Journal of Chromatography, 478 (1989) 169–179
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 629

SEPARATION OF THE FOUR OPTICAL ISOMERS OF A DIHYDROPYRIDINE CALCIUM CHANNEL ANTAGONIST

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(First received April 5th, 1989: revised manuscript received May 17th, 1989)

SUMMARY

An isocratic high-performance liquid chromatography (HPLC) method is described for the separation of the four optical isomers of RS-93522-004, a racemic dihydropyridine-based drug containing two chiral centers. The drug is derivatized using (−)-camphanic acid chloride and the resulting four bis-camphanate diastereomers are separated on an achiral silica gel HPLC column. The precision, accuracy, and linearity of the method has been evaluated as applied to the determination of the isomer ratio of RS-93522-004. The method has also been evaluated to determine the limit of quantitation for the individual bis-camphanate diastereomers. Determination of the optical purity of the chiral derivatizing agent has also been addressed. No difference in the relative reactivity of the four individual RS-93522-004 optical isomers towards (−)-camphanic acid chloride is observed and the determination of the ratio of the RS-93522-004 bis-camphanate diastereomers has been shown to be unaffected by derivatization reaction yield. Attempts to resolve the optical isomers of several RS-93522-004 derivatives using various chiral HPLC columns are briefly discussed.

INTRODUCTION

In recent years, greater emphasis has been placed on the evaluation of differences in pharmacological effects of enantiomeric pharmaceutical compounds and the potential enantioselective metabolism of single isomers of racemic drug mixtures. This has led to the development of a wide array of chromatographic methods designed to separate and quantitate enantiomeric drug isomers[1].

Enantiomeric separations using high-performance liquid chromatography (HPLC) have been achieved by a variety of methods, which have led to two frequently used approaches. The first utilizes the classical technique of pre-column derivatization of enantiomers with chiral agents and separation of the resulting diastereomers on achiral stationary phases[2]. Inexpensive normal or reversed-phase HPLC columns are employed for this purpose. However, in this technique, important consideration must be given to enantiomeric purity of the chiral derivatizing agent and potential differences in reactivity of enantiomers towards the chiral reagent. The second in-

0021-9673/89/$03.50

volves use of chiral stationary phases (CSPs)[3]. This approach is desirable because enantiomeric separations can be made directly, without pre-column derivatization steps, and because of the availability of several different types of commercial CSPs. However, commercially available CSP columns are expensive and, in some cases, derivatization of the analyte is still required to improve chromatographic properties and enhance enantiomeric selectivity.

RS-93522-004, 2-[4-(2,3-dihydroxypropoxy)phenyl]ethyl methyl 1,4-dihydro-2,6- dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (**1**), is a synthetic dihydropyridine-based calcium channel antagonist under development for treatment of hypertension. It contains two chiral centers and is synthesized as a racemic mixture of four isomers consisting of two enantiomeric sets of diastereomers. Results from animal studies have shown the isomers with the (*S*)-configuration at C-4 of the dihydropyridine moeity display significantly greater pharmacological effects than the (4*R*)-isomers. Thus, the goal was to develop a method to separate and quantitate the individual isomers of RS-93522-004. Such a method would be of great utility for drug substance control, for the determination of the isomeric purity of single isomers, and as a means to probe for enantioselective metabolism of administered racemic drug.

The vast majority of the literature involving the two chromatographic strategies mentioned above describe separations of enantiomeric compounds containing only a single asymmetric carbon. Reports of the concurrent separation of both enantiomeric and diastereomeric isomers of racemic compounds containing multiple chiral centers are limited[4,5], and involve the separation of diastereomeric derivatives produced from derivatization of the optical isomers with chiral agents. Examples utilizing CSPs for this purpose are rare[6] and suggest the general inability of CSPs to simultaneously distinguish between both enantiomeric and diastereomeric components of isomeric mixtures. Column switching has been one approach used for such separations.[7] This technique utilizes conventional reversed-phase HPLC for separation of diastereomeric isomers followed by chiral HPLC separation of the enantiomeric components of an individual diastereomeric pair. This approach, however, suffers from the necessity for specialized equipment and still requires the use of costly CSP columns.

Several examples of the enantiomeric separation of dihydropyridine-based drugs using CSPs have been reported[8–12] yet all involve compounds containing only a single asymmetric carbon. Kern *et al.*[13] evaluated several commercially available CSPs to achieve separation of the four optical isomers of RS-93522-004, without success, and has reported a reversed-phase HPLC method which separates the diastereomeric components of RS-93522-004. Attempts, made in this study, to separate the optical isomers of a number of RS-93522-004 derivatives with the use of CSPs were also unsuccessful. This work describes the derivatization of RS-93522-004 with (−)-camphanic acid chloride and evaluates a normal-phase HPLC system which separates the resulting four bis-camphanate diastereomers. To our knowledge, no examples of enantiomeric resolution for this class of drugs using pre-column derivatization with chiral reagents has been reported.

EXPERIMENTAL

Apparatus

A Spectra-Physics (San Jose, CA, U.S.A.) 8100XR chromatograph, equipped

with a Valco (Houston, TX, U.S.A.) 20-μl fixed-loop injector, was used with a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 UV absorbance detector set at 229 nm and 0.02 a.u.f.s. The oven temperature was set at 50°C. The detector output was monitored using a Spectra-Physics SP4270 integrator.

Materials

RS-93522-004 and its individual optical isomers were synthesized by the Institute of Organic Chemistry, Syntex Research, Palo Alto, CA, U.S.A. HPLC grade solvents were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) Hydrochloric acid was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.) and pyridine was obtained from Aldrich (Milwaukee, WI, U.S.A.). (−)-Camphanic acid chloride (98+%) was obtained from Aldrich, and (*R*)-(+)-1-(1-naphthyl)ethylamine (99.9+%) and (*S*)-(−)-1-(1-naphthyl)ethylamine (99.9+%) were obtained from Norse Laboratories (Newbury Park, CA, U.S.A.)

Chromatographic conditions

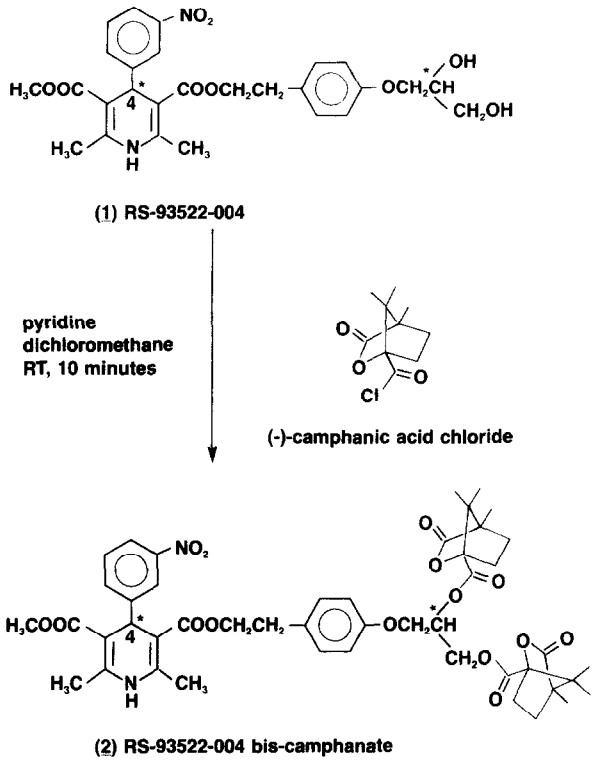
A Nucleosil silica (5-μm, 25 cm × 4.6 mm I.D.) column was purchased from Alltech Assoc. (Deerfield, IL, U.S.A.) and used with a guard column (20 mm × 2.0 mm I.D.) packed with Alltech pellicular silica. The mobile phase consisted of 0.1% acetonitrile and 4% 2-propanol in isooctane (2,2,4-trimethylpentane). The addition of 0.1% acetonitrile is required to improve peak shape and reduce peak tailing. The flow-rate was maintained at 2.0 ml/min and the column pressure was approximately 2000 p.s.i. The sample concentration was approximately 0.3 mg/ml and the sample loading approximately 6 μg. HPLC solvents were filtered through 0.45-μm filters and the mobile phase was degassed by purging with helium.

Derivatization procedure

The derivatization reaction is shown in Scheme 1. A sample of 20 mg of RS-93522-004 or its single optical isomers and 40 mg of (−)-camphanic acid chloride are placed in a dry 12 × 75 mm test tube. The material is dissolved in 1 ml of anhydrous dichloromethane, and 30 μl of anhydrous pyridine is added to the solution. The test tube is then stoppered, shaken to mix the solution, and allowed to stand for 10 min at room temperature. A volume of 1 ml of 0.1 *M* hydrochloric acid is added to the test tube to quench the reaction. The organic layer is transferred with a pasteur pipette to a round bottomed flask and evaporated to dryness. The resulting residue is dissolved in approximately 5 ml of tetrahydrofuran and diluted with mobile phase to 100 ml to obtain a sample solution of approximately 0.3 mg/ml.

Modified derivatization procedure

A modified derivatization procedure was performed as above except the reaction was carried out with 30 mg of RS-93522-004 and 80 mg of (−)-camphanic acid chloride dissolved in 8 ml of anhydrous dichloromethane. Aliquots (1 ml) of the reaction mixture were taken at 0.5-min intervals for 5 min, and a final aliquot was taken at 240 min. Each aliquot was quenched with 0.5 ml of 0.1 *M* hydrochloric acid at the time of sampling. The HPLC analysis of each aliquot was performed using a Spherisorb ODS II (5 μm, 25 cm × 4.6 mm I.D.) column purchased from Alltech Assoc. (Deerfield, IL, U.S.A.) with a mobile phase consisting of methanol–water



Scheme 1. Derivatization of RS-93522-004 with (−)-camphanic acid chloride. RT = reaction time.

(80:20). The column oven temperature was maintained at 40°C with a flow-rate of 1.0 ml/min and UV detection at 229 nm. The sample concentration was approximately 1.0 mg/ml in methanol and the sample loading was approximately 20 μg with a 20-μl sample loop.

Purity determination of (−)-camphanic acid chloride

Approximately 20 mg of (−)-camphanic acid chloride, as obtained from the manufacturer, was placed in a dry 12 × 75 mm test tube and dissolved in 0.5 ml of anhydrous dichloromethane. To this solution was added 20 μl of anhydrous pyridine and 20 μl of (*R*)-(+)-1-(1-naphthyl)ethylamine [(*R*)-NEA] or (*S*)-(−)-1-(1-naphthyl)-ethylamine [(*S*)-NEA]. The test tube was then stoppered, shaken to mix the solution, and allowed to stand for 30 min at room temperature. The reaction mixture was then quenched with 1 ml of 0.1 *M* hydrochloric acid. The organic layer was transferred with a pasteur pipette to a round bottomed flask and evaporated to dryness. The residue contained the corresponding NEA camphanamide diastereomers. The structure of each NEA camphanamide derivative was confirmed by both ^{1}H NMR and mass spectrometric (MS) analyses.

The NEA camphanamide derivatives were chromatographed using a Nucleosil

silica (5 μm, 25 cm × 4.6 mm I.D.) column with a mobile phase of hexane–ethyl acetate–acetonitrile (70:30:0.1) and UV detection at 254 nm. The flow-rate was maintained at 1.0 ml/min with a column oven temperature of 40°C. The sample concentration was approximately 0.5 mg/ml in ethyl acetate and the sample loading was approximately 10 μg with a 20-μl sample loop. The (*S*)-NEA camphanamide and (*R*)-NEA camphanamide derivatives were found to elute at 6.2 and 7.4 min, respectively.

The enantiomeric purity of (−)-camphanic acid chloride, as obtained from the manufacturer, was determined to be >99.3% by normal-phase HPLC analysis of the diastereomeric camphanamide components produced from its reaction with (*R*)-NEA, as described in the discussion of results.

RESULTS AND DISCUSSION

Chromatographic system development

In a previous report by Kern *et al.*,[13] several normal and reversed-phase HPLC methods were evaluated for their ability to separate the optical isomers of several derivatives of RS-93522-004. The derivatizing agents used in this work were all achiral. In addition, a number of commercially available CSPs were evaluated to separate the optical isomers of underivatized RS-93522-004 and the reported derivatives. However, none of the CSPs investigated resolved either the individual enantiomers or diastereomers of RS-93522-004 or its derivatives. The diastereomeric components of RS-93522-004 were found to separate as the bis-3,5-dinitrobenzoate esters by reversed-phase HPLC.

In the present work, RS-93522-004 was derivatized with a number of chiral acid chloride reagents to give the corresponding bis-esters. These included (−)-camphanic acid chloride, (+)-camphor-10-sulfonyl chloride, (−)-menthyloxyacetic acid chloride, (*S*)-(−)-N-(trifluoroacetyl)prolyl chloride, (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate, (*S*)-N-1-(2-naphthylsulfonyl)-2-pyrrolidine carbonyl chloride. In all cases, an excess of the derivatizing reagent was used to obtain the bis-derivatives in order to minimize the number of potential reaction products. The bis-ester derivatives were chromatographed on several commercially available CSPs including Pirkle covalent phenylglycine DNB, Pirkle covalent naphthylalanine, Cyclobond β-cyclodextrin, Resolvosil BSA, Enantiopak α-1-glycoprotein, and Daicel OT(+). The derivatives of (−)-menthyloxyacetic acid chloride and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate were chromatographed only on the Cyclobond column. In most cases, no isomer separation was achieved and separation of diastereomeric components was accomplished in only a few instances[14,15]. This led to the evaluation of various normal and reversed phase HPLC systems. The normal-phase HPLC system reported here ultimately attained near baseline resolution of the four bis-camphanate diastereomers of RS-93522-004 (Fig. 1, Table I).

Identity of optical isomers

The four optical isomers of RS-93522-004 were individually derivatized following the described reaction conditions to give the corresponding bis-camphanate diastereomers. Their structures were confirmed by ^{1}H NMR, MS, and UV analyses, and their isomeric purity was established by the chromatographic method described here.

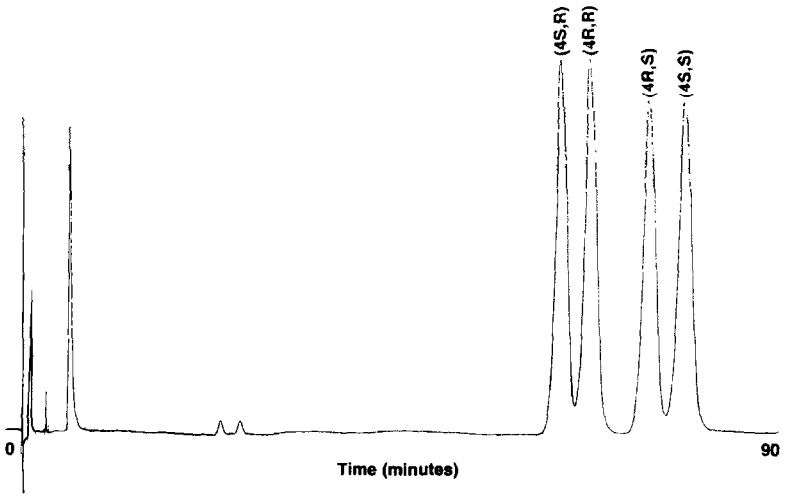


Fig. 1. HPLC separation of the RS-93522-004 bis-camphanate diastereomers.

The order of elution of the derivatized isomers was determined by chromatographic coinjection of the individual bis-camphanate diastereomers with the derivatized racemic drug. The order of elution was determined to be: the (4*S*,*R*) isomer first, the (4*R*,*R*) isomer second, the (4*R*,*S*) isomer third, and the (4*S*,*S*) isomer last (see Fig. 1).

Limit of quantitation

The limit of quantitation for individual bis-camphanate diastereomers was determined by chromatographing spiked solutions containing known amounts of the (4*R*,*R*) bis-camphanate isomer (peak 2) in the presence of the (4*S*,*R*) bis-camphanate isomer (peak 1). The concentration of the (4*S*,*R*) bis-camphanate isomer was held constant at 0.074 mg/ml (*i.e.* one-fourth of the specified concentration of 0.3 mg/ml) and the (4*R*,*R*) bis-camphanate isomer was spiked at levels equivalent to 0.25, 0.5, 1.0, 2.0, and 3.0% of the concentration of the former. The results appear in Table II. The limit of quantitation of the (4*R*,*R*) bis-camphanate isomer was found to be 0.5%, or 0.0075 μg for a given sample injection, under these chromatographic conditions. It should be noted that the (4*R*,*R*) bis-camphanate isomer elutes as a minor backside

TABLE I

SEPARATION OF THE RS-93522-004 BIS-CAMPHANATE DIASTEREOMERS

k' and R_s are the capacity factor and resolution, respectively. Chromatographic conditions are given in the Experimental section.

Isomer	k'	R_s
4*S*,*R*	37.6	
		1.28
4*R*,*R*	39.6	
		2.49
4*R*,*S*	43.7	
		1.41
4*S*,*S*	46.1	

TABLE II

ACTUAL AND OBSERVED PERCENTAGES OF THE RS-93522-004 (4*R*,*R*) BIS-CAMPHANATE DIASTEREOMER SPIKED INTO THE (4*S*,*R*) BIS-CAMPHANATE DIASTEREOMER

Actual (%)	*Observed (%)*
0.25	nd
0.50	0.51
1.00	1.15
2.00	2.13
3.00	3.07

peak following the major (4*S*,*R*) bis-camphanate isomer peak (see Fig. 2). Therefore, in this instance, the quantitation limit of 0.5% represents a worst case of detectability. In addition, the limit of quantitation can be applied to the remaining bis-camphanate diastereomers of RS-93522-004 because, at the detection wavelength of 229 nm, there is no observed difference in their extinction coefficients.

Purity of chiral reagent

An important consideration in the validation of the method is to insure that the chiral derivatizing agent is of sufficient enantiomeric purity so as not to affect the accurate quantitation of the RS-93522-004 bis-camphanate single isomers. The enantiomeric purity of (−)-camphanic acid chloride, as obtained from the manufacturer, was determined by normal-phase HPLC analysis of the diastereomeric camphanamide components produced from its reaction with (*R*)-(+)-1-(1-naphthyl)ethylamine [(*R*)-NEA], as described in the Experimental section. (−)-Camphanic acid chloride was derivatized with (*R*)-NEA and (*S*)-(−)-1-(1-naphthyl)ethylamine [(*S*)-NEA] to establish the HPLC retention times of the resulting diastereomeric (*R*)-NEA and (*S*)-NEA camphanamide derivatives. The (*S*)-NEA camphanamide and (*R*)-NEA camphanamide derivatives were found to elute at 6.2 and 7.4 min, respectively. Normal-phase HPLC analysis of the (*R*)-NEA camphanamide derivative showed the peak eluting at 6.2 min to integrate to 0.7% by area normalization. The contributions to the area of this peak come from both the (*R*)-NEA derivative of

Fig. 2. HPLC chromatogram illustrating the limit of quantitation of the RS-93522-004 (4*R*,*R*) bis-camphanate diastereomer.

(+)-camphanic acid chloride, which arises from enantiomeric impurity of the (−)-camphanic acid chloride reagent, and from the enantiomeric (*S*)-NEA derivative of (−)-camphanic acid chloride, which arises from (*S*)-NEA contamination of the (*R*)-NEA reagent. Therefore, the enantiomeric purity of (−)-camphanic acid chloride was determined to be at least 99.3%.

This result was supported by derivatization of the RS-93522-004 (4*S*,*R*) isomer, available in high isomeric purity, with (−)-camphanic acid chloride and analysis using the HPLC procedure described here. Only the (4*S*,*R*) bis-camphanate diastereomer main peak was observed in the resulting HPLC chromatogram. No other diastereomeric components were observed which could be attributed to (+)-camphanate derivatives arising from enantiomeric impurity of the chiral derivatizing reagent. It should also be noted that the absence of the other bis-camphanate diastereomer peaks suggests that, under the derivatization reaction conditions, racemization of RS-93522-004 does not occur.

Relative reactivity of optical isomers

The potential for the individual optical isomers of RS-93522-004 to react with (−)-camphanic acid chloride at different rates during derivatization was evaluated by determining the bis-camphanate diastereomer ratios at incomplete reaction yields. To achieve intermediate reaction yields, the derivatization conditions were modified. Approximately four times the volume of dichloromethane specified was used in the derivatization, which slowed the reaction rate considerably. Under these conditions, the reaction did not reach completion after 4 h, and reaction mixtures containing differing yields of RS-93522-004, mono-camphanate ester, and bis-camphanate ester were obtained. Aliquots of the reaction mixture were taken at intervals and quenched to prevent further reaction. A reversed-phase HPLC method was developed to determine the percent of underivatized RS-93522-004, the percent of mono-camphanate ester, and the percent of bis-camphanate ester present in each quenched reaction aliquot (see Experimental). The yield of the bis-camphanate ester ranged from approximately 12 to 70% under these reaction conditions. The percent yield data is tabulated in Table III.

Each quenched reaction aliquot was also assayed using the described method to determine the ratio of the RS-93522-004 bis-camphanate diastereomers. The diastereomer ratios remained relatively constant at intermediate reaction yields as shown in Table IV. These data demonstrate that the ratio of the bis-camphanate diastereomers is unaffected by the derivatization reaction yield and that quantitative derivatization is not required. These data also support the determination that there is no difference in the relative reactivity of the four optical isomers of RS-93522-004 towards (−)-camphanic acid chloride.

Precision, accuracy, and linearity

The precision, accuracy, and linearity of the HPLC method were evaluated as applied to the determination of the isomer ratio of RS-93522-004. The precision was evaluated by performing six replicate injections of a single solution of the bis-camphanate diastereomers obtained from the reaction of RS-93522-004 with (−)-camphanic acid chloride. In a typical derivatization reaction, the resulting four bis-camphanate diastereomers are approximately equimolar with a composite concentration

TABLE III

PERCENT YIELD DATA

Reaction time (min)	*RS-93522-004 (%)*	*Mono-camphanate (%)*	*Bis-camphanate (%)*
0.5	14.3	73.9	11.8
1.0	13.7	74.7	11.5
1.5	11.9	75.5	12.6
2.0	7.1	76.4	16.5
3.0	2.7	74.6	22.8
5.0	1.6	69.0	29.4
240	0.5	29.8	69.7

of about 0.3 mg/ml. The area normalized percentages of the four individual bis-camphanate diastereomer peaks were determined and the results obtained were within 0.8% relative standard deviation, indicating good precision of the chromatographic method. The method reproducibility was assessed by performing duplicate injections from six separate reactions of RS-93522-004 and (−)-camphanic acid chloride and determining, individually, the area normalized percentages of the four bis-camphanate diastereomer peaks. The results obtained for each bis-camphanate diastereomer peak were within 0.4% relative standard deviation, indicating the reproducibility of the method.

The accuracy was assessed by derivatization of an accurately weighed mixture of the individual RS-93522-004 (4*S*,*S*), (4*R*,*R*), (4*R*,*S*), and (4*S*,*R*) isomers, in a ratio of approximately 1:1:1:1, with (−)-camphanic acid chloride, and subsequent determination of the diastereomer ratio of the bis-camphanate derivatives by the described HPLC method. Within the expected precision of the method, the observed bis-camphanate diastereomer ratio agreed well with the theoretical isomer ratio, as shown in Table V, thus demonstrating the accuracy of the method.

To determine the linearity of the method, sample solutions of the bis-camphanate derivatives at concentrations of approximately 5, 50, 85, 100, 115, and 200% of the specified concentration of 0.3 mg/ml were prepared. The total area of the four bis-camphanate diastereomer peaks *versus* known concentration were obtained and

TABLE IV

RS-93522-004 BIS-CAMPHANATE DIASTEREOMER RATIOS

% Yield	*Peak 1*	*Peak 2*	*Peak 3*	*Peak 4*
11.5	22.4	26.7	23.5	27.4
12.6	22.1	26.9	24.0	27.0
16.5	23.5	26.8	22.3	27.4
22.8	22.0	27.6	23.2	27.2
29.4	24.1	27.6	21.7	26.6
69.7	22.9	28.4	22.0	26.7
Mean	22.8	27.3	22.8	27.1
S.D.	0.8	0.6	0.9	0.3

TABLE V

RS-93522-004 BIS-CAMPHANATE DIASTEREOMER RATIOS

Peak	*Experimental area (%)*	*Theoretical area (%)*
4*S*,*R*	30.8	30.6
4*R*,*R*	25.5	25.6
4*R*,*S*	20.4	20.0
4*S*,*S*	23.3	23.9

used to prepare a linear regression line formula. Acceptable linearity was observed over the range tested, following the derived linear equation $y = 1.004x + 0.191$, where y = observed response and x = expected response. The average deviation from a theoretical calibration line having a slope of 1.00, expressed as the standard error of estimate, was 0.43%. The correlation coefficient found was 0.999, indicating the method is linear in the examined range of concentration. In addition, the relationship between the area ratios of (peak 1 + peak 2)/(peak 3 + peak 4) *versus* known concentration was also examined. A plot of area ratio *versus* concentration gave a slope of 0.053 demonstrating that the area ratio of the peaks remained relatively constant in the examined range of concentration. The individual area ratios of peaks 1/2 and peaks 3/4 *versus* concentration were similarly examined with parallel results.

CONCLUSION

A simple, normal-phase HPLC procedure has been described which separates the four optical isomers of RS-93522-004, a racemic dihydropyridine-based drug containing two asymmetric carbon centers, as their diastereomeric bis-camphanate derivatives. The method has been shown to be accurate, precise, and sensitive. In addition, no difference in the relative reactivity of the four individual RS-93522-004 optical isomers towards (−)-camphanic acid chloride is observed, and the determination of the ratio of optical isomers in RS-93522-004 is unaffected by derivatization reaction yield.

ACKNOWLEDGEMENTS

The authors thank Ms. Lilia Kurz for obtaining the NMR spectra, Mr. Greg Witcop for obtaining the mass spectra, Mr. John R. Kern and Mr. Keith Avitabile for their valuable insights and assistance with the chromatography, and Mr. Gary L. Hedden for the synthesis of the optical isomers of RS-93522-004.

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