β-cyclodextrin stationary phase (Cyclobond-I) was also investigated both in the reversed-phase and normal-phase modes. Separation of the derivatized or underivatized enantiomers was not obtained on this stationary phase.

The phenylglycine stationary phase (Regis) used in this method to separate the derivatized enantiomers was found to be extremely stable. The efficiency, selectivity and other chromatographic properties of the phenylglycine column remained unchanged after 200 injections of the derivatized enantiomers. On several occasions, the back pressure of the column increased after 20–40 injections. This problem was eliminated by replacing the inlet frit of the column with a new one or by sonicating the old frit in acetone – 6 *M* nitric acid (50:50) for a few minutes. Two phenylglycine columns from Regis (with different lot numbers) were tested for separation and selectivity of the enantiomers. The resolution and selectivity factors of the two columns were almost identical. The results of this experiment indicate that the separation of the enantiomers is reproducible from column to column when purchased from the same manufacturer.

CONCLUSION

The reversed-phase HPLC method (to separate the derivatized enantiomers) presented here is relatively simple, sensitive and highly reproducible. The pre-chromatographic derivatization of the acid functional group of the chiral compound with an achiral amine into an amide is reproducible, simple and does not convert one enantiomer to another during derivatization. The derivatized sample of the chiral compound needs no extra clean-up steps. This minimized the manipulative error and resulted in good reproducibility. The attachment of a naphthyl group with the chiral compound aided the separation of the enantiomers and also enhanced the UV signal and hence increased the sensitivity. The α value of D-phenylglycine and L-phenylglycine stationary phases (Regis) were almost identical (1.24) for the derivatized *S*- and *R*-enantiomers. These results indicate that the separation of the derivatized enantiomers is reproducible from column to column having different spatial config-

uration of the stationary phase. This method may be useful to separate (with minor manipulation of the chromatographic conditions) the enantiomers of other derivatives of nipecotic acid or many other compounds which have functional groups and spatial configuration similar to the chiral compound analyzed in this experiment.

REFERENCES

1 A. Sioufi, G. Kaiser, F. Leroux and J. P. Dubois, *J. Chromatogr.*, 450 (1988) 221.
2 M. Gazdag, G. Szepesi and K. Milhalyfi, *J. Chromatogr.*, 450 (1988) 145.
3 B. Blessington and N. Crabb, *J. Chromatogr.*, 454 (1988) 450.
4 T. Arai, H. Koike, K. Hirata and H. Oizumi, *J. Chromatogr.*, 448 (1988) 439.
5 L. R. Gelber and J. L. Neumeyer, *J. Chromatogr.*, 257 (1983) 317.
6 H. Furukawa, *Chem. Pharm. Bull.*, 23 (1975) 1625.
7 J. N. LePage, W. Lindner, G. Davies, D. Seitz and B. L. Karger, *Anal. Chem.*, 51 (1979) 433.
8 N. Nimura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
9 E. Gil-Av, A. Tishbee and P. E. Hare, *J. Am. Chem. Soc.*, 102 (1980) 5115.
10 W. H. Pirkle and R. Däppen, *J. Chromatogr.*, 404 (1987) 107.
11 D. W. Armstrong, X. Yang, S. M. Han and R. A. Menges, *Anal. Chem.*, 59 (1987) 2594.
12 D. W. Armstrong, *Anal. Chem.*, 59 (1987) 84A.
13 R. W. Souter, *Chromatographic Separations of Stereoisomers*, CRC Press, Boca Raton, FL, U.S.A., 1985.
14 J. D. Morrison (Editor), *A Symmetric Synthesis*, Vol. I, Academic Press, New York, 1983.
15 M. Zeif and L. J. Crane (Editors), *Chromatographic Chiral Separations*, Marcel Dekker, New York, 1988.
16 I. W. Wainer, *Trends Anal. Chem.*, 6 (1987) 125.
17 I. W. Wainer, *A Practical Guide to the Selection and Use of HPLC Chiral Stationary Phases*, in press.
18 J. Gal, *LC·GC, Mag. Liq. Gas Chromatogr.*, 5 (1987) 106.
19 W. H. DeCanp, *Chirality*, 1 (1989) 2.
20 W. H. Pirkle and B. C. Hamper, *J. Chromatogr.*, 450 (1988) 199.
21 L. Nygard, *Report No. A2690-S*, Ed. 1 (unpublished), NOVO Industri, Copenhagen, 1988.