ISSN 0021-9673

VOL. 450 NO. 2 OCTOBER 21, 1988

including

1st Int. Symp. on Separation of Chiral Molecules Paris, May 31–June 2, 1988

JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER

JOURNAL OF CHROMATOGRAPHY

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; drug level monitoring and pharmacokinetic studies; clinical toxicology; analytical studies in occupational medicine.

Submission of Papers. Papers in English, French and German may be submitted, in three copies. Manuscripts should be submitted to: The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands. Review articles are invited or proposed by letter to the Editors. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Subscription Orders. Subscription orders should be sent to: Elsevier Science Publishers B.V., P.O. Box 211, 1000 AE Amsterdam, The Netherlands, Tel. 5803 911, Telex 18582 ESPA NL. The *Journal of Chromatography* and the *Biomedical Applications* section can be subscribed to separately.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications* and *Cumulative Author and Subject Indexes, Vols. 401–450*) has 37 volumes in 1988. The subscription prices for 1988 are:

J. Chromatogr. (incl. *Cum. Indexes, Vols. 401–450*) + *Biomed. Appl.* (Vols. 424–460):
Dfl. 6290.00 plus Dfl. 962.00 (p.p.h.) (total *ca.* US$ 3537.50)

J. Chromatogr. (incl. *Cum. Indexes, Vols. 401–450*) only (Vols. 435–460):
Dfl. 5070.00 plus Dfl. 676.00 (p.p.h.) (total *ca.* US$ 2803.00)

Biomed. Appl. only (Vols. 424–434):
Dfl. 2145.00 plus Dfl. 286.00 (p.p.h.) (total *ca.* US$ 1185.75).

Our p.p.h. (postage, package and handling) charge includes surface delivery of all issues, except to subscribers in Argentina, Australia, Brasil, Canada, China, Hong Kong, India, Israel, Malaysia, Mexico, New Zealand, Pakistan, Singapore, South Africa, South Korea, Taiwan, Thailand and the U.S.A. who receive all issues by air delivery (S.A.L. — Surface Air Lifted) at no extra cost. For Japan, air delivery requires 50% additional charge; for all other countries airmail and S.A.L. charges are available upon request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 423) are available at Dfl. 230.00 (plus postage). Claims for missing issues will be honoured, free of charge, within three months after publication of the issue. Customers in the U.S.A. and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 52 Vanderbilt Avenue, New York, NY 10017. Tel. (212) 916-1250.

Abstracts/Contents Lists published in Analytical Abstracts, ASCA, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Referativnyi Zhurnal and Science Citation Index.

See inside back cover for Publication Schedule, Information for Authors and information on Advertisements.

0021-9673/88/$03.50

Printed in The Netherlands

VOL. 450, NO. 2 JOURNAL OF CHROMATOGRAPHY OCTOBER 21, 1988

CONTENTS

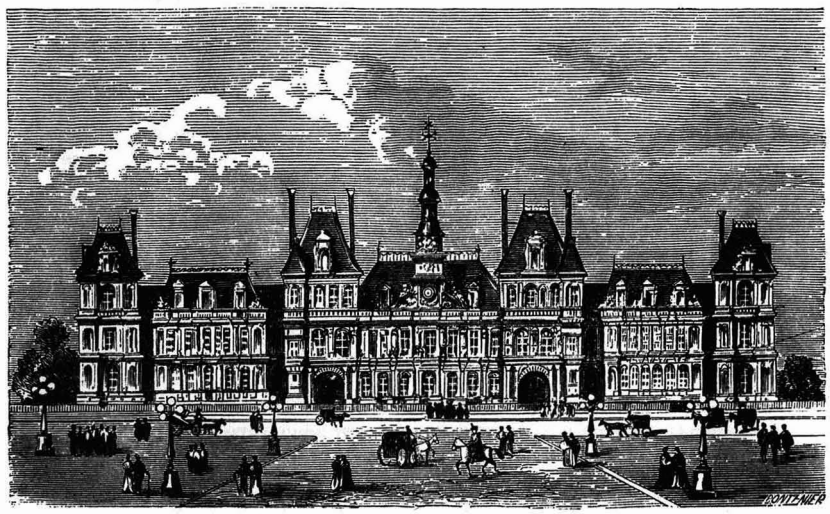
(Abstracts/Contents Lists published in Analytical Abstracts, ASCA, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Referativnyi Zhurnal and Science Citation Index)

FIRST INTERNATIONAL SYMPOSIUM ON SEPARATION OF CHIRAL MOLECULES, PARIS, MAY 31–JUNE 2, 1988

(Continued overleaf)

In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*)

SPECIAL ISSUE



Paris, the old (then new) Town Hall, ca. 1875

FIRST INTERNATIONAL SYMPOSIUM ON SEPARATION OF CHIRAL MOLECULES

Paris, May 31–June 2, 1988

CONTENTS

FIRST INTERNATIONAL SYMPOSIUM ON SEPARATION OF CHIRAL MOLECULES, PARIS, MAY 31–JUNE 2, 1988

Journal of Chromatography, 450 (1988) 143
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 757

PREFACE

The *First International Symposium on Separation of Chiral Molecules* was held in the Maison de la Chimie, Paris, from May 31st to June 2nd, 1988. The symposium, which was attended by more than 500 participants from about 30 countries, was organized by the Société Française de Chimie.

The symposium has allowed fruitful discussions between specialists in various separation techniques, such as crystallization, enzymatic methods and chromatography.

D. BAUER

Journal of Chromatography, 450 (1988) 145–155
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 723

SOME ASPECTS OF THE SELECTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE SEPARATION OF CHIRAL COMPOUNDS IN PHARMACEUTICAL ANALYSIS

M. GAZDAG*, G. SZEPESI and K. MIHÁLYFI
Chemical Works of Gedeon Richter Ltd., P.O. Box 27, H-1475 Budapest (Hungary)

SUMMARY

The resolution of norgestrel enantiomers using cyclodextrin inclusion complexation was investigated. For the purity testing of levonorgestrel the separation method was optimized with regard to the simultaneous determination of enantiomeric and non-isomeric impurities at low concentrations. The dependence of the selectivity and efficiency of the separation on the nature and concentration of the organic solvent (methanol or acetonitrile) in the eluent and on column temperature was studied in order to establish the optimal conditions for the separation. The optimal method was validated and proved to be suitable for the determination as little as 0.1% of enantiomeric impurity in levonorgestrel.

INTRODUCTION

In the last few years there has been great interest in the improvement of high-performance liquid chromatographic (HPLC) methods suitable for the separation of enantiomeric compounds. Several methods have been developed, based mainly on four basic principles: (a) transformation of enantiomeric compounds to their diastereomeric derivatives; (b) the use of chiral stationary phases; (c) the use of a chiral ion pair reagent in the mobile phase; or (d) the use of cyclodextrin inclusion complexes by applying chemically bonded cyclodextrin columns or cyclodextrin-containing eluents.

In pharmaceutical analysis, when enantiomeric separations are required (such as the control of the resolution procedure in drug production, the control of the optical purity of enantiomeric active ingredients, the preparative-scale preparation of pure enantiomers and the determination of the enantiomeric ratio of racemic drugs in biological media) no method based on the above-mentioned principles has preference and an HPLC method should be selected by taking into consideration both the aim of the analysis and the power and performance of the method. In this paper we discuss in detail the principles that can be advantageously considered when selecting a method. The method validation procedure is demonstrated on the example of the determination of the enantiomeric composition of D- and L-norgestrel using cyclodextrin inclusion complexation for the separation.

0021-9673/88/$03.50

As a necessary continuation of our recent work[1] on the separation of the enantiomers of D,L-norgestrel, it was required to optimize further and validate the method for its possible application to the determination of the optical purity of D-norgestrel. In this work the separation and determination of impurities originating from the synthesis and the enantiomeric impurity (L-isomer) present in very low concentrations (less than 1%) were achieved.

EXPERIMENTAL

A Hewlett-Packard 1090A liquid chromatograph equipped with an HP-1040A diode-array detector, a thermostated column compartment, an autosampler, an HP-85B personal computer, an HP-9121 disk drive, an HP-2225A Thinkjet printer and an HP-7470A plotter was used. Separations were performed on a prepacked Ultrasphere ODS (5 μm) column (250 × 4.6 mm I.D.) (Beckman). The eluents were prepared from HPLC-grade solvents (E. Merck) and were degassed prior to use.

Levonorgestrel and D,L-norgestrel were prepared at the Chemical Works of Gedeon Richter in USP XXI quality. Levonorgestrel samples with and without spiking with 2% of D,L-norgestrel (samples containing less than 0.1 and 1% of L-norgestrel impurity, respectively) were used for the model experiments. γ-Cyclodextrin (γ-CD) was obtained from Chinoin and was used without further purification.

Other experimental conditions (such as eluent composition, flow-rate, temperature and detection) are given in the figure captions.

RESULTS AND DISCUSSION

D,L-Norgestrel is official in the USP XXI[2] and D-norgestrel in the USP XXI and BP 1980[3,4] as levonorgestrel. The optical purity of levonorgestrel is checked merely by measuring its optical rotation, enabling only an approximate estimation of the enantiomeric composition of a levonorgestrel sample. For these experiments a levonorgestrel sample containing two main impurities (designated peaks 1 and 2 on the chromatograms) with total concentrations less than 1% was used. This sample did not contain L-norgestrel impurity (based on our later experimental data, its concentration was calculated to be less than 0.1%), so it was spiked with 1% of L-norgestrel in order to perform model experiments.

A chromatogram of the model sample in the absence of γ-CD in the eluent is shown in Fig. 1. An excellent separation of other impurities was achieved, but the enantiomeric impurity was not separated.

To establish better conditions for the determination of small amounts of isomeric impurity without affecting the accurate determination of other impurities originating from the synthesis and also occurring at low concentrations it was necessary to re-optimize and validate the separation system described previously[1], considering the special requirements of purity testing. In our recent work it was shown that the incorporation of γ-CD in the eluent at a concentration of 0.01 mol/l is sufficient for the enantiomeric separation of norgestrel isomers. Using other CDs (α- and β-CD) these isomers cannot be resolved, so our recent results obtained for the dependence of capacity ratios on the γ-CD concentration and the effect of the cavity size in the CD ring on the selectivity of separation were adopted in this study[1].

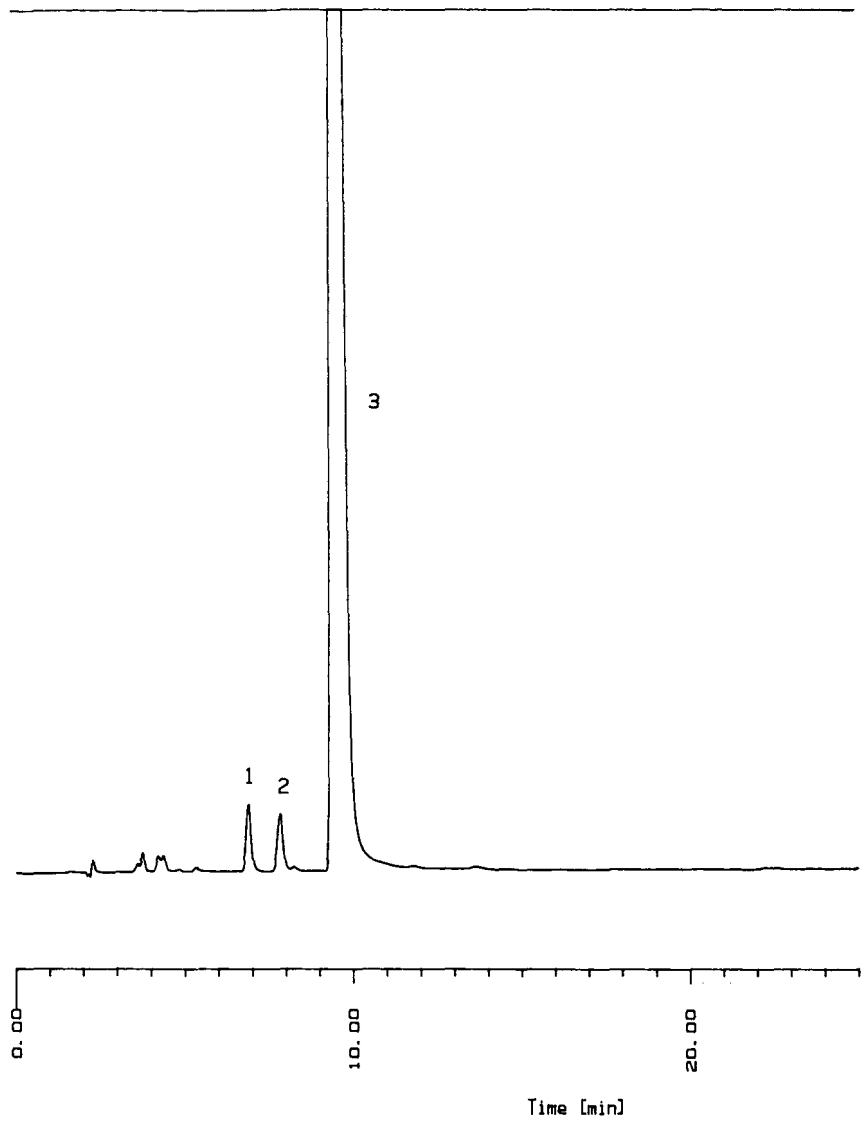


Fig. 1. Chromatogram of a model sample in the absence of cyclodextrins. Conditions: column, Ultrasphere ODS; eluent, methanol–water (7:3); flow-rate, 1 ml/min; detection, 244 nm; injection, 20 μl of 0.05% methanolic solution. Compounds: 1 and 2 = non-isomeric impurities; 3 = levonorgestrel + L-norgestrel.

Effect of the nature and concentration of organic solvents on the selectivity and efficiency of the separation

Figs. 2 and 3 show the dependences of the capacity ratios on the concentration of the organic solvent [methanol (Fig. 2) and acetonitrile (Fig. 3)] in the eluent. The changes in the selectivity and resolution of the separations are also indicated.

It can be concluded from Figs. 2 and 3 that a better selectivity and resolution for

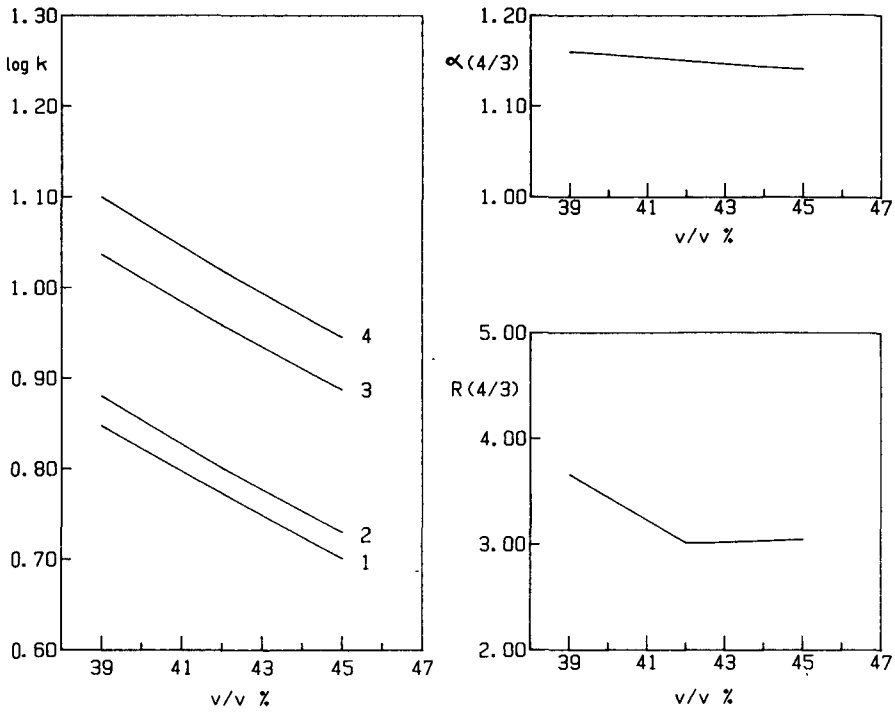


Fig. 2. Dependence of capacity ratios on methanol concentration in the eluent. Conditions as in Fig. 1 except the eluent, which was methanol–water (45:55) containing 0.01 mol/l of γ-CD. The sample was dissolved in methanol. Temperature, 27.0°C. Compounds: 3 = levonorgestrel; 4 = L-norgestrel; others as in Fig. 1.

norgestrel isomers (peaks 3 and 4) can be achieving using methanol–water than acetonitrile–water eluents but the opposite applies for the other impurities (peaks 1 and 2).

The chromatograms obtained using the optimal eluent systems are shown in Fig. 4A (45% methanol) and Fig. 4B (30% acetonitrile).

Dependence of separation characteristics on temperature

To optimize the separation system further, the dependence of the selectivity and efficiency of the separation on the column temperature was investigated and the results are shown in Figs. 5 (methanol–water) and Fig. 6 (acetonitrile–water) for the optimal eluent compositions.

The following general conclusions can be drawn:

(a) Only small changes in the selectivity and resolution occur on increasing the column temperature, with the exception of the resolution of peaks 1 and 2 with methanol–water as the eluent, where a significant increase in separation selectivity with increase in the column temperature was achieved.

(b) Using methanol–water as the eluent a slight change in the capacity ratios of the compounds (with the exception of peak 2) occurred on increasing the column

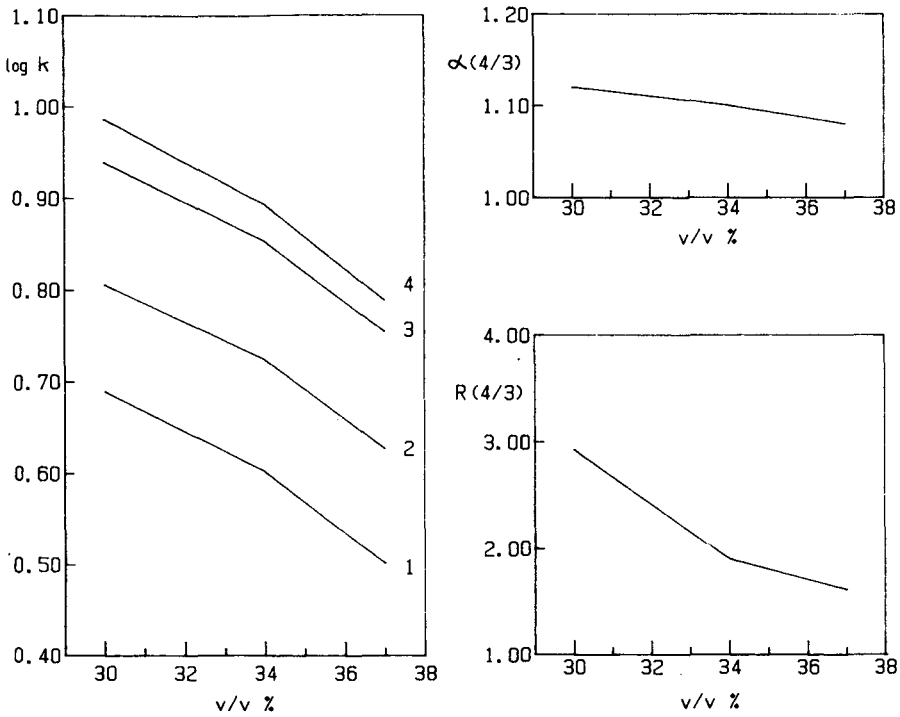


Fig. 3. Dependence of capacity ratios on acetonitrile concentration in the eluent. Conditions as in Fig. 2 except the eluent, which was acetonitrile–water (3:7) containing 0.01 mole/l of γ-CD. The sample was dissolved in acetonitrile. Temperature, 27.6°C.

temperature. This change differs for the various compounds (negligible for the L-isomer, increasing for the D-isomer and peak 2, decreasing for peak 1). With acetonitrile–water as the eluent this change in the capacity ratios is unidirectional, showing a significant increase for each compound without a substantial decrease in the selectivity and efficiency of the separation at elevated column temperature. As the use of elevated column temperatures in general results in a decrease in retention in chromatography and, similarly, if the equilibrium constant of inclusion complex formation increases with increasing temperature, leading to lower capacity factors indicating stronger formation of more polar inclusion complexes, this trend in the changes can be adequately explained by increasing competition of the organic solvent with the solutes for the preferred location in the hydrophobic cavity. This assumption seems to be supported by the fact that different extents of the changes in capacity ratios were observed for the different compounds in methanol–water and acetonitrile–water as eluents.

The chromatograms obtained at different column temperatures are shown in Figs. 7 (methanol–water) and 8 (acetonitrile–water).

Validation of the separation method for purity testing

Current concepts of the HPLC method validation procedure have been reported

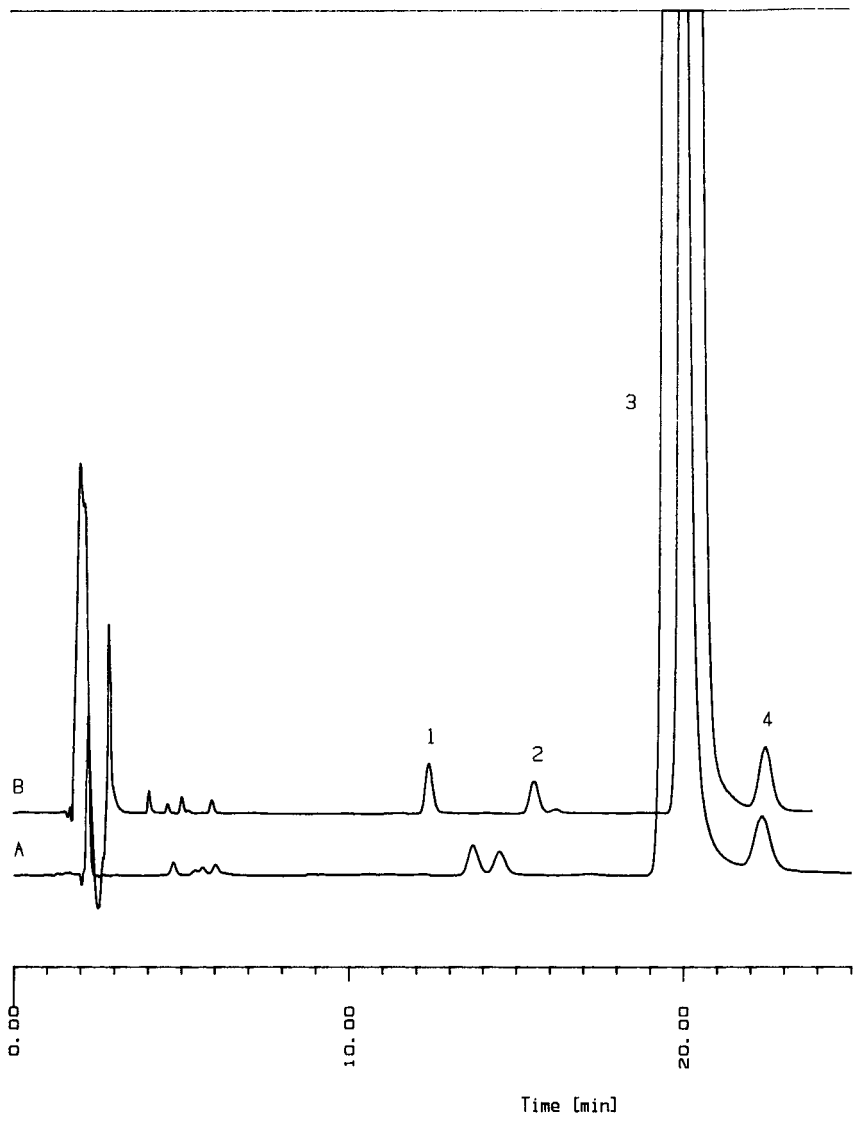


Fig. 4. Chromatograms obtained with the optimal eluent systems. Organic solvent: (A) methanol; (B) acetonitrile. Other conditions as in (A) Fig. 2 and (B) Fig. 3.

in the *Pharmacopeial Forum* in 1986[5] and discussed in detail in other publications[6–8]. According to this guideline, demonstration of the following data elements is required for HPLC method validation for purity testing: accuracy, precision, limit of determination, selectivity, range, linearity and "ruggedness". According to our classification[9], the validation data can be divided into three main groups:

(a) Statistically evaluated data elements such as accuracy, precision, reproduci-

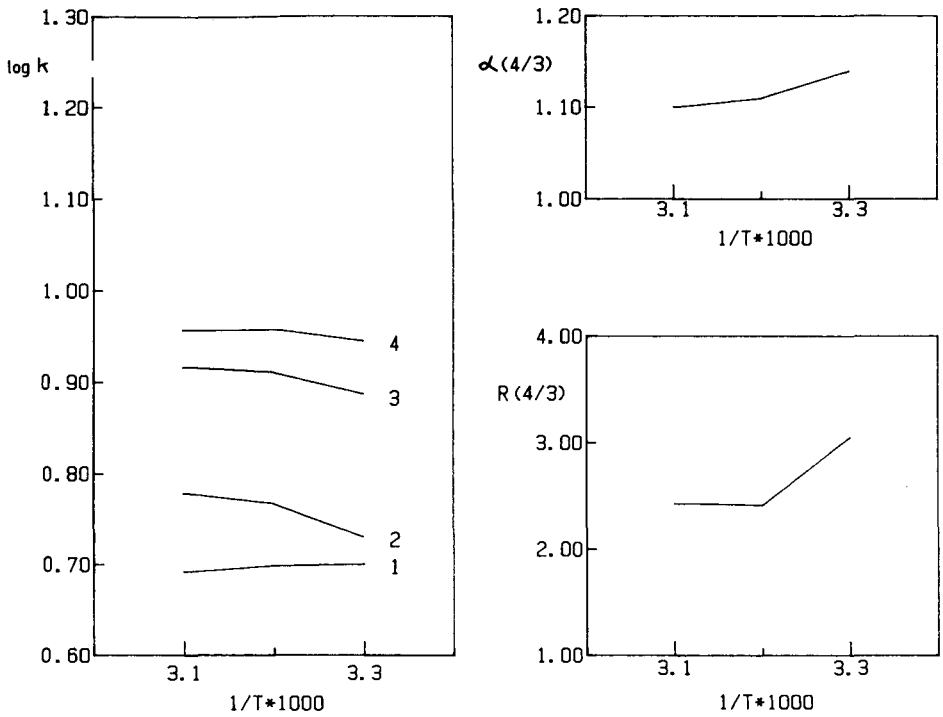


Fig. 5. Dependence of the separation characteristics on temperature with methanol–water as eluent. Conditions as in Fig. 4A.

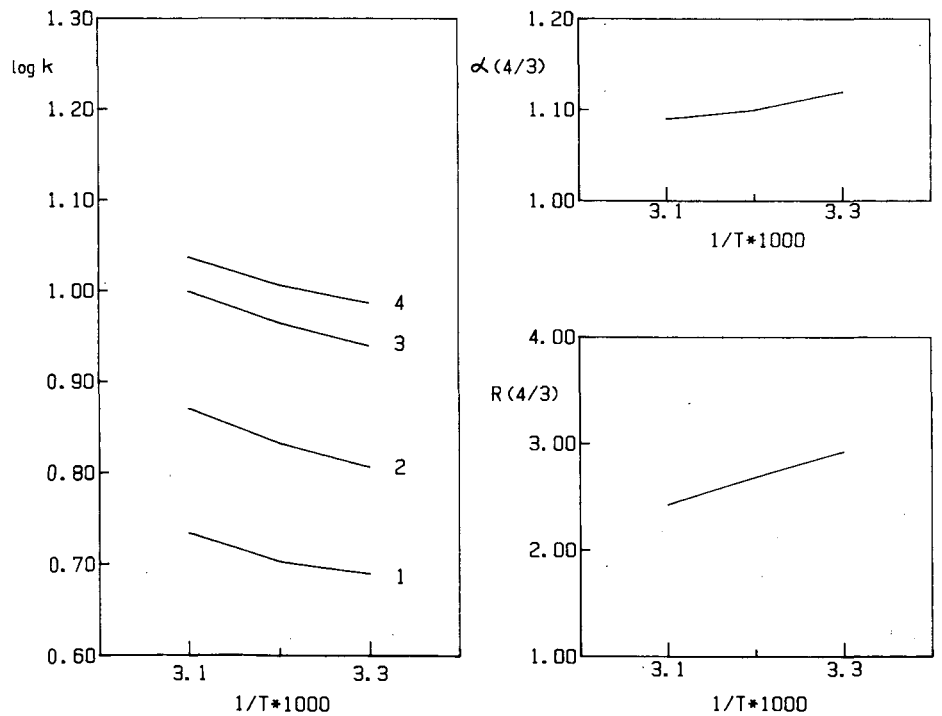


Fig. 6. Depencence of the separation characteristics on temperature with acetonitrile–water as eluent. Conditions as in Fig. 4B.

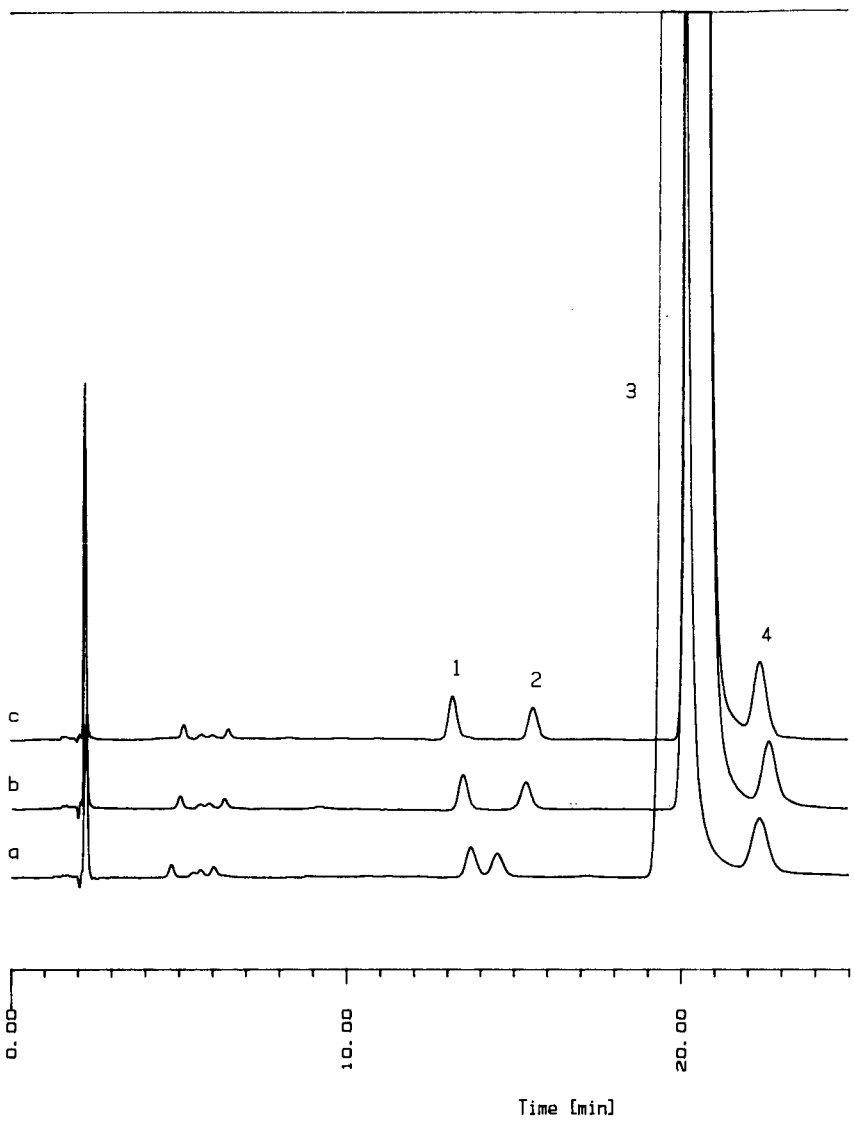


Fig. 7. Chromatograms obtained at different column temperatures using methanol–water as eluent. Temperature: (a) 27.0°C; (b) 40.0°C; (c) 50.0°C. Other conditions as in Fig. 4A.

bility (repeatability), day-to-day reproducibility, inter-laboratory reproducibility ("ruggedness") and detector linearity and range.

(b) System suitability data contain measures of the resolving power of the HPLC system and comprise criteria established for accepting or rejecting analytical results. The following data elements belong to the term "system suitability" and can be controlled each day for a preselected sample (mainly for standard solutions used for quantitation) prior to the use of method for routine analysis to ensure that the system is

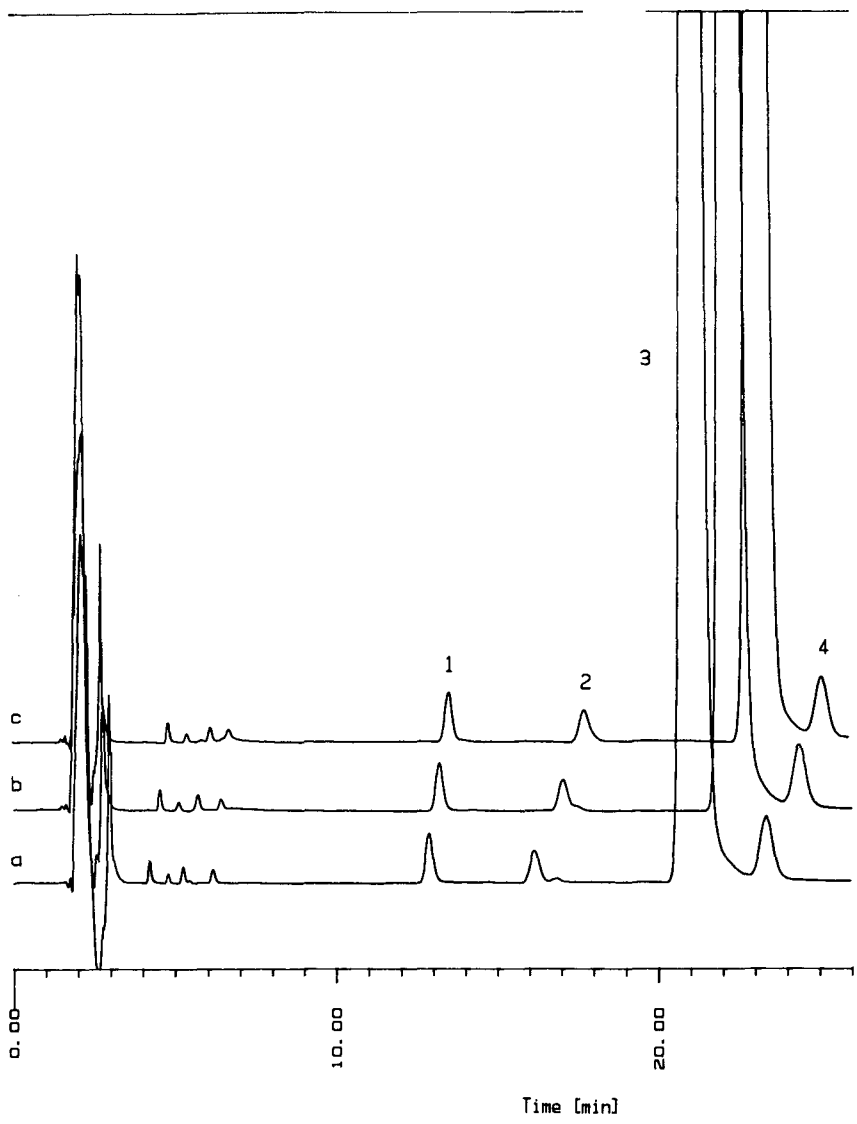


Fig. 8. Chromatograms obtained at different column temperatures using acetonitrile–water as eluent. Temperature: (a) 27.6°C; (b) 40.0°C; (c) 50.0°C. Other conditions as in Fig. 4B.

performing up to specified standards: minimal required value of resolution measured between two adjacent peak pairs to control the separation performance; approximate capacity ratio for one known component to calculate the possible retention of others and to control the detectability of late-eluting compounds; maximal allowable value of the peak asymmetry factor to control the condition of the separation column; approximate peak height measured for one known component chromatographed at a prescribed concentration using fixed detection and amplification parameters to

control the condition of detection; maximal allowable value of the relative standard deviation to control the precision of the HPLC method.

(c) Additional data to characterize the performance of the separation system, such as the column loadability, depending on the size of column hardware and the type of stationary phase in to column, the lowest detectable amount for one or more components and detection limit, and peak purity test to verify the homogeneity of a chromatographic peak.

Various definitions of the parameters for HPLC method validation and their determination have been published[5–8].

Validation data for the determination of isomeric and non-isomeric impurities in levonorgestrel are given in Table I of the data elements mentioned above, the following are not mentioned in Table I: (i) ruggedness (inter-laboratory reproducibility), owing to a lack of the necessary experiments; (ii) column loadability, as it is a function of the γ-CD concentration used in the eluent; for the experiments the column was loaded with 10 μg of levonorgestrel without any change in the separation characteristics; (iii) detection limit, as the investigations were focused on the determination of lowest detectable amount of the L-isomer; and (iv) peak purity, as the separation system in the

TABLE I

METHOD VALIDATION DATA

Conditions: column, Ultrasphere ODS; eluent, methanol–water (45:55) containing 0.01 mol/l γ-CD; flow-rate, 1 ml/min; detection, 244 nm; injection, 20 μl of 0.05% methanolic solution. Compounds: 1 and 2 = non-isomeric impurities; 3 = levonorgestrel; 4 = L-norgestrel.

Parameter	*Limit*	*Found*
Accuracy (determined for 1% L-norgestrel content		±1.22%
Precision (for 5 injections):		
Peak 1 (level: less than 1%)		±2.71%
Peak 2 (level: less than 1%)		±2.23%
Levonorgestrel	Max. ±2.0%	±0.51%
L-Norgestrel (level: less than 1%)		±3.35%
Day-to-day reproducibility:		
Peak 1 (level: less than 1%)		±2.25%
Peak 2 (level: less than 1%)		±2.42%
Levonorgestrel	Max. ±2.0%	±0.52%
L-Norgestrel (level: less than 1%)	Max. ±5.0%	±3.42%
Range:		
Levonorgestrel		10 ng–10 μg
L-Norgestrel		10 ng–10 μg
Detector linearity equations:		
Levonorgestrel: $y = -0.251 + 37.233x$; R(square) = 1.000		
L-Norgestrel: $y = -24.276 + 37.667x$; R(square) = 1.000		
Peak asymmetry (for levonorgestrel)	Max. 1.5	1.04
Required resolution (determined for compounds 3 and 4)	Min. 2.0	3.05
Capacity ratio (for levonorgestrel):	~7.5	7.7
Lowest detectable amount:		
L-Norgestrel	0.1%	0.1%
Others	0.05%	0.05%
Relative standard deviation (levonorgestrel)	Max. 2%	0.51%

absence of γ-CD was investigated in that respect for non-isomeric impurities and it was proved that the peak of levonorgestrel is pure, and only the enantiomeric impurity can co-elute with the main component. In the presence of γ-CD this impurity can be satisfactorily separated.

CONCLUSIONS

From the experimental data in Table I the following conclusions can be drawn. γ-CD forms a strong inclusion complex with D- and L-norgestrel and as little as 0.1% of enantiomeric impurity in levonorgestrel can be determined with adequate accuracy and precision. The method is suitable for the simultaneous determination of other impurities at low concentrations, providing the same quantitative data as can be obtained by reversed-phase chromatography. The detector signal is proportional to the concentration over a wide concentration range, linear correlations between the concentrations and detector responses (peak areas) being obtained both for L- and D-isomers.

The results also indicate the advantageous application of methanol–water as the eluent, giving excellent resolution between the enantiomers of norgestrel and an acceptable separation of the other impurities. The temperature dependence of the capacity ratios indicates a slight deterioration of the separation characteristics at elevated temperature, which can be explained by the increasing disturbance of inclusion complex formation by the presence of an organic solvent in the eluent.

REFERENCES

1 M. Gazdag, G. Szepesi and L. Huszár, *J. Chromatogr.*, 351 (1986) 128.
2 *United States Pharmacopeia XXI*, U.S. Pharmacopeial Convention, Rockville, MD, 1985, p. 746.
3 *United States Pharmacopeia XXI*, U.S. Pharmacopeial Convention, Rockville, MD, 1985, Suppl. 1, p. 1738.
4 *British Pharmacopoeia 1980, Addendum 1983*, H. M. Stationary Office, London, 1984, p. 240.
5 *Pharmacopoeial Forum*, U.S. Pharmacopeial Convention, Rockville, MD, 1986, p. 1241.
6 D. R. Williams, *BioPharm*, (1987) 34.
7 E. Debesis, J. P. Boehlert, T. E. Givand and J. C. Sheridan, *Pharm. Tech.*, (1982) 120.
8 E. B. Sheinin, *Pharm. Tech.*, (1986) 82.
9 M. Gazdag, G. Szepesi and K. Mihályfi, *J. Chromatogr.*, submitted for publication.

Journal of Chromatography, 450 (1988) 157–162
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 693

HEXAHELICENE CHIRAL STATIONARY PHASE

I. PHASE SYNTHESIS AND USE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS

S. A. MATLIN* and V. E. STACEY
Chemistry Department, The City University, Northampton Square, London EC1V 0HB (U.K.)
and
W. J. LOUGH
Beecham Pharmaceuticals, Medicinal Research Centre, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD (U.K.)

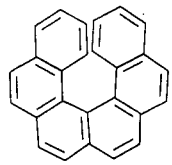
SUMMARY

Hexahelicen-7-ylacetic acid methyl ester was synthesized and resolved on a column of (+)-2-(2,4,5,7-tetranitro-9-fluorenylideneamino-oxy)propionic acid on aminopropyl silica. Following hydrolysis, the (+)-hexahelicen-7-ylacetic acid was bonded by carbodiimide coupling to aminopropyl silica. The resulting chiral bonded phase was shown to be effective for the resolution of nitroaryl-containing enantiomers.

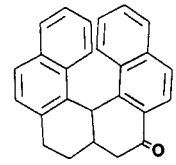
INTRODUCTION

The helicenes are polynuclear hydrocarbons which are chiral by virtue of a helical twist resulting from molecular overcrowding[1]. The first example[2], hexahelicene (I), was originally resolved by crystallization of its diastereomeric charge transfer complex with one enantiomer of 2-(2,4,5,7-tetranitro-9-fluorenylideneamino-oxy)propionic acid (TAPA)[3]. More recently, a variety of helicenes have been resolved on high-performance liquid chromatographic (HPLC) phases containing TAPA[4–6], an atropisomer of a binaphthyl-2,2-diyl hydrogen phosphate[7,8], N-2,4-dinitrophenyl α-aminoamide phases[9,10] and riboflavin[11], in many cases with high separation factors (α). The observation that several chiral HPLC phases, containing a wide range of structural classes of enantiomers and usually including an electron-deficient centre such as a nitroaryl ring, are capable of resolving helicene enantiomers prompted us to consider a reversal of roles. It was anticipated that the inclusion of a helicene enantiomer in a bonded stationary phase would provide an HPLC packing with broad resolving power for several classes of enantiomers. This hypothesis received encouragement from the demonstration by Kim *et al.*[12] that an enantiomer of hexahelicene-7,7-dicarboxylic acid, coated on silica, will resolve N-2,4-dinitrophenyl amino acid esters by HPLC. In the present work, the objective was to prepare a chemically bonded, rather than physically coated, stationary phase which would be

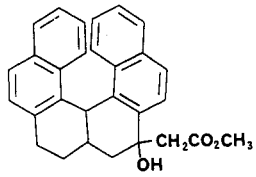
0021-9673/88/$03.50



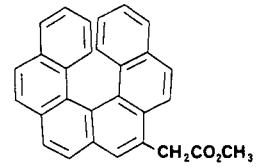
I



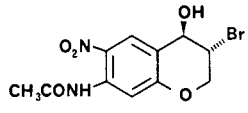
II



III



IV



V

stable to hydrolysis and solvent stripping, permitting a wide range of mobile phases to be employed.

EXPERIMENTAL

Synthesis of hexahelicen-7-ylacetic acid

A 2 g mass of 3,4,4,5,6,12*c*-hexahydrophenanthro[3,4-*c*]phenanthren-3-one[2] (II), 11.6 ml of lithium bis(trimethylsilyl)amide and 0.92 ml of methyl acetate were allowed to react[13] together for 2 h, and the solution was diluted with water and extracted with dichloromethane. After drying and evaporation, 3,4,4,5,6,12*c*-hexahydro-3-hydroxyphenanthro[3,4-*c*]phenanthrene-3-acetic acid methyl ester was obtained, yield 2.2 g (92%). The 1.68 g of this hydroxyester was dehydrated[14] by dissolving in dichloromethane and drying the solution onto 4.8 g of iron(III) chloride–silica. After standing for 2 days in a desiccator, 1.2 g (75%) of the resulting unsaturated ester was eluted from the solid with dichloromethane. Final dehydrogenation was achieved by melting 1.0 g of the unsaturated ester with 0.16 g of sulphur at 230° for 5 h, dissolving the cooled residue in dichloromethane and filtering through silica. Evaporation afforded hexahelicen-7-ylacetic acid methyl ester, one peak on HPLC, and M^+ *m/z* 400 (electron impact mass spectrometry).

Resolution of the ester was effected by preparative HPLC on a 50 cm × 22 mm

I.D. column packed with TAPA/aminopropylsilica (APS) (see below), using a mobile phase of acetonitrile–dichloromethane–*n*-hexane (4:10:86, v/v/v), eluted at 20 ml/min, monitored at 254 nm. The (+)-enantiomer was eluted first, with baseline separation.

After resolution, the ester was hydrolysed in quantitative yield by stirring a 0.5% (w/v) solution in methanol containing excess sodium hydroxide, for 24 h. The acid was recovered by dilution with water, acidification with concentrated hydrochloric acid and extraction into dichloromethane.

Synthesis of bonded phases

TAPA/APS. A 100 g mass of aminopropyl silica (from 5 μm Hypersil silica, as described previously[15]) was washed with methanol–acetic acid, then dilute ammonia (pH 9), and suspended in 200 ml of HPLC-grade tetrahydrofuran (THF). Then 11 g of (+)-TAPA dissolved in 50 ml of THF were added and the mixture was stirred for 48 h. The solid was filtered off, washed with THF until the filtrate was colourless and then dried *in vacuo* (yield 106 g). Microanalysis showed that 25.8% of the available amino groups had reacted to form the TAPA salt.

Hexahelicen-7-ylactic acid/APS. A 0.47 g mass of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-4-toluenesulphonate[8] (CDI; Aldrich Chemicals) was dissolved in 50 ml of far-UV grade acetonitrile and to this was added dropwise 0.44 g of (+)-hexahelicen-7-ylacetic acid in 50 ml of the same solvent, followed by 4.0 g of aminopropyl silica. After stirring overnight, the solid was filtered off, washed several times with acetonitrile–dichloromethane, then dried *in vacuo*. Microanalysis showed that 33.5% of the available amino groups had reacted with the acid.

Column packing

General apparatus and methodology for column packing analytical and preparative columns have been described previously[15–18]. Both the TAPA/APS and hexahelicene bonded phases were high-pressure slurry packed using isopropanol–*n*-hexane (5:95, v/v) as mobile phase.

HPLC apparatus

Separations were performed using a Waters 6000A pump, a Rheodyne 7125 injection valve fitted with a 20-μl sample loop, a column (25 cm × 0.45 cm I.D.) with zero dead volume fittings (HPLC Technology, U.K.), a Cecil 2112 variable-wavelength UV detector fitted withan 8-μl flow-cell and a Linseis recorder. Alternatively, a microbore HPLC system was used, consisting of a Kontron 420 pump, a Rheodyne 7410 injection valve fitted with a 1-μl loop, a 25 cm × 0.1 cm I.D. column, a Kontron 432 variable-wavelength UV detector and an LDC CI 10B computing integrator and printer/plotter.

Mobile phases were prepared using vacuum and ultrasonically degassed HPLC-grade solvents (Rathburn Chemicals, Walkerburn, U.K.).

RESULTS AND DISCUSSION

The ketone (II), an intermediate in Newman's synthesis[2] of hexahelicene, reacted with the anion derived from methyl acetate and the resulting product (III) was dehydrated and dehydrogenated to furnish racemic hexahelicen-7-ylactic acid methyl

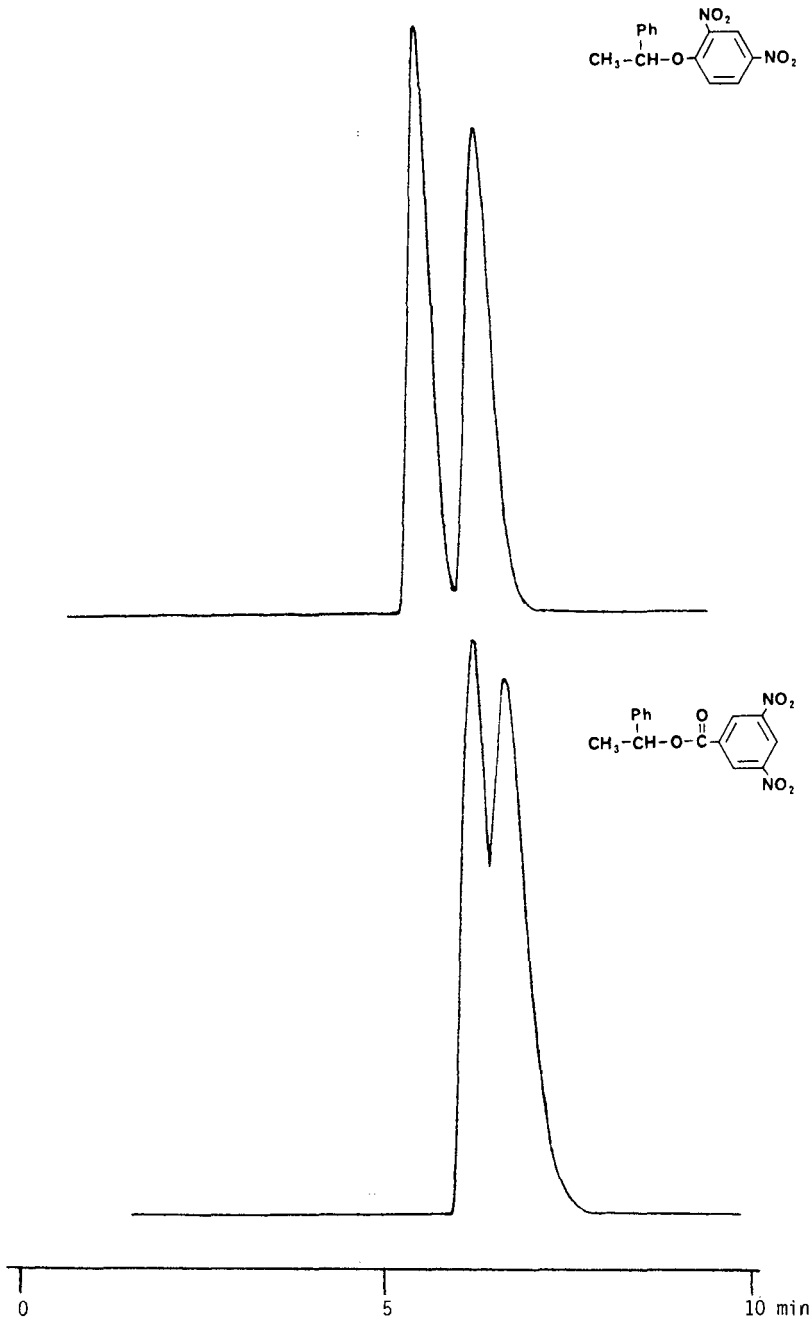


Fig. 1. HPLC resolution of enantiomers of the 2,4-dinitrophenyl ether and 3,5-dinitrobenzoate ester of 1-phenylethanol. Column, 25 × 0.45 cm I.D. hexahelicen-7-ylacetyl aminopropyl Hypersil (5 μm); mobile phase, isopropanol–*n*-hexane (10:90, v/v) at 2.0 ml/min, monitored at 254 nm × 0.1 a.u.f.s.; injections, 20 μl of solution, *ca.* 0.3 mg/ml in mobile phase. Ph = Phenyl.

ester (IV). This was resolved into its enantiomers by preparative HPLC on a column containing (+)-TAPA as the salt form on aminopropyl silica. The (+) isomer eluted first. Hydrolysis afforded (+)-hexahelicen-7-ylacetic acid, which was coupled to aminopropyl silica using a water-soluble carbodiimide reagent (CDI) to form the amide bond. Microanalysis showed that one third of the available aminopropyl groups had reacted and it is believed that essentially all of the hexahelicenyl material is present as the amide, rather than salt form. This follows from the method of synthesis and is supported by (i) the lack of column bleed when substantial amounts of isopropanol were used in the mobile phase and (ii) the long-term reproducibility of both retention times and resolution when the same separation was repeated after months of use. The bonded phase was packed into a conventional HPLC column and also a microbore column.

Preliminary evaluation indicates that the new phase is effective for the resolution of a variety of compounds containing one or more chiral centres and an electron-deficient aromatic ring. 1-Phenylethanol was chosen as a chiral compound having a reactive functional group at the chiral centre. The racemic alcohol was not itself resolved on the hexahelicenyl bonded phase when eluted with isopropanol–*n*-hexane, but its 2,4-dinitrophenyl derivative gave almost baseline separation within 7 min when eluted with 20% 2-propanol in hexane on the packed column (Fig.1). Under the same conditions, the 3,5-dinitrobenzoate ester eluted with a similar retention time as a pair of partially resolved peaks (Fig.1).

As a more complex example, the nitrochroman (V) contains two chiral centres and was synthesized as a racemic bromohydrin from the corresponding achiral chromene. The enantiomers were resolved virtually to baseline in a few minutes (capacity factors 3.7, 4.7) on the microbore column eluted with isopropanol–*n*-hexane (10:90, v/v). Following this result, an extensive series of chiral nitrochroman derivatives and analogues have been investigated to determine the specific structural requirements for enantiomeric separation. Full details will be reported elsewhere[19].

ACKNOWLEDGEMENTS

We thank the SERC and Beecham Pharmaceuticals for a CASE award and City University for equipment funds for HPLC.

REFERENCES

1 R. H. Martin, *Angew. Chem. Int. Ed. Engl.*, 13 (1974) 649.
2 M. S. Newman and D. Lednicer, *J. Am. Chem. Soc.*, 78 (1956) 4765.
3 M. S. Newman, W. B. Lutz and D. Lednicher, *J. Am. Chem. Soc.*, 77 (1955) 3420.
4 F. Mikes, G. Boshart and E. Gil-Av, *J. Chem. Soc., Chem. Comm.*, (1976) 99.
5 F. Mikeš, G. Boshart and E. Gil-Av, *J. Chromatogr.*, 122 (1976) 205.
6 W. J. C. Prinsen and W. H. Laarhoven, *J. Chromatogr.*, 393 (1987) 377.
7 F. Mikeš and G. Boshart, *J. Chem. Soc., Chem. Comm.*, (1978) 173.
8 F. Mikeš and G. Boshart, *J. Chromatogr.*, 149 (1978) 455.
9 S. A. Matlin, A. Tito-Lloret, W. J. Lough, D. G. Bryan, T. Browne and S. Mehani, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4(1981) 81.
10 C. H. Lochmüller and R. R. Ryall, *J. Chromatogr.*, 150 (1978) 511.
11 Y. H. Kim, A. Tishbee and E. Gil-Av, *J. Am. Chem. Soc.*, 102 (1980) 5915.
12 Y. H. Kim, A. Tishbee and E. Gil-Av, *J. Chem. Soc., Chem. Comm.*, (1982) 1336.

13 R. W. Rathke, *J. Am. Chem. Soc.*, 92 (1970) 3222.
14 A. Fadel and J. Salaun, *Tetrahedron*, 41 (1985) 413.
15 S. A. Matlin and J. S. Tinker, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 507.
16 S. A. Matlin and L. Chan, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 570.
17 S. A. Matlin and L. Chan, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 23.
18 S. A. Matlin, A. Belenguer, R. G. Tyson and A. N. Brookes, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 86.
19 J. M. Evans, W. J. Lough, R. S. Oliver, S. A. Matlin and V. E. Stacey, in preparation.

Journal of Chromatography, 450 (1988) 163–168
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 851

CHIRAL STATIONARY PHASE DERIVED FROM L-LACTIC ACID FOR THE OPTICAL RESOLUTION OF N-(3,5-DINITROBENZOYL)AMINO ACID METHYL ESTERS

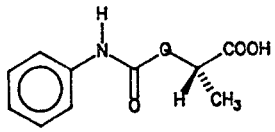
P. SALVADORI*, D. PINI, C. ROSINI, G. UCCELLO-BARRETTA and C. BERTUCCI
Centro CNR Macromolecole Stereordinate ed Otticamente Attive, Dipartimento di Chimica e Chimica Industriale, Via Risorgimento 35, I-56100 Pisa (Italy)

SUMMARY

A new chiral stationary phase is obtained by linking a simple derivative of L-lactic acid, (*S*)-(−)-(2-phenylcarbamoyloxy)propionic acid, to γ-aminopropylsilanized silica gel. N-(3,5-Dinitrobenzoyl)amino acid methyl esters, were separated on the new phase with separation factors between 1.2 and 1.5.

INTRODUCTION

A great variety of chiral stationary phases (CSPs) for the chromatographic resolution of racemates have been prepared in the last few years (see, *e.g.*, refs. 1 and 2). Often, the linkage of an optically active residue, such as a polymer of natural[3–6] or synthetic[7,8] origin, and a low-molecular-weight organic molecule[2] to a chemically modified silica support provides the chiral gel to be employed in the optical resolution. In our study aimed at preparing chiral phases derived from simple, optically active molecules that are easily available and inexpensive, we have introduced[9–14] a new family of phases derived from *Cinchona* alkaloids. In this context our attention has been attracted by a simple derivative of L-lactic acid, (*S*)-(−)-(2-phenylcarbamoyloxy)propionic acid (carbamalactic acid) (CBL) (I), that was recently used as resolving agent for amines[15]. The most interesting features of this compound are: (1) easy preparation in high yield from ethyl lactate, an inexpensive, optically pure, and commercially available product; (2) presence of different functional groups directly linked to a chiral center; (3) presence of a carboxyl group, which can be exploited to immobilize this chiral compound on γ-aminopropylsilanized silica gel.



I

In this paper we will discuss the preparation and use of such a CSP for the resolution of 3,5-dinitrobenzamides of α-amino acid methyl esters, *i.e.* simple deriv-

0021-9673/88/$03.50

atives of an important class of compounds, which, both in L and D form, are widely applied in food and pharmaceutical[16] industry, in organic chemistry[17] and in biochemistry.

EXPERIMENTAL

The chromatographic separations were carried out with a JASCO Twinkle apparatus connected to a JASCO Uvidec-100V UV detector. Optical rotary power was measured with a Perkin-Elmer 241 polarimeter (1-dm cell).

(S)-(−)-(2-Phenylcarbamoyloxy)propionic acid (carbamalactic acid) (I)

An 11.8-g (0.10-mol) amount of ethyl (*S*)-lactate and 15.5 g (0.13 mol) of phenylisocyanate in 20 ml of *n*-heptane were refluxed for 2 h. After evaporation of the solvent, the residue was stirred for 2 days with 10% aqueous sodium carbonate at room temperature; subsequent acidification of the solution with hydrochloric acid (1:1) allows to precipitate compound I. Recrystallization of crude I from chloroform gave 18.0 g (0.086 mol; 86% yield) of pure I, m.p. 141°C, $[\alpha]_D^{25}$ − 12.6 (c = 1.15, ethanol).

Preparation of the chiral stationary phase

Method A. LiChrosorb NH_2 (Merck, 5 μm, 4 g) was treated with 1.3 g (6.2 mmol) of acid I and 2 g (8.1 mmol) of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in 60 ml of methylene chloride. The slurry was stirred at room temperature for 48 h. After centrifugation, the solid was thoroughly washed with methanol until the disappearance of the absorption at 235 nm (A < 0.05, 1 cm cell), and dried under vacuum. An 0.104-g amount of chiral stationary phase CBL was hydrolyzed with 10% sodium hydroxide in water–methanol (1:1); the content of chiral residue, evaluated by spectrophotometry, was about 2.5% (w/w).

Method B. To a solution of 1.5 g (7.2 mmol) of acid I in 40 ml of dimethylformamide (DMF) were added 5 g of LiChrosorb NH_2 (Merck, 5 μm), 1.7 g (7.2 mmol) of benzotriazol-1-yl diethyl phosphate, prepared in quantitative yield as reported[18], and 1 ml (7.2 mmol) of triethylamine. The mixture was stirred at room temperature for 48 h. After centrifugation, the solid was washed and dried as reported in Method A. The content of chiral residue was about 2.9% (w/w).

This material was used for slurry packing (by conventional techniques) of 250 × 4.6 mm I.D. columns.

(R)(S)-N-3,5-Dinitrobenzoyl-α-amino acids

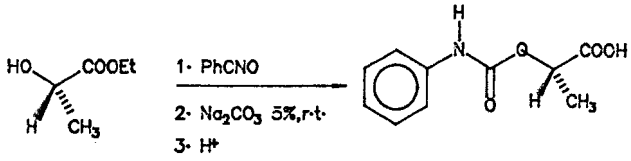
Typically, a solution of 1 mmol of amino acid, 1 mmol of 3,5-dinitrobenzoyl chloride and 3 mmol of propylene oxide in 30 ml of tetrahydrofuran, was stirred at room temperature for 15 h. The solvent was removed and the residue recrystallized from acetonitrile. In all cases, the yield was higher than 80%.

Methyl esters of (R)(S)-N-3,5-dinitrobenzoyl-α-amino acids

The (*R*)(*S*)-N-3,5-dinitrobenzoyl-α-amino acid was dissolved in a large excess of absolute methanol. The solution, saturated with hydrogen chloride gas, was refluxed for 1 h; the solvent was removed under vacuum and the residue recrystallized from acetonitrile. The yields were in the range 70–75%.

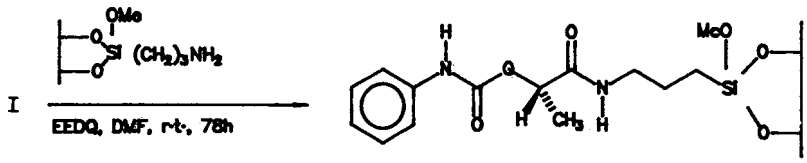
RESULTS AND DISCUSSION

Compound I ($[\alpha]_D^{25}$ − 12.6; c = 1.15, ethanol) was prepared[15] from ethyl L-lactate by reaction with phenylisocyanate in isooctane and successive saponification with aqueous dilute sodium carbonate:



Et = Ethyl; Ph = phenyl; r.t. = room temperature

Particular care was taken in the hydrolysis step: the use of a dilute solution of sodium carbonate at room temperature avoided the partial racemization of compound I observed in preliminary experiments carried out at 50–70°C. Linkage to the chromatographic support was accomplished by reacting compound I with γ-aminopropylsilanized silica gel, in the presence of a suitable coupling agent, forming an amide linkage:

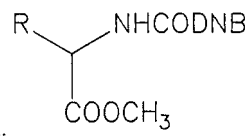


Me = Methyl

The use of EEDQ as the coupling agent should guarantee[19] that no racemization of the optically active acid takes place. After centrifugation, the solid was washed with methanol until the recovered washing solution did not show any appreciable absorption due to compound I (235 nm). The content of chiral residue present in the CSP was evaluated by hydrolysing a known amount of CSP in a methanolic sodium hydroxide solution and measuring the absorbance at 235 nm; this procedure provided a content of about 2.5% (w/w) (*i.e.*, 12 mmol per 100 g of support). The immobilization reaction was repeated with another condensing agent, to determine the dependence of the percentage of chiral residue introduced on the procedure used for making the amide linkage. 1-Benzotriazol-1-yl-diethylphosphate (BDP) was chosen as coupling agent because of its easy preparation and the high yields in amide formation recently reported[18]. The silica gel obtained contained *ca.* 2.9% chiral agent, determined as described above, indicating that the nature of the coupling agent did not affect to a large extent the amount of the chiral compound linked to the silica support. This support was then slurry packed, using conventional techniques, into a stainless-steel column (12.5 × 0.4 cm I.D.). The 3,5-dinitrobenzamides of several amino acids were prepared by reacting the amino acid with 3,5-dinitrobenzoyl chloride in tetrahydrofuran, in the presence of an excess of propylene oxide, as recently described[20]. Esterification was carried out by using ethereal diazomethane or methanol in the presence of hydrogen chloride gas.

TABLE I

SEPARATION OF 3,5-DINITROBENZOYLAMINO ACID METHYL ESTERS ON THE CSP DERIVED FROM L-LACTIC ACID



Ind = Indolyl; DNB = 3,5-dinitrobenzoyl.

Compound	*R*	k'_1	α	R_s	*A.C.**	*Eluent: hexane–2-propanol*
1	Methyl	8.20	1.31	1.2	–	95:5
2	*n*-Propyl	5.00	1.45	1.8	–	95:5
3	Isopropyl	4.75	1.31	1.0	*S*	95:5
4	*n*-Butyl	3.00	1.50	1.7	–	95:5
5	Isobutyl	5.25	1.57	2.0	–	95:5
6	*sec.*-Butyl	16.50	1.38	1.0	–	99:1
7	*tert.*-Butyl	10.50	1.21	1.0	–	99:1
8	$CH_3SCH_2CH_2$	8.80	1.47	1.8	*S*	95:5
9	Phenyl	6.80	1.26	1.0	*S*	95:5
10	Benzyl	6.60	1.30	1.3	–	95:5
11	Ind-CH_2	5.50	1.36	1.6	*S*	80:20
12	CH_3OOC-$(CH_2)_3$	8.60	1.39	1.5	*S*	70:30
13	DNB-NH-$(CH_2)_4$	2.00	1.25	1.0	–	80:20

* Absolute configuration of the most retained enantiomer.

The results obtained in separating the above compounds are summarized in Table I, and Fig. 1 shows the resolution of the 3,5-dinitrobenzoyl methionine methyl ester as an example. All of the compounds examined were separated with separation factors (α) greater than 1.2, and with resolution factors (R_s) greater than 1 (Table I). Seven of the essential amino acids (Val, Leu, Ile, Met, Phe, Trp and Lys) have been successfully separated on the present phase. By using samples enriched in the L form,

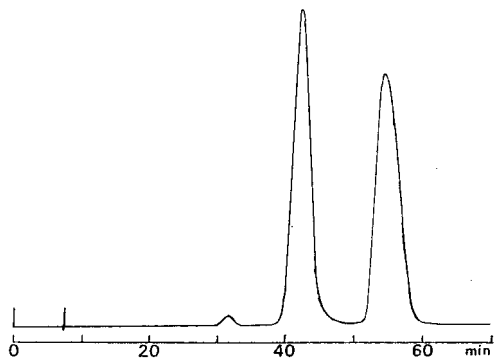
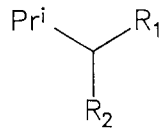


Fig. 1. Resolution of N-(3,5-dinitrobenzoyl)methionine methyl ester on the CSP. Eluent: hexane–2-propanol (95:5), flow-rate: 0.5 ml/min.

TABLE II

DEPENDENCE OF k'_1 AND α ON STRUCTURE, FOR COMPOUNDS OF FORMULA



Bz = Benzoyl; DNB = 3,5-dinitrobenzoyl; Pr^i = isopropyl. Eluent: hexane–2-propanol (95:5).

R_1	R_2	k'_1	α
NHDNB	$COOCH_3$	4.75	1.31
NHDNB	CN	9.50	1.05
NHBz	$COOCH_3$	3.75	1.00

it has been established that this enantiomer is the most retained on this CSP derived from L-lactic acid. The dependence of the capacity factor (k') and of α on the structure of the amino acid derivative cannot be described in a simple way. However, considering only the compounds where the R moiety is an alkyl group, it seems that α reaches its maximum value when R is a primary group, branching at the carbon in α position reducing the resolution. Additional interesting information can be obtained from Table II, which shows the dependence of k'_1 (capacity factor of the first eluted enantiomer) and α on the structure for a series of compounds related to valine. The most relevant results are as follows: (i) the presence of a 3,5-dinitrobenzoyl group on the substrate is essential for the chiral recognition; in fact its replacement with a benzoyl group reduces k'_1 and no resolution is achieved —this suggests the importance of interactions between π-acidic and π-basic groups in the chiral discrimination process; (ii) replacement of an ester function with another polar group such as –CN, which has, however, different chemical and geometrical characteristics, strongly reduces α —this might be a consequence of the absence of the carbonyl group in the compound listed in row 2, indicating the important rôle of this group in the chiral recognition process. To summarize and accepting the three-point rule[2] it can be stated that the interactions which are involved in the chiral recognition are: (1) a π–π interaction between the π-acidic 3,5-dinitrobenzoyl ring of the substrate and the phenyl group of the CSP; (2) hydrogen bond between the acidic N-H proton of the CSP and the carboxyl group of the esters of the substrate; (3) the third, discriminating interaction could be a steric repulsion (present in only one of the two possible diastereoisomeric adducts) involving the R group linked to the asymmetric center of the substrate.

CONCLUSIONS

A simple derivative of L-lactic acid, carbamalactic acid, can be used for preparing a new CSP by reacting it with γ-aminopropylsilanized silica gel; the chiral residue is covalently bonded to the chromatographic support. With this CSP and an organic mobile phase (*e.g.*, hexane–2-propanol), baseline separation of 3,5-dinitrobenzoyl-amino acid methyl esters can be obtained. The support has a high stability as demonstrated by the reproducibility of the α values after several months of use.

REFERENCES

1 R. W. Souter, *Chromatographic Separation of Stereoisomers*, CRC Press, Boca Raton, FL, 1985.
2 W. H. Pirkle and T. C. Pochapsky, *Adv. Chromatogr.*, 27 (1987) 352.
3 S. Allenmark, *J. Liq. Chromatogr.*, 9 (1986) 425.
4 J. Hermansson and M. Eriksson, *J. Liq. Chromatogr.*, 9 (1986) 621.
5 G. Schill, I. W. Wainer and S. A. Barkan, *J. Liq. Chromatogr.*, 9 (1986) 641.
6 A. Mannschreck, H. Koller and R. Wernicke, *Kontakte (Darmstadt)*, (1985) 40.
7 J. N. Kinkel, W. Frenkel and G. Blaschke, *Kontakte (Darmstadt)*, (1987) 3.
8 Y. Okamoto, *ChemTech*, (1987) 176.
9 C. Rosini, C. Bertucci, D. Pini, P. Altemura and P. Salvadori, *Tetrahedron Lett.*, (1985) 3361.
10 C. Rosini, P. Altemura, D. Pini, C. Bertucci, G. Zullino and P. Salvadori, *J. Chromatogr.*, 348 (1985) 79.
11 D. Pini, C. Rosini, C. Bertucci, P. Altemura and P. Salvadori, *Gazz. Chim. Ital.*, 116 (1986) 603.
12 C. Bertucci, C. Rosini, D. Pini and P. Salvadori, *J. Pharm. Biomed. Anal.*, 5 (1987) 171.
13 P. Salvadori, C. Rosini, D. Pini, C. Bertucci, P. Altemura, G. Uccello-Barretta and A. Raffaelli, *Tetrahedron*, 43 (1987) 4969.
14 C. Rosini, C. Bertucci, D. Pini, P. Altemura and P. Salvadori, *Chromatographia*, 24 (1987) 671.
15 E. Brown and F. Viot, *Tetrahedron Lett.*, (1985) 4451.
16 Y. Izumi, I. Chibata and T. Ito, *Angew. Chem., Int. Ed. Engl.*, 17 (1978) 178.
17 J. Martens, *Topics Curr. Chem.*, 125 (1984) 167.
18 K. Sunggak, C. Heung and K. K. Young, *Tetrahedron Lett.*, (1985) 1341.
19 B. Belleau and G. Malek, *J. Am. Chem. Soc.*, 90 (1966) 1651.
20 W. H. Pirkle and M. Ho, *J. Org. Chem.*, 49 (1984) 3043.

Journal of Chromatography, 450 (1988) 169–174
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 794

Note

Separation and identification of enantiomers by high-performance liquid chromatography with a chiral column and a polarimetric detector as applied to deltamethrin

C. MEINARD* and P. BRUNEAU

Division Scientifique Roussel UCLAF, Procida-CRBA St Marcel, 13011 Marseille Cedex (France)

The resolution of racemates has been widely studied. The physico-chemical properties of optical antipodes are identical, differing only in their opposite rotations in polarized light. The main methods of isolating these optically pure compounds are fractional recrystallization, stereospecific synthesis and enzymatic synthesis.

Gas chromatography has been attempted but has several limitations: the compound studied must show a certain volatility, high temperatures often cause racemization of the stationary phase, which results in a loss of enantioselectivity of the column with time, and preparative analysis is not generally possible in this event.

High-performance liquid chromatography (HPLC) with chiral supports has allowed the resolution of racemates. The specificity of these types of columns with respect to the substances to be studied has led to numerous theoretical studies on the separation mechanisms, thus permitting a better understanding and optimization of these separations[1–3].

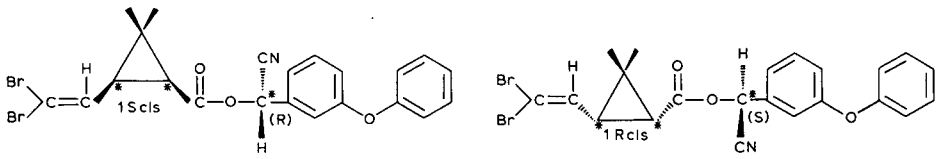
UV detection is necessary in this type of analysis as it is much more sensitive than polarimetric detection. UV detection makes it possible to identify and quantify isomers. Polarimetric detection allows the characterization of isomers through their action on polarized light, but it is much less sensitive unless a suitable computerized system is connected which amplifies the signal and reduces the background noise[4].

Recently, HPLC, owing to its high resolving power and the availability of various detection systems, has been used for the identification of diastereoisomers with a UV detector, the characterization of enantiomers with a polarimetric detector, allows the separation and identification of the optical antipodes, their characterization and their eventual quantification.

Pyrethroids often need to be subjected to diastereoisomeric and enantiomeric analysis. The resolution of racemates and the identification of optical antipodes is delicate and requires specific methods such as polarimetry or chiral phases in HPLC.

In this work, different techniques were applied to deltamethrin (RU 22974), its optical antipode (RU 40767) (Fig. 1) and the racemate (RU 43501). The difficulty lies in finding a chiral phase that will allow the resolution of the racemate studied. Much research has been carried out in this field and according to the classification established by Lienne *et al.*[5] we obtained seven classes of chiral supports which act differently and haved various powers of separation as regards the chemical structures to be separated.

0021-9673/88/$03.50



RU 40767 RU 22974

Fig. 1. Structure of deltamethrin (RU 22974) and its optical antipode (RU 40767).

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model 414 LC pump (Kontron, Zürich, Switzerland), a PU 4021 multi-channel UV detector equipped with a PU 4850 video chromatography control centre (Pye Unicam, Cambridge, U.K.) and a Model 241 LC polarimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) with a 0.65 mm I.D. microflow cell having a 10-cm optical pathway. The polarimeter was used at 302 nm and the resulting recorded range was $\pm 0.5°$ full-scale. The two detectors were connected by a stainless-steel capillary tube. A Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop was used.

Analytical conditions

All solvents were of spectrographic grade from Fluka (Buchs, Switzerland).

First, a standard LiChrosorb Si 60 (5 μm) column (250 mm × 4 mm I.D.) (Merck, Darmstadt, F.R.G.) was used. The mobile phase consisted of a mixture of 1900 ml of hexane, 45 ml of acetonitrile, 100 ml of pentane, 10 ml of dioxane and 1.5 ml of 2-propanol and the flow-rate was 1.3 ml/min.

Second, a Chiralpack $OT_{(+)}$ (5 μm) chiral column (250 mm × 4 mm I.D.) (Daicel Chemical Industries, Japan) thermostated at 10°C was used. The mobile phase was methanol–acetic acid (99:1) and the flow-rate was 0.5 ml/min.

RESULTS AND DISCUSSION

The analysis of deltamethrin and of its diastereoisomers and enantiomers has been reported previously[6,7].

Identification of enantiomers

In the first step, coupling a UV spectrometer and a polarimeter with a standard LiChrosorb Si 60 column allowed the identification and quantification of these enantiomers (Fig. 2). This method, which uses the ratio of the signals obtained from the polarimetric and UV detectors, allows a quantitative study to be performed. UV detection gives the sum of the signals of the two optical antipodes and the polarimeter the difference. Hence from the difference in signals we can determine the two optical antipodes in a mixture without separating them (Fig. 3). Baseline resolution of the racemate was not possible with the LiChrosorb Si 60 column.

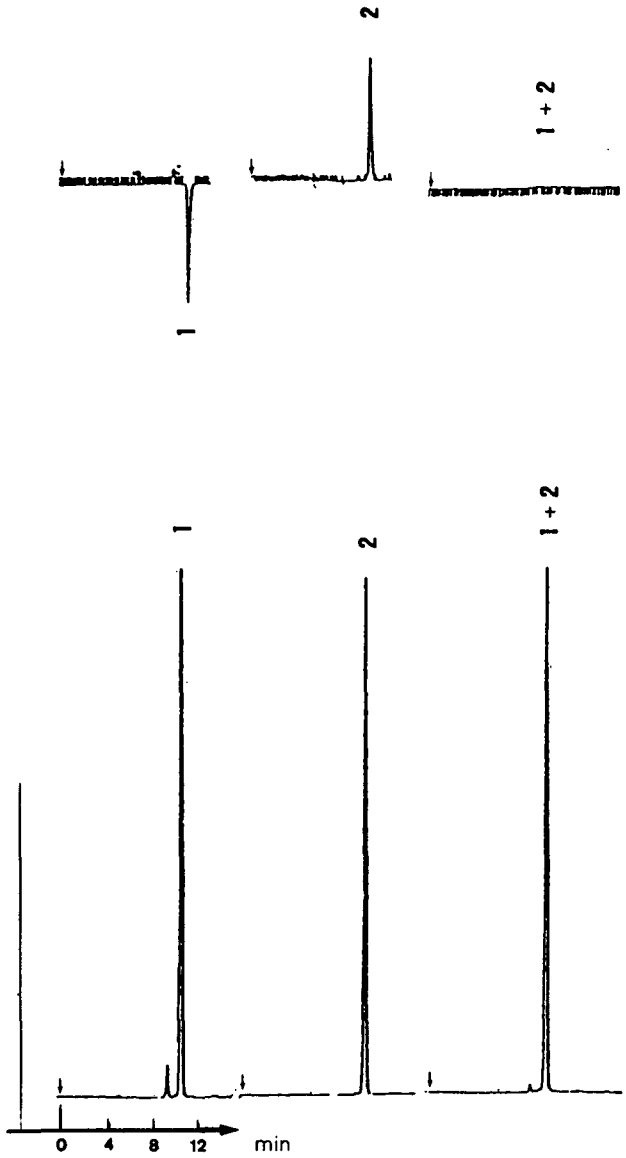


Fig. 2. Chromatograms of deltamethrin, optical antipodes and racemate obtained with a LiChrosorb SI 60 column; (A) UV detection; (B) polarimetric detection. Peaks: 1 = deltamethrin; 2 = optical antipode; 1 + 2 = racemate.

Separation of enantiomers

Our study concerned deltamethrin and its optical antipode (Ru 40767) (Fig. 1). The resolution of the racemate was achieved using the Daicel Chiralpack $OT_{(+)}$ column designed by Okamato *et al.*[8] (Figs. 4 and 5), and the enantiomers were quantitated with an accuracy of ± 5%.

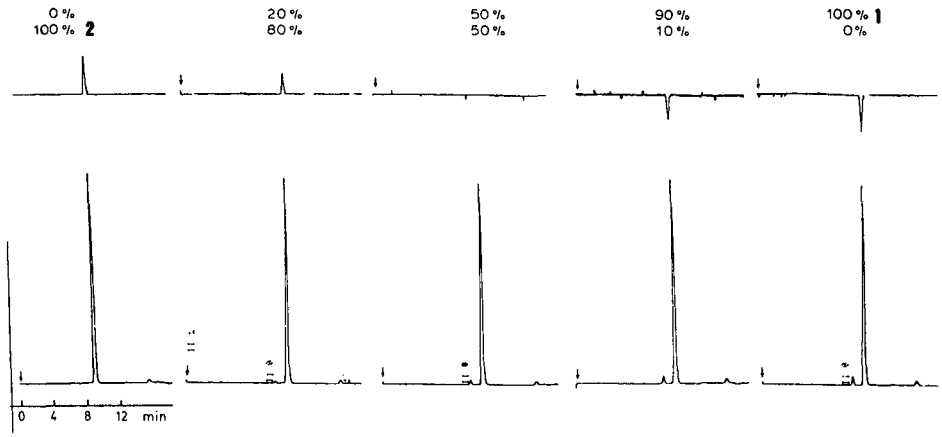


Fig. 3. Chromatograms obtained with different ratios of the enantiomers with UV (bottom) and polarimetric (top) detection. Peaks: 1 = deltamethrin; 2 = optical antipode.

The support consists of an isotactic polymer of triphenylmethyl methacrylate (PTrMA) (Fig. 6), presenting chiral centres and fixed on a macroporous silica gel. This polymer is made optically active by anionic polymerization in the presence of chiral catalysts which give a helical structure to the polymer chains[8,9].

This "impregnated" chiral stationary phase gives low capacity factors (k') and therefore short retention times, $T_r = T_0(1 + k')$. The enantioselectivity and resolution are high.

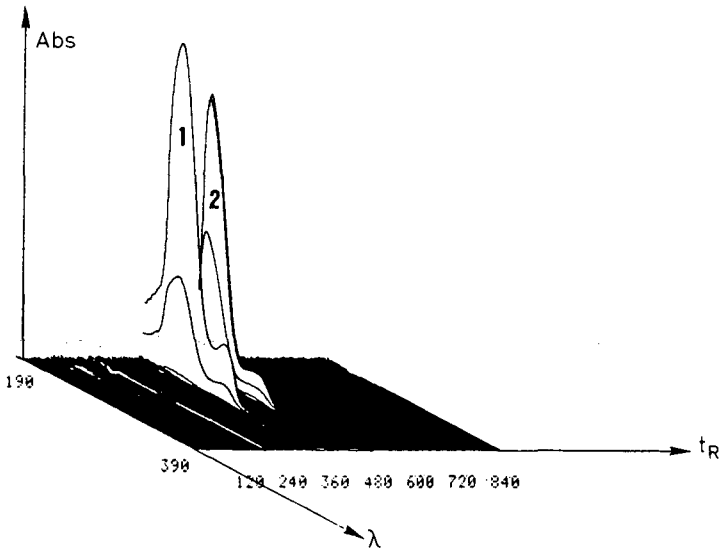
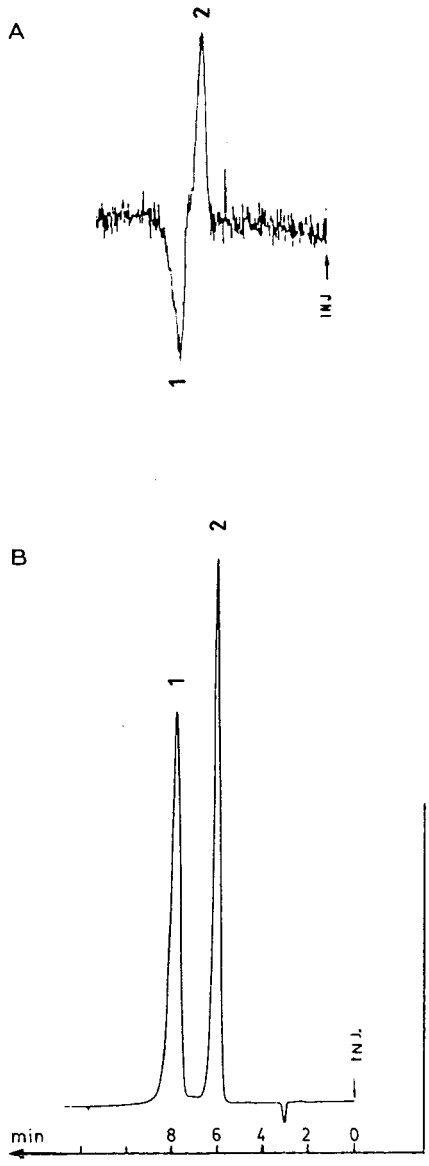


Fig. 4. Resolution of the racemate RU 43501 by HPLC with a Chiralpack $OT_{(+)}$ column, as described. Peaks: 1 = deltamethrin; 2 = optical antipode. t_R is retention time in s; λ = wavelength in nm.



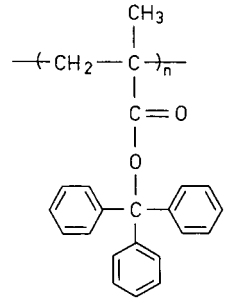


Fig. 5. Resolution of the racemate RU 43501 by HPLC with a Chiralpack $OT_{(+)}$ column: (A) polarimetric detection; (B) UV detection. Peaks: 1 = deltamethrin; 2 = optical antipode.

Fig. 6. Structure of the polymer of triphenylmethylmethacrylate (PTrMA).

PTrMA shows chirality owing to its helicity with a high specific rotatory power, $[\alpha]_D^{20} \geqslant 250$ in tetrahydrofuran. The recommended solvents are hexane, 2-propanol, ethanol, acetonitrile, methanol and water. Polar solvents usually give a better resolution. Thermostating of this type of column is sometimes required for better separation.

The importance of these hydrophobic interactions between solutes and the chiral stationary phase and the superimposition of these interactions on inclusion phenomena and steric overloading of the PTPMA group play a significant role in elucidating the mechanism of the phase. The explanation of this phenomenon and the description and production of this polymer have been reported[8–11].

CONCLUSIONS

This example shows the importance of these new HPLC chiral supports, which, when coupled with two types of detectors, UV and polarimetric, lead to the resolution of a racemate as well as the identification, separation, characterization and quantification of enantiomers in a mixture. They provide valuable help in research as regards determinations of structures and absolute configuration.

REFERENCES

1 W. H. Pirkle and R. Däppen, *J. Chromatogr.*, 404 (1987) 107.
2 D. W. Armstrong, *Anal. Chem.*, 59 (1987) 84A.
3 D. W. Armstrong, X. Yang, S. M. Han and R. A. Menges, *Anal. Chem.*, 59 (1987) 2594.
4 B. S. Scott and D. L. Dunn, *J. Chromatogr.*, 319 (1985) 419.
5 M. Lienne, M. Caude, A. Tambute and R. Rosset, *Analusis*, 15 (1987) 431.
6 C. Meinard, J. C. Suglia and P. Bruneau, *J. Chromatogr.*, 176 (1979) 140.
7 C. Meinard, P. Bruneau and J. Perronnet, *J. Chromatogr.*, 349 (1985) 109.
8 Y. Okamoto, S. Honda, I. Okamoto and H. Yuki, *J. Am. Chem. Soc.*, 103 (1981) 6971.
9 Y. Okamoto and K. Hatada, *J. Liq. Chromatogr.*, 9 (1986) 369.
10 M. Kamachi, K. Hatada and S. I. Nozakura, *Polym. J.*, 13 (1981) 919.
11 H. Kageyama, Y. Hayashi, S. Harada, Y. Kai and N. Kasai, *Makromol. Chem.*, 186 (1985) 203.

Journal of Chromatography, 450 (1988) 175–182
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 708

CYCLODEXTRIN–CHIRAL SUBSTRATE INTERACTIONS

HIGH FIELD NUCLEAR MAGNETIC RESONANCE AND MOLECULAR GRAPHICS STUDIES

A. W. COLEMAN* and G. TSOUCARIS

Laboratoire de Physique, ER CNRS 180, 4 Avenue J. B. Clément, 92296 Chatenay Malabry (France)

H. PARROT, H. GALONS and M. MIOCQUE

Laboratoire de Chimie Organique, UA CNRS 496, 4 Avenue J. B. Clément, 92296 Chatenay Malabry (France)

and

B. PERLY, N. KELLER and P. CHARPIN

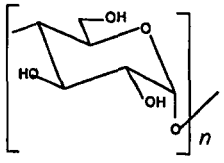
Cea-cen, Département de Physicochimie, UA CNRS 331, 91191 Gif-sur-Yvette (France)

SUMMARY

The inclusion compounds of (+)-, (−)- and (±)-menthol have been studied by 500-MHz nuclear magnetic resonance (NMR) spectroscopy in deuterium oxide and by molecular graphics techniques. The molecular graphics modelling suggests a slightly reduced degree of Van der Waals interactions in the case of (−)-menthol coupled with greater chemical inequivalence of the CH_3 groups of the isopropyl function. These results have been confirmed by ^1H NMR studies which show the CH_3 protons to be sensitive to the external environment.

INTRODUCTION

The cyclodextrins provide a cylindrical cavity for the inclusion of organic host molecules, and the saccharide molecules give a chiral environment. They have been used extensively as enzyme mimics[1] and more recently their use in asymmetric solid state reactions has been studied[2].

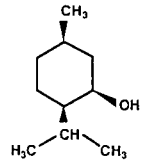


n = 6, 7 or 8

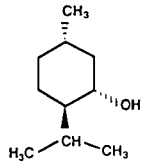
We are studying the chemical modification of the cyclodextrins to allow the complexation of metal complexes and their subsequent use in asymmetric synthesis. However, in order to design such compounds, knowledge of the inclusion and release

0021-9673/88/$03.50

of suitable organic substrates is vital. Menthol has been shown to undergo chiral separation on formation of inclusion compounds with β-cyclodextrin (β-CD), although the separation (5%) was low[3]. As the molecule possesses three potentially non-equivalent methyl groups we decided to study its inclusion compounds with β-cyclodextrin by 500-MHz NMR spectroscopy and molecular graphics.



(1*R*,2*S*,5*R*)-(−)-menthol



(1*S*,2*R*,5*S*)-(+)-menthol

EXPERIMENTAL

Chemicals

β-CD was obtained from Roquette. The (−)-, (+)- and (±)-menthol were obtained from Aldrich without purification. Deuterium oxide was prepared by CEA (Saclay, France).

Preparation of inclusion compounds

A sample of β-CD (2.0 g) was suspended in water (50 ml) and heated at 60°C with stirring. Menthol (0.4 g) was added to the solution and the heat source was removed. After stirring for 1 h at room temperature, the precipitate was collected and washed with diethyl ether.

Molecular graphics studies

The inclusion compounds of (+)- and (−)-menthol were studied using the SYBYL molecular graphics program[4]. The complexes were constructed via the Building subroutine using previously minimized structures for host and substrate molecules. During the course of the construction, optimum inclusion was obtained at a depth in which the hydroxyl group of menthol was at the same height as oxygen linking the saccharide residues and with an O–O distance of 2.5 Å. On this basis we propose a possible hydrogen bond which would stabilize the structure and, in consequence, this orientation was fixed. The structure was then energetically minimized using the Maximum 2 subroutine over 40 cycles, to yield final Van der Waals energies of -51.4 kcal mol^{-1} for β-CD–(+)-menthol and -52.1 kcal mol^{-1} for β-CD–(−)-menthol. The model structures are given in Figs. 1 and 2 respectively with the CH_3 groups of the isopropyl functions marked. The models suggest an inequivalence of the CH_3 groups in both complexes.

High field NMR studies

The 500-MHz NMR studies were undertaken using an upgraded Bruker WM 500 spectrometer with a process controller and an array processor for (+)-, (−)- and (±)-menthol complexes of β-CD in using [$^2H_6$] dimethyl sulphoxide and deuterium

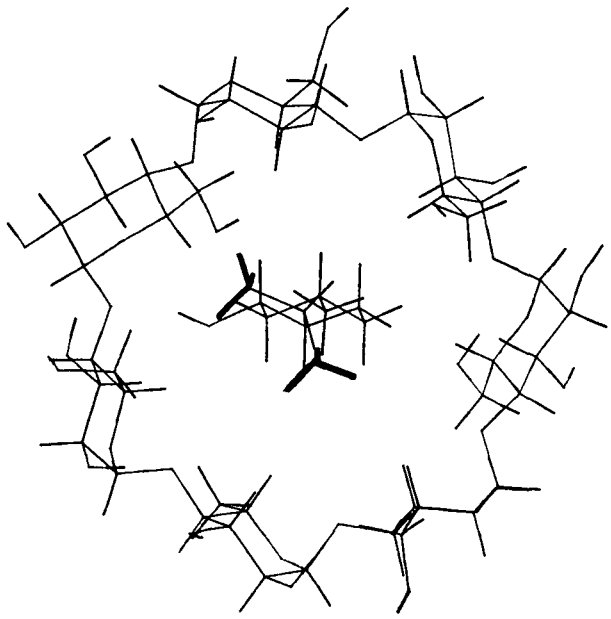


Fig. 1. Molecular graphics model of the β-CD–(+)-menthol inclusion complex.

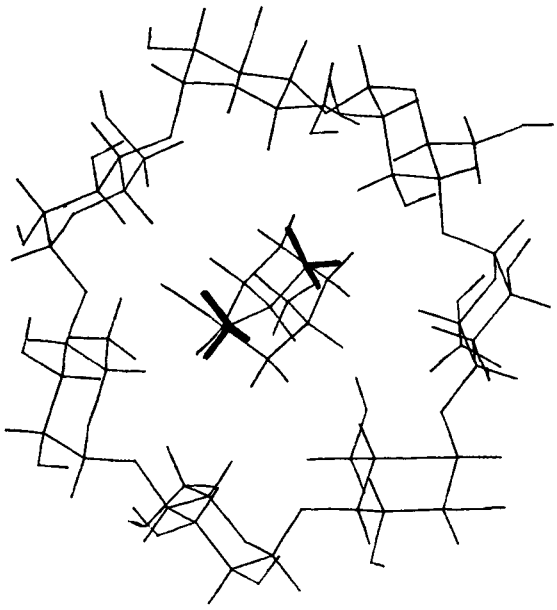


Fig. 2. Molecular graphics model of the β-CD–(−)-menthol inclusion complex.

oxide as solvents. In the case of deuterium oxide, the hydroxyl functions of β-CD were previously exchanged with deuterium and the compounds were prepared in deuterium oxide.

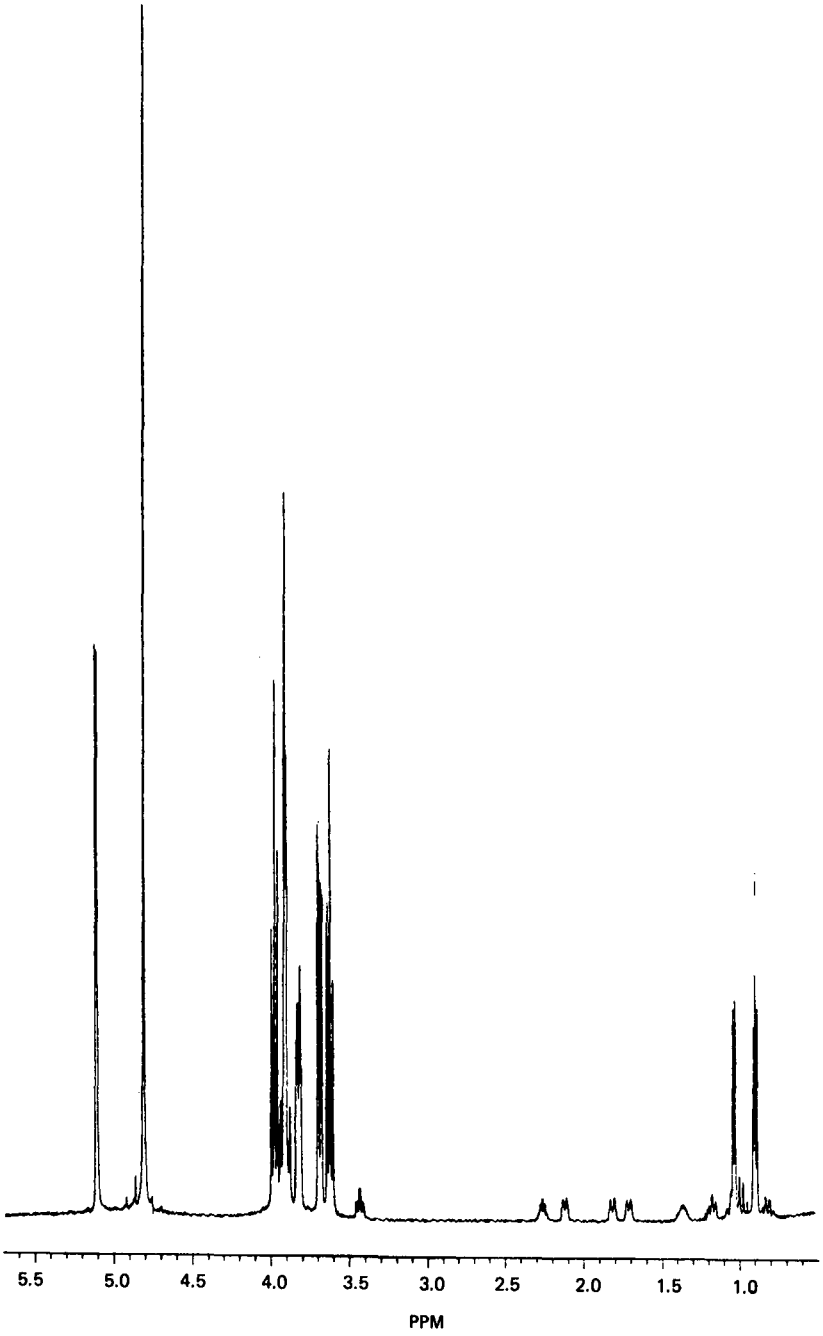


Fig. 3. ^{1}H NMR spectrum of the (+)-menthol–β-CD inclusion complex.

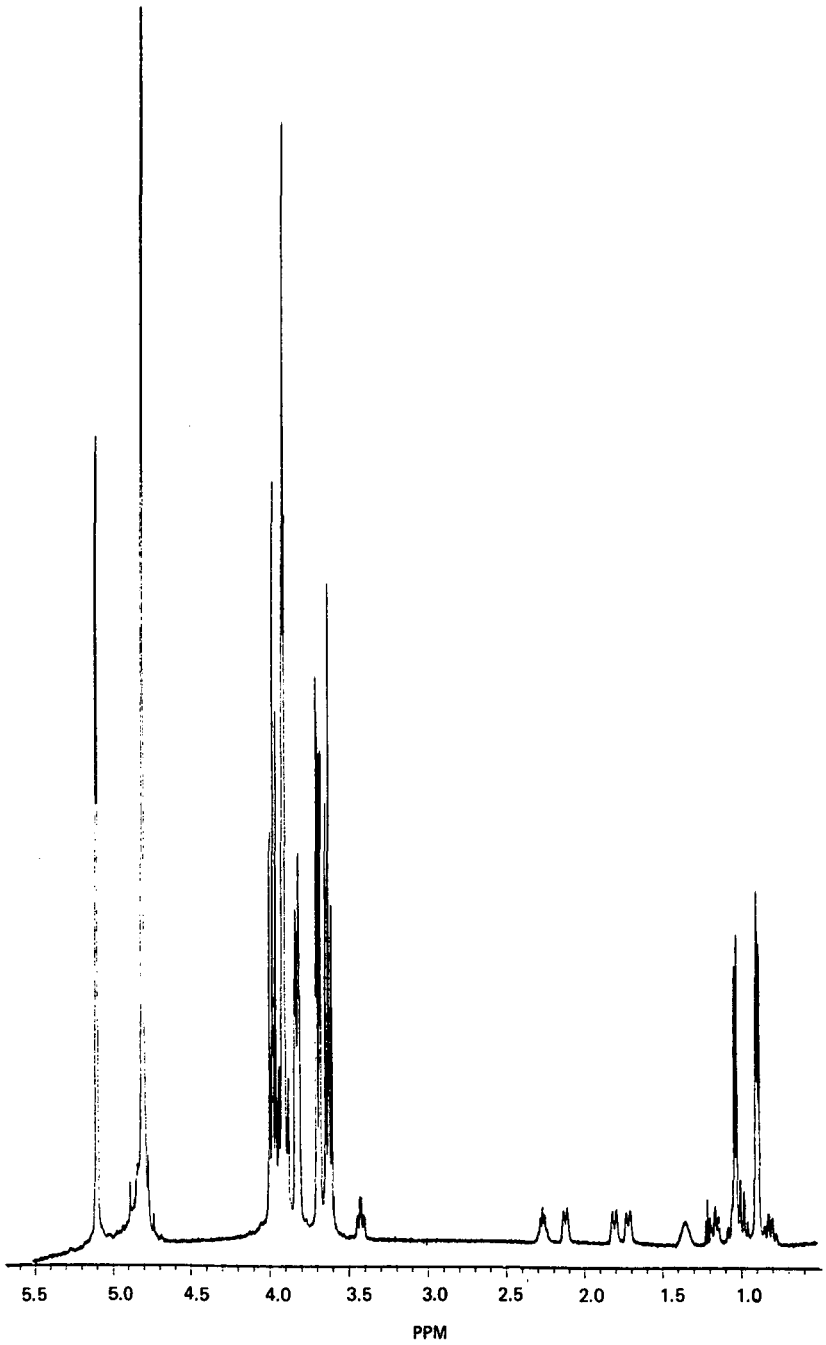


Fig. 4. ^{1}H NMR spectrum of the (−)-menthol–β-CD inclusion complex.

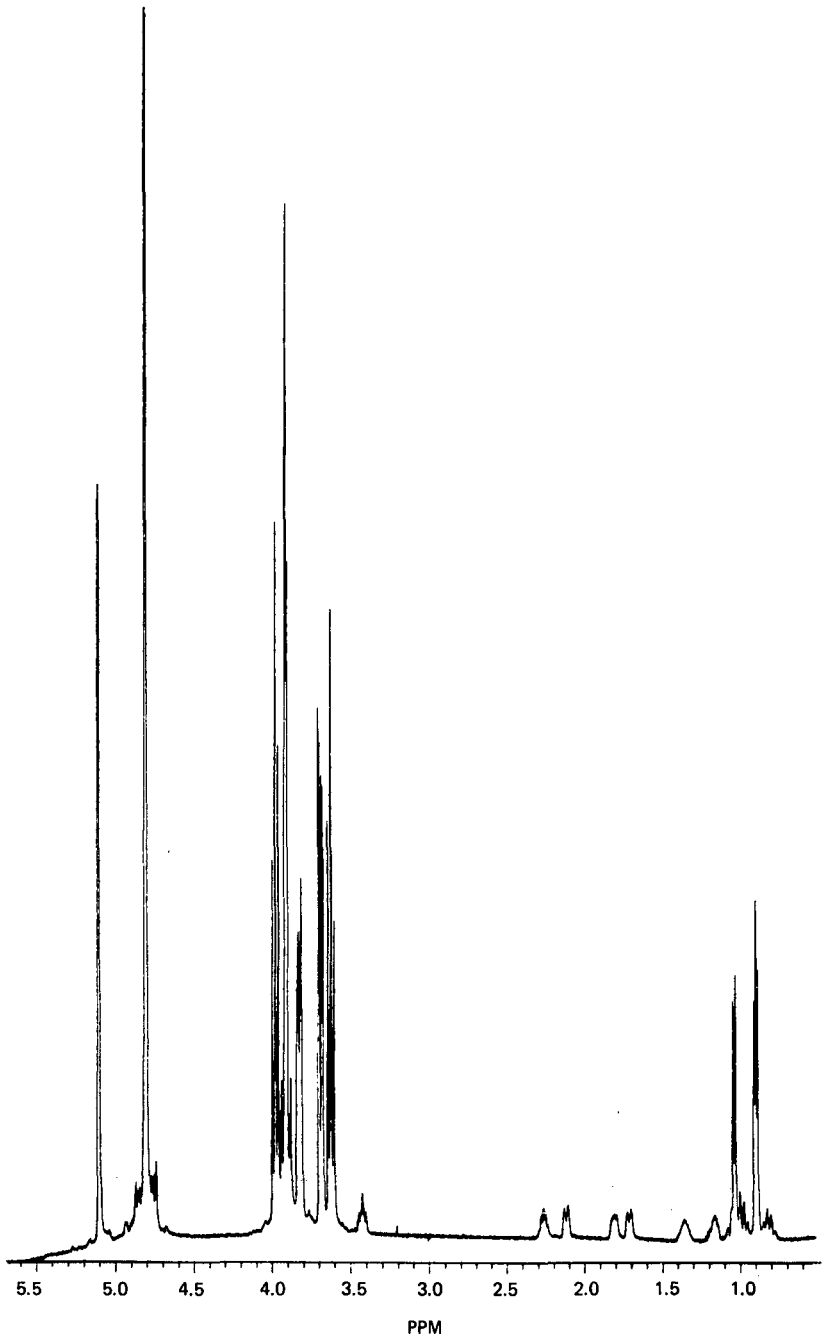


Fig. 5. ^{1}H NMR spectrum of the (±)-menthol–β-CD inclusion complex.

RESULTS AND DISCUSSION

For the spectra obtained in [$^2H_6$]dimethyl sulphoxide the formation of a 1:1 inclusion compound was confirmed. As inclusion complexes in this solvent are usually litte dissociated, further information was expected. However, in the case of (−)-menthol, considerable broadening of the β-CD peaks was observed; a possible explanation of this is that a slow exchange between the menthol inclusion complex and the dimethyl sulphoxide inclusion complex is taking place, confirming the greater stability suggested by molecular graphics.

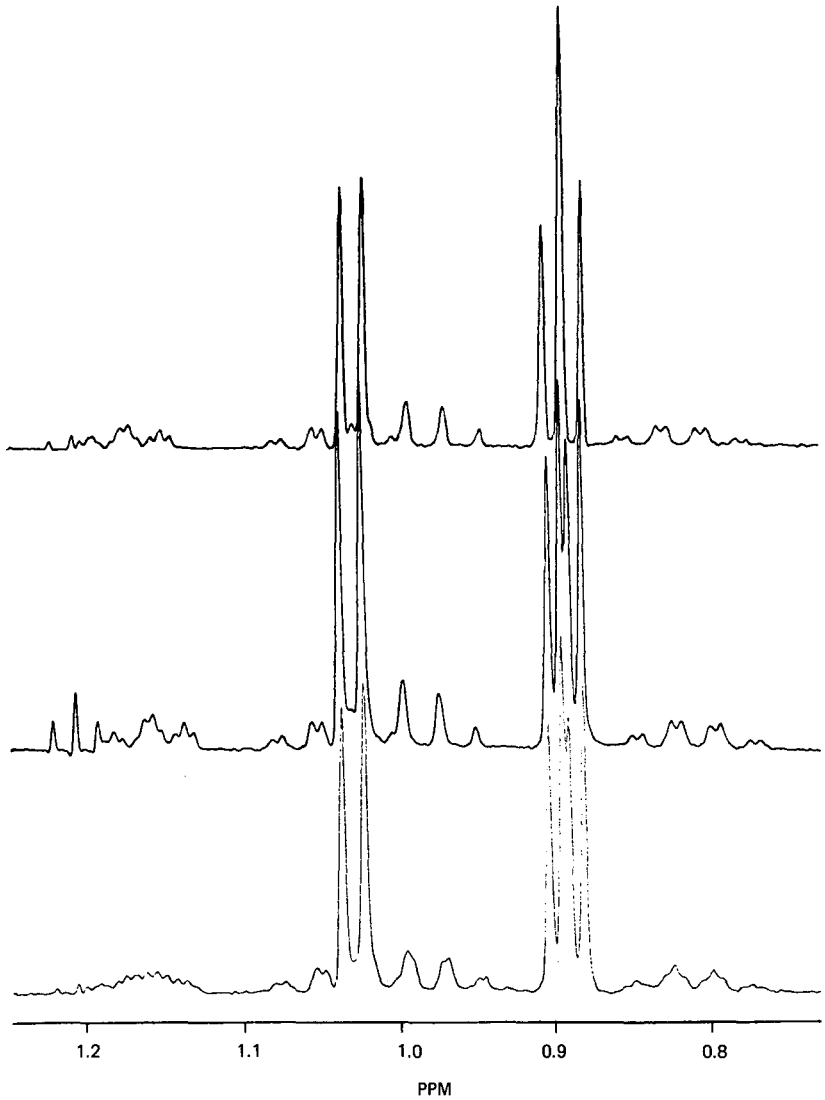


Fig. 6. Expansion of the methyl region in the ^1H NMR spectra of the inclusion complexes of (−)- (middle), (+)- (top) and (±)-menthol (bottom) β-CD.

The very low solubility of β-CD inclusion complexes in water has until now prevented the study of such species by NMR spectroscopy. The advent of ultra high field instruments with their higher sensitivity should now permit such experiments, and we give the 500-MHz spectra of (+)-menthol–β-CD, (−)-menthol–β-CD and (±)-menthol–β-CD in Figs. 3, 4 and 5 respectively. The doublet of the single CH_3 group is shifted from 0.7 to 1.03 ppm; the molecular graphics models place this group in the region of the primary hydroxyl groups of the cyclodextrin, which is consistent with this shift. In Fig. 6 is shown an expansion of the methyl region of the spectra of the three compounds; in the case of the CH_3 of the isopropyl groups a clear inequivalence is seen, which is more marked for (−)-menthol, again in agreement with the differing environments suggested by molecular graphics. In the case of the racemic mixture an excess of the (−)-menthol complex is demonstrated, in accord with results from NMR spectroscopy in [$^2H_6$] dimethyl sulphoxide and molecular graphics.

In conclusion, the combination of high field NMR spectroscopy and molecular graphics has allowed us to carry out a fundamental study of the interactions in molecular inclusion complexes leading to chiral separation. The NMR technique is a particularly potent probe in such investigations, allowing an highly detailed analysis of the compounds, whilst the molecular graphics of such compounds are somewhat less sophisticated. The results obtained here are promising for the future use of such methods in the design of specific chiral hosts for asymmetric separations.

REFERENCES

1 I. Tabushi, *Pure Appl. Chem.*, 58 (1986) 1529.
2 Y. Tanaka, H. Sakuraba, Y. oka and N. Nakanishi, *J. Inclusion Phenom.*, 2 (1985) 841.
3 D. Warsch and F. Vogtle, *Top. Curr. Chem.*, 140 (1987) 21.
4 *SYBYL Molecular Graphics Program*, Tripos, St. Louis, MO, 1988.

Journal of Chromatography, 450 (1988) 183–189
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 700

CHIRAL MICHAEL ADDITIONS OF ACETAMIDOMALONATE TO α-ENONES

NUCLEAR MAGNETIC RESONANCE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS

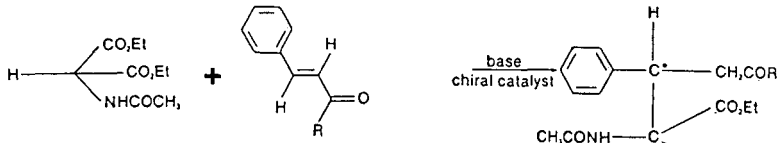
E. DELEE, I. JULLIEN* and L. LE GARREC
Recherche Syntex France, Leuville-sur-Orge, 91310 Montlhéry (France)
and
A. LOUPY, J. SANSOULET and A. ZAPARUCHA
Laboratoire des Réactions Sélectives sur Supports, Bât. 410, Université Paris-Sud, 91405 Orsay Cedex (France)

SUMMARY

Solid–liquid phase transfer catalysis without addition of solvent was used to induce asymmetric Michael addition. Determinations of enantiomer ratios by chiral liquid chromatography and by NMR chiral shifts using europium salts are compared. The effect of chiral phase transfer catalyst structure, addition of solvent and addition of molecular sieves are discussed. The best enantiomeric excess are obtained with N-benzyl-N-methylephedrinium salts in the absence of solvent and in the presence of molecular sieves.

INTRODUCTION

It was shown previously that solid–liquid phase transfer catalysis (PTC) without added solvent[1,2] efficiently promotes reactions of anionic nucleophiles on activated double bonds (Michael additions)[3,4]. For instance, it has been shown that, under these conditions, N-acetylamidomalonate (1) reacts easily with different α-enones[4] (Fig. 1).



1 2 a R = C_6H_5 b R = CH_3 3 d + l

Fig. 1. Scheme of the reaction. Et = Ethyl.

0021-9673/88/$03.50

During the course of this reaction, one asymmetric centre is created if the electrophile involved (2) is β-substituted. Thus, performing the reaction in the presence of a chiral ammonium salt can induce some asymmetric Michael addition. Few examples of such PTC asymmetric inductions have been described and they seem to be limited[5,6].

EXPERIMENTAL

Equimolar amounts of reactants 1 and 2 were mixed in the presence of catalytic amounts (6 mol.%) of base (potassium hydroxide or carbonate) and a chiral tetraalkylammonium salt, but in the absence of organic solvent. The mixture was heated for 1 h at 60°C under mechanical agitation. Organic products were then removed by addition of methylene chloride and filtration on Florisil[7]. For the sake of comparison, experiments could be performed in the presence of organic solvent or of molecular sieves.

The products were identified and characterized by gas chromatography and NMR spectroscopy. Enantiomers were determined by chiral high-performance liquid chromatography (HPLC) and NMR shifts using europium salts.

Assays for optical purity

Optical rotations. The relative *d* and *l* attributions of enantiomers 3 were obtained by determining the optical rotation of enantiomerically enriched samples (c = 2, acetone). The $[\alpha]_D^{25}$ values are 0 (racemic), +8°l (*d:l* = 81:19) and −7°l (*d:l* = 27:73).

Chiral liquid chromatography. A direct HPLC separation of enantiomers without any derivatization or isolation of compounds 3 (3a,R = C_6H_5; 3b,R = CH_3) was achieved using a chiral EnantioPac column. This well known protein-type (α_1-acid glycoprotein) column[8] has been used for compounds of many different chemical structures[9–11]. The optimized conditions employed for the separation of 3a enantiomers were as follows: eluent, 2-propanol–phosphate buffer (pH 7) (10:90, v/v), the

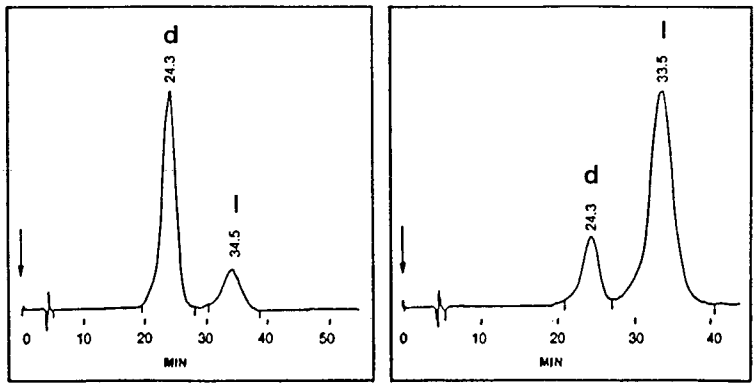


Fig. 2. HPLC resolution (R_s = 2.24) of compounds 3a on EnantioPac column. *d:l* = 81:19 (left) or 16:84 (right).

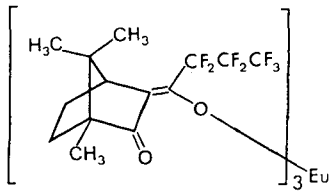


Fig. 3. Europium derivative used in NMR resolution: (+)- or (−)-[Eu(hfc)$_3$].

buffer composition being 4 m*M* NaH_2PO_4 + 4 m*M* Na_2HPO_4 + 10 m*M* NaCl; flow-rate, 0.25 (or 0.20) ml/min; temperature, 20°C; sample load, 1 nmol; UV detection, 243 nm, 0.02 a.u.f.s.

Under the above conditions, we obtained a resolution factor[12] (R_s) between 3.12 and 1.52 for samples 3a (depending on the flow-rate and the age of the column). Examples of the chromatograms obtained are given in Fig. 2.

The enantiomer ratios obtained by HPLC are given in Table I. They were evaluated by area normalization and comparison with a racemic mixture, which led to a relative area ratio of 50.8:49.2.

The chiral HPLC separation of compounds 3b (R = CH_3) is more difficult on an Enantiopac column. The HPLC conditions employed for the separation of 3b enantiomers were as follows: mobile phase, 2-propanol–phosphate buffer (pH 7) (2:98, v/v); flow-rate, 0.30 ml/min; temperature, 20°C; sample load, 1 nmol; UV detection, 207 nm, 0.02 a.u.f.s.

The enantiomer ratios were evaluated from peak-height ratios as complete resolution was not achieved, and are given in Table I.

NMR chiral shift. The enantiomeric excess was also obtained by NMR chiral shift studies[13,14] on enantiomeric mixtures of 3a or 3b containing about 10 mol.-% of tris[3-(heptafluoropropylhydroxymethylene)-(+)- or (−)-camphorato]europium-(III) derivative (Fig. 3). The shift was observed on the methyl signal of the acetyl moiety with the *l* enantiomer shifting to lower field relative to the *d* enantiomer when (−)-europium salt was used (Fig. 4c).

We can emphasize the reverse behaviour with the following systems: the same chiral europium derivative, (+)-[Eu(hfc)$_3$], interacting with mixtures of 3a with respective *l*/*d* composition 80:20 and 20:80 (*cf.*, Fig. 4a and b); and enantiomeric chiral (+)- and (−)-europium salts complexing the same sample [*l*:*d* = 80:20] of 3a (*cf.*, Fig. 4a and c).

RESULTS

The effects of different factors on the reaction were studied (see Fig. 1): effect of the phase transfer catalyst structure with TEBA ($Et_3\overset{+}{N}CH_2Ph$, Cl^-) (a non-chiral catalyst; Et = Ethyl, Ph = phenyl) 4 [(+)- or (−)-N-benzyl-N-methylephedrinium salts] and 5 [N-benzyl salts from (−)-cinchonidine or (+)-cinchonine] (see Fig. 5 for structures); effect of the presence of solvent by addition of carbon tetrachloride or toluene, generally advocated in asymmetric Michael additions[6,15]; and effect of addition of molecular sieves as their presence has been claimed to enhance the

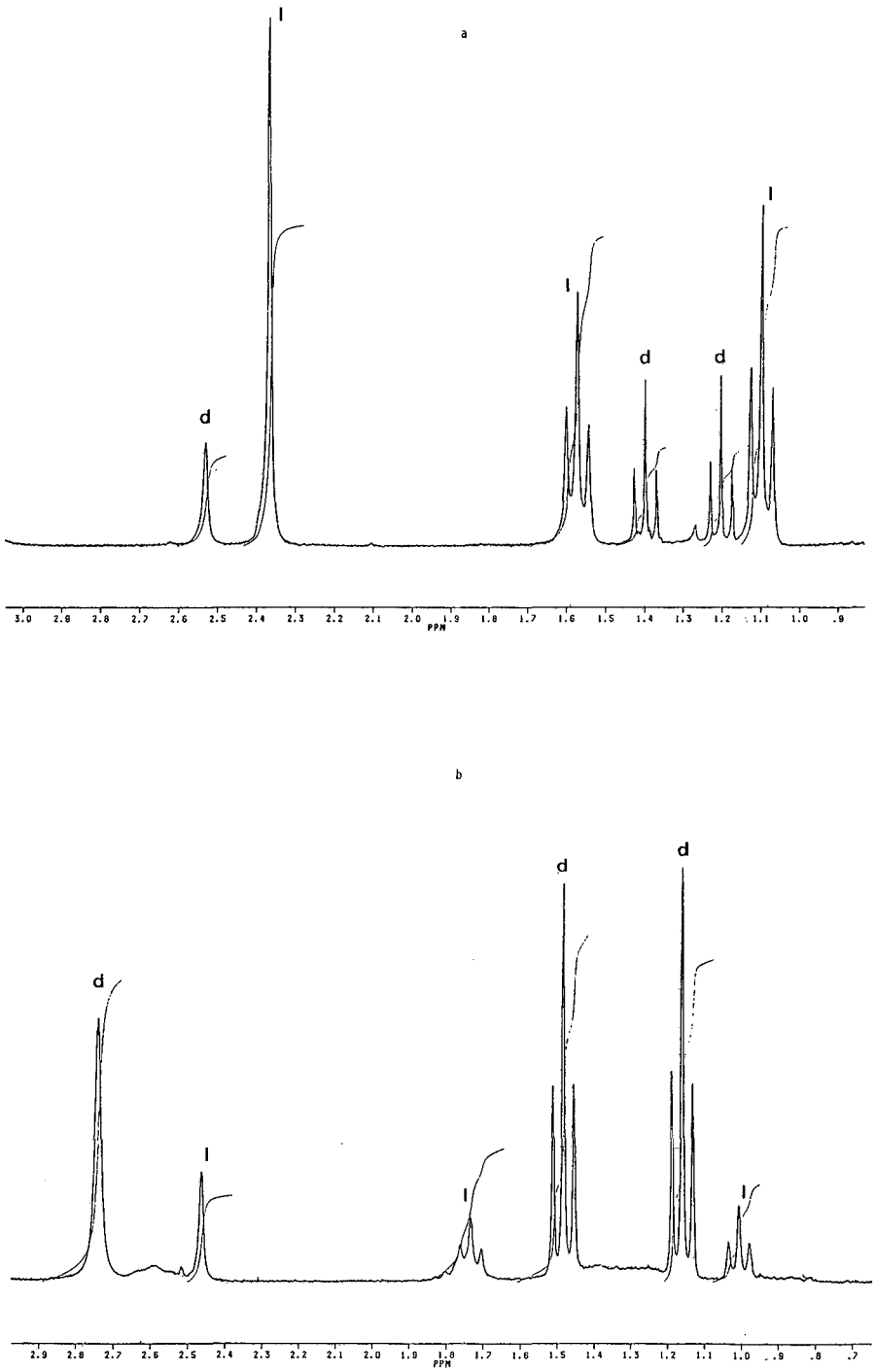

a
l
d
l
d
d
l
3.0 2.8 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 .9
PPM
b
d
l
l
d
d
l
2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 .9 .8 .7
PPM

Fig. 4.

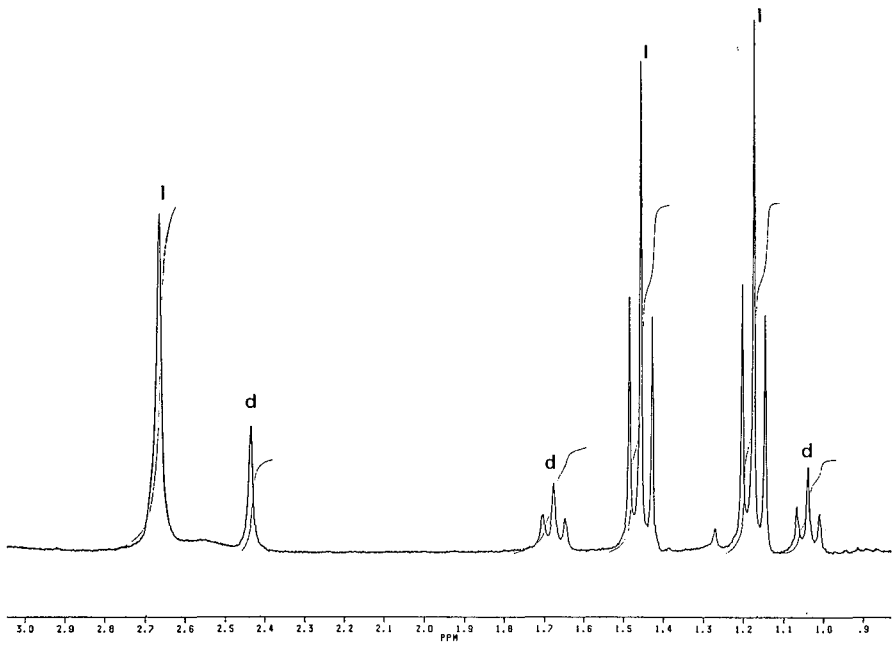


Fig. 4. NMR resolution of compounds 3a obtained at 250 MHz with (+)- or (−)-[Eu(hfc)$_3$] derivative. (a) *l*:*d* = 80:20 in presence of (+)-[Eu(hfc)$_3$]; (b) *l*:*d* = 20:80 in presence of (+)-[Eu(hfc)$_3$]; (c) *l*:*d* = 80:20 in presence of (−)-[Eu(hfc)$_3$].

enantiomeric excess for different kinds of asymmetric reactions[16,17]. Some results are reported in Table I.

Although the chemical yields are limited (40–60%), probably connected with the possible reversibility of Michael addition, the enantiomeric excess (ee) leads to satisfactory results. It appears that N-benzyl salts derived from (+)- or (−)-N-methylephedrine are the most efficient (ee $\geqslant$ 60%) when compared with cinchonidinium or cinchoninium salts (ee $\approx$ 30%). Electron-attracting substituents on the benzyl moiety, advocated in the asymmetric alkylation of indanone derivatives[6], had unfavourable effects in this work.

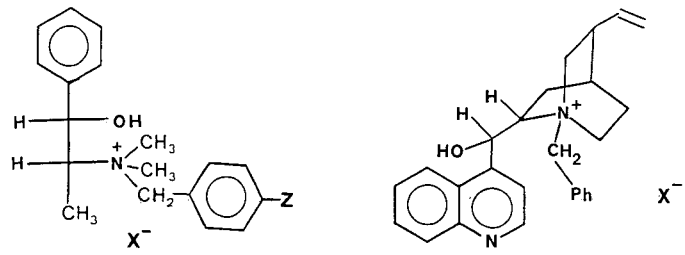


Fig. 5. Formulae of the chiral catalysts used: 4 = (+)- or (−)-N-benzyl-N-methylephedrinium salts; 5 = N-benzyl salts from (−)-cinchonidine or (+)-cinchonine.

TABLE I

CHIRAL MICHAEL ADDITION OF ACETAMIDOMALONATE I TO α-ENONE 2: SUMMARY OF RESULTS

Compound	*Catalyst**		*Additional compound*	*Chemical yield (% 3 isolated)*	*Enantiomers (l:d)*	
	Z	X^-			*HPLC*	*NMR*
2a, $R=C_6H_5$	TEBA		–	71	50:50	50:50
	4(−)-H	Br^-	–	56	80:20	78:22
	(−)-H	Cl^-	–	57	84:16	80:20
	(+)-H	Br^-	–	48	19:81	19:81
	(−)-H	Br^-	Mol. sieves 3Å	62	83:17	84:16
	(−)-H	Br^-	Mol. sieves 4Å	53	82:18	79:21
	(−)-H	Br^-	CCl_4 (1 or 5 ml)	53	No sample	56:44
	(−)-H	Br^-	Toluene (1 or 5 ml)	51	59:41	64:36
	(−)-H	Br^-	Mol. sieves 3A + toluene	57	No sample	68:32
	5(−)	Cl^-	–	51	64:36	66:34
	(+)	Cl^-	–	57	33:67	30:70
	4(−)-NO_2	Br^-	–	58	62:38	60:40
	-CN	Br^-	–	54	67:33	67:33
	-CF_3	Br^-	–	42	73:27	Crude sample
2b, $R=CH_3$	TEBA		–	55	50:50	50:50
	4(−)-H	Br^-	–	48	57:43	56:44

* TEBA = triethylbenzylammonium; 4 and 5 = chiral catalysts (see Fig. 5).

Organic solvents should be omitted, as the enantiomeric excess is noticeably improved in the absence of carbon tetrachloride or toluene (ee $\geqslant$ 60% compared with only 20–30%). Addition of molecular sieves has a small beneficial effect.

CONCLUSION

As will be detailed in a forthcoming paper[7], and as far as asymmetric synthesis is concerned, three main conclusions can be drawn, that the enantiomeric excess can be increased under PTC conditions (i) by omitting an organic solvent during the reaction step, *i.e.*, operating under solid–liquid PTC without solvent[1,2], as the presence of carbon tetrachloride or toluene reduces asymmetric induction considerably; (ii) by choosing the most appropriate PTC catalyst, N-benzylammonium salts derived from (+)- or (−)-N-methylephedrine being the most efficient; and (iii) by addition of molecular sieves, which seems to have a slightly positive effect.

A final point can be made with respect to the important role of a phenyl group α to the carbonyl function in an α-enone. Its presence seems to be fundamental in order to observe good asymmetric induction as the enantiomeric excess decreases from 2a to 2b. The enantiomer ratios obtained by NMR and HPLC are similar. However, some advantages of chiral HPLC determinations appear in connection with the possibility of analysing crude mixture (NMR studies require a previous purification to obtain pure 3a or 3b as 1 is troublesome, giving a complex with europium salts). Nevertheless, this last method may be the only possible route when a separation is poor or impossible by chiral HPLC.

REFERENCES

1 G. Bram, A. Loupy and J. Sansoulet, *Isr. J. Chem.*, 26 (1985) 291.
2 A. Loupy, J. Sansoulet and F. Vaziri-Zand, *Bull. Soc. Chim. Fr.*, (1987) 1027; and references cited therein.
3 G. Bram, J. Sansoulet, H. Galons, Y. Bensaid, C. Combet-Farnoux and M. Miocque, *Tetrahedron Lett.*, (1985) 4601.
4 G. Bram, J. Sansoulet, H. Galons and M. Miocque, *Synth. Commun.*, 18 (1988) 367.
5 R. S. E. Conn, A. V. Lovell, S. Karady and L. M. Weinstock, *J. Org. Chem.*, 51 (1986) 4710.
6 U. H. Dolling, D. L. Hughes, A. Bhattacharya, K. M. Ryan, L. M. Weinstock and E. J. J. Grabowski, in C. M. Starks (Editor), *Phase Transfer Catalysis — New Chemistry, Catalysts and Applications*, (*ACS Symposium Series*, Vol. 326), American Chemical Society, Washington, DC, 1987; and references cited therein.
7 A. Loupy, C. Merienne, J. Sansoulet and A. Zaparucha, *Tetrahedron Lett.*, submitted for publication.
8 J. Hermansson and M. Eriksson, *J. Liq. Chromatogr.*, 9 (1986) 621.
9 G. Schill, I. W. Wainer and S. A. Barkan, *J. Chromatogr.*, 365 (1986) 73.
10 E. Delee, L. Le Garrec, I. Jullien, S. Beranger, J. C. Pascal and H. Pinhas, *Chromatographia*, 24 (1987) 357.
11 L. Le Garrec, E. Delee, J. C. Pascal and I. Jullien, *J. Liq. Chromatogr.*, 10 (1987) 3015.
12 B. Testa, *Xenobiotica*, 16 (1986) 265.
13 F. Inagaki and T. Miyazawa, *Prog. Nucl. Magn. Reson. Spectrosc.*, 14 (1981) 67.
14 L. M. Sweeting, D. C. Crans and G. M. Whitesides, *J. Org. Chem.*, 52 (1987) 2273; and references cited therein.
15 K. Hermann and H. Wynberg, *J. Org. Chem.*, 44 (1979) 2238.
16 R. M. Hanson and K. B. Sharpless, *J. Org. Chem.*, 51 (1986) 1922.
17 Y. Hirai, T. Terada and T. Yamazaki, *J. Am. Chem. Soc.*, 110 (1988) 958.

Journal of Chromatography, 450 (1988) 191–197
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 697

DIRECT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF DIHYDROPYRIDINE ENANTIOMERS

E. DELEE, I. JULLIEN* and L. LE GARREC
Recherche Syntex France, Leuville-sur-Orge, 91310 Montlhéry (France)

SUMMARY

A direct high-performance liquid chromatographic enantioseparation on a chiral stationary phase using a protein as chiral selector was achieved for some dihydropyridines with different structural groups (amine, amide, acid and hydroxyl groups). The protein involved was α_1-acid glycoprotein and the work was mainly carried out with an EnantioPac column. An improved version of this chiral column (Chiral-AGP) was compared with the previous one and gave interesting results.

INTRODUCTION

To establish the optical purity of new drugs and to monitor their asymmetric synthesis, simple methods are required. The aim was to obtain a direct separation without a lengthy sample preparation (derivatization or extraction) and to determine optical enantiomers. We also required a high-performance liquid chromatographic (HPLC) method that would separate many different structural families without major changes in conditions. The utilization of aqueous mobile phases with the potential for application also to biological fluids is important. These requirements were met with a protein-type chiral stationary phase column, the protein involved being α_1-acid glycoprotein (AGP). This protein consists of polymers composed of naturally occurring chiral amino acid subgroups and the binding that occurs can often be stereospecific.

We previously described[1,2] the resolution of β-amino alcohol enantiomers on the EnantioPac (AGP) column and we have also used this column to monitor a solid–liquid phase transfer catalysis without addition of solvent, inducing asymmetric Michael addition of acetamidomalonate to alphaenone[3]. In this paper we give results for dihydropyridines (DHP). This work was mainly carried out on a well known EnantioPac column, but some preliminary results on a second version of AGP (Chiral-AGP) were also obtained.

To improve the resolution, the mobile phase was optimized and the concentration of 2-propanol (organic modifier), the flow-rate, the ionic strength, the pH and the temperature were adjusted.

0021-9673/88/$03.50

EXPERIMENTAL

Columns

Both chiral columns used contained AGP. The first is commercially available as EnantioPac (LKB), with a broad applicability[4–6]. The advantages and limitations of this protein-type column have been discussed in detail[7–9]. We used a 100 × 4.0 mm (I.D.) cartridge column system with a matrix based on a diethylaminoethylsilica gel (10 μm particle size) on which the plasma protein is immobilized by ionic bonding.

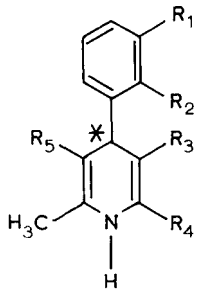
The second column, an improved version of EnantioPac (Chiral-AGP), is claimed by the manufacturer to overcome some limitations of the EnantioPac column. We used a pilot-plant scale column (100 × 4.0 mm I.D.), without a cartridge, with AGP immobilized on porous, spherical silica particles (5 μm particle size). The surface chemistry of the silica is also different to that of EnantioPac.

DHP sample preparation

As the DHP tested (see structures in Table I) were not water-soluble, we usually prepare stock solutions in 2-propanol–water (1:1) and dilute them with the corresponding mobile phase. We injected about 1 nmol of each DHP (10 μl; Shimadzu autosampler).

TABLE I

STRUCTURES OF DIHYDROPYRIDINES RESOLVED ON THE ENANTIOPAC COLUMN



Compound	R_1	R_2	R_3	R_4	R_5
A	NO_2	H	COOH	CH_3	$COOCH_3$
B	NO_2	H	$COO(CH_2)_3Br$	CH_3	$COOCH_3$
C	NO_2	H	$COO(CH_2)_3O$–C$_6$H$_4$–$(CH_2)_2OH$	CH_3	$COOCH_3$
D	NO_2	H	$COOCH_2CH_3$	CH_3	$COOCH_3$
E	H	CF_3	NO_2	CH_3	$COOCH_3$
F	H	Cl	SO_2–C$_6$H$_4$–OCH_3	CH_3	$COOCH_3$
G	H	Cl	$COO(CH_2)_2$–C$_5$H$_4$N	CH_3	$CONH\text{-}C_6H_5$
H	H	Cl	$COOCH_2CH_3$	CH_2OH	$COOCH_3$
I	Cl	Cl	$COO(CH_2)_2N(CH_3)_2$	CH_3	$COOCH_3$

HPLC system

The temperature of the column was usually maintained at 20°C with a circulating water-jacket and a thermostated water–alcohol bath. The mobile phase was 2-propanol–phosphate buffer (4 m*M* NaH_2PO_4 + 4 m*M* Na_2HPO_4 + 10 m*M* NaCl), the proportions of the components and the flow-rates (between 0.15 and 0.35 ml/min; LKB Model 2150 pump) being given in Table II. The column effluent was monitored at 227 nm with a variable-wavelength detector (Kratos Model 783). The integrator was a Shimadzu CR3A.

RESULTS AND DISCUSSION

The optimized mobile phase composition and flow-rate for each DHP tested on the EnantioPac column are given in Table II. They represent a summary of different trials in which we optimized the separation with respect to the percentage of 2-propanol and the pH of mobile phase in particular.

The resolution factor[10], R_s, is also given in Table II for each DHP.

Fig. 1 gives some examples of chromatograms obtained for DHP with different functional groups. When authentic enantiomers were available, we identified each enantiomer as (+) or (−) and calculated their optical purities by peak-area normalization in comparison with the racemic DHP.

In addition for one DHP (C) with a hydroxyl group, we studied the influence of several factors on the enantioseparation. The results are summarized in Fig. 2 and show the importance of the concentration of organic modifier and the pH. Small alterations in these two parameters change the enantioselectivity of DHP drastically. Temperature is also an important parameter for resolution, whereas flow-rate is more important for the column lifetime than for resolution (a low flow-rate of less than 0.5 ml/min is recommended for the EnantioPac column). We optimized the above parameters to obtain a compromise between good resolution and as short a run time as possible.

All the DHP resolved, have binding groups and a ring structure coupled direct-

TABLE II

HPLC CONDITIONS AND RESOLUTION FACTORS ON THE ENANTIOPAC COLUMN

t_2 = retention time of second eluted peak; R_s = resolution factor.

Compound	*Mobile phase*			t_2 *(min)*	R_s
	2-Propanol (%)	*Buffer pH*	*Flow-rate (ml/min)*		
A	1	5.95	0.35	51.5	1.03
B	18	6.97	0.25	25.1	0.78
C	14	6.97	0.25	48.6	1.41
D	15	6.97	0.25	25.9	1.22
E	15	6.97	0.30	32.2	1.08
F	10	4.05	0.15	101.9	1.60
G	15	6.97	0.30	65.8	3.78
H	8	6.97	0.25	78.7	1.33
I	10	6.97	0.25	49.8	1.41

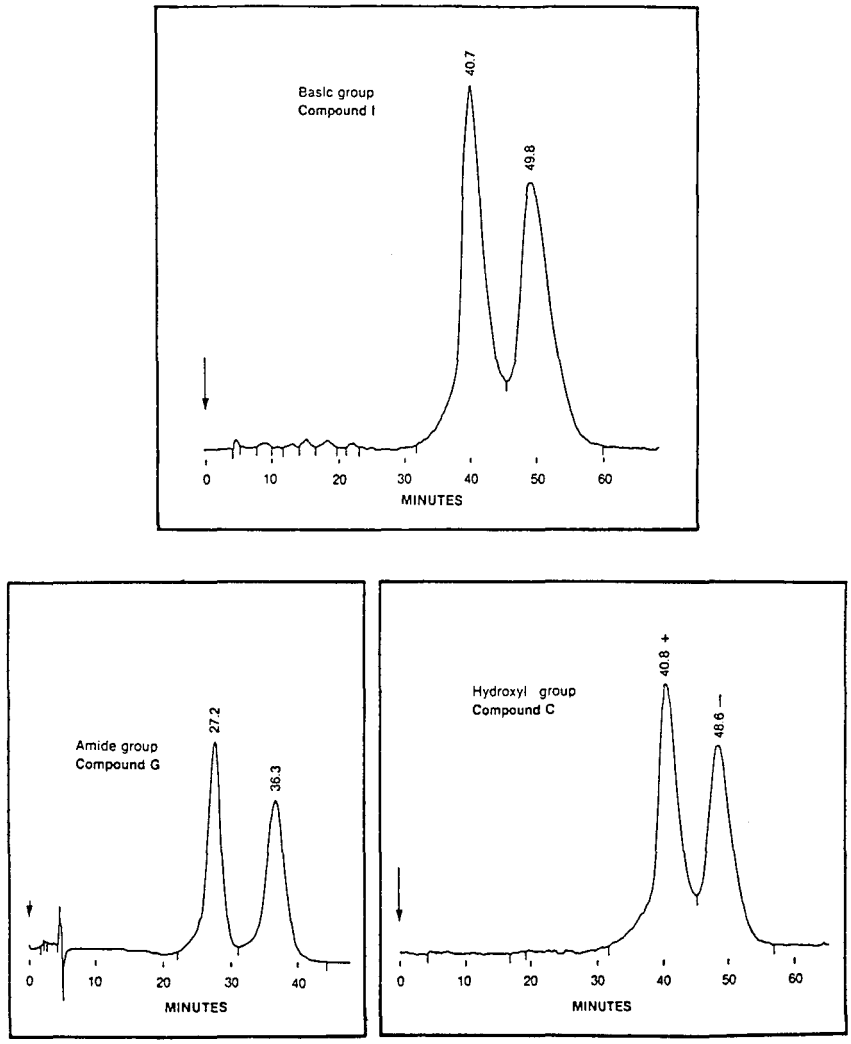


Fig. 1. Examples of dihydropyridine enantiomer resolution on EnantioPac column (compounds I, G and C).

ly to the single chiral atom. However, we obtained a poor resolution with other DHP having the same functional groups but other substituents. This result corroborates the hypothesis that the separation mechanism includes not only hydrophobic interactions and interactions of polar groups but also steric effects[4,5].

A comparison of results obtained with the Chiral-AGP and EnantioPac columns is given in Table III for compounds A, B, D, E and I using the HPLC conditions as in Table II. For all the compounds except A (for which the HPLC conditions may not be optimal) the time of analysis was reduced and the resolution was increased when the Chiral-AGP was used (see the chromatograms in Fig. 3). In addi-

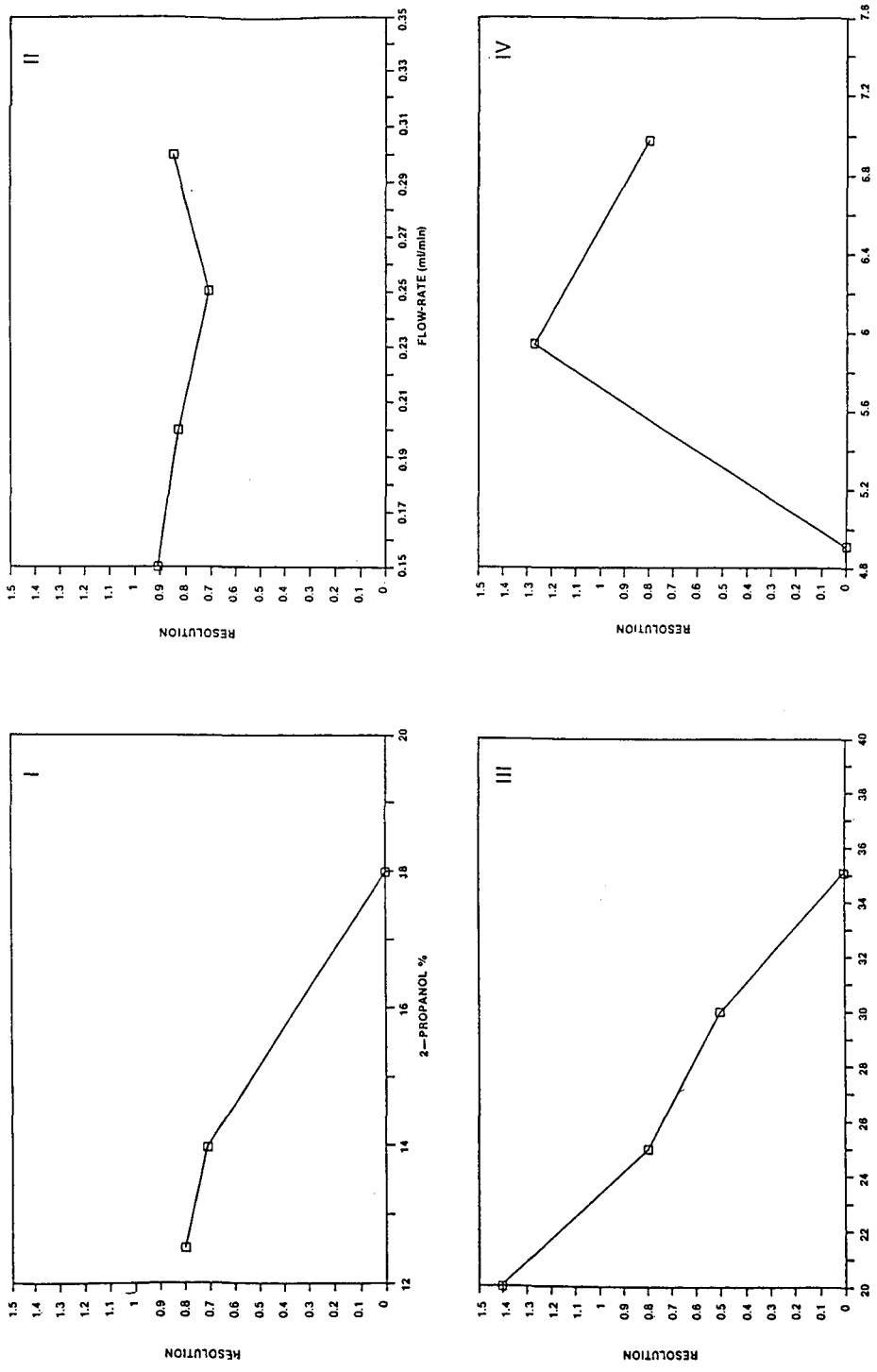


Fig. 2. Influence of percentage of 2-propanol (I), flow-rate (II), temperature (III) and pH (IV) on enantioselectivity of compound C. EnantioPac column. other conditions: (I), flow-rate, 0.25 ml/min; temperature, 25°C; pH 6.97; (II), 14% 2-propanol; temperature, 25°C; pH 6.97; (III), 12.5% 2-propanol; flow-rate, 0.25 ml/min; pH 6.97; (IV), 12% 2-propanol; flow-rate, 0.25 ml/min; temperature, 20°C.

TABLE III

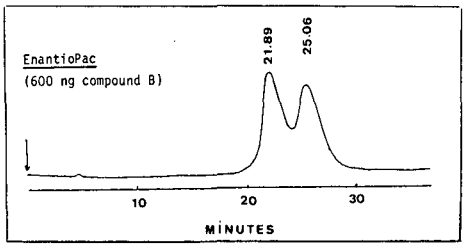
COMPARISON BETWEEN ENANTIOPAC AND CHIRAL-AGP COLUMNS

HPLC conditions as in Table II. t_1 and t_2 = retention times (min) of the first and the second eluted peaks, respectively; R_s = resolution factor.

Compound	*EnantioPac column*			*Chiral-AGP column*		
	t_1	t_2	R_s	t_1	t_2	R_s
A	42.9	51.5	1.03	26.0	71.9	1.50
B	21.9	25.1	0.78	12.7	16.0	1.18
D	21.6	25.9	1.22	12.4	15.2	1.81
E	28.4	32.2	1.08	12.8	14.8	1.20
I	40.7	49.8	1.41	39.3	46.8	2.19

tion, the flow-rate could be increased to 0.5 ml/min, allowing a reduction in the run time. These first results are very promising and we also hope that the column lifetime will be increased.

It should be noted that as the chiral stationary phases are similar in nature their mechanisms of separation are the same and Chiral-AGP probably will not resolve enantiomers that cannot be separated on the EnantioPac column.



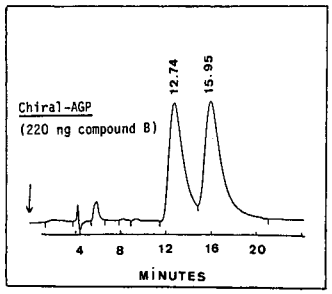


Fig. 3. Comparison of the enantiomer resolution of compound B on the EnantioPac and Chiral-AGP columns using the same HPLC conditions (see Table II).

CONCLUSION

The wide applicability of AGP columns to the direct enantiomeric separation of dihydropyridines with an aqueous mobile phase makes it an economic means of supporting stereospecific synthesis, although the first EnantioPac type has to be handled very carefully and has some limitations (column lifetime, low capacity, flow-rate restriction). The newer Chiral-AGP has given very promising results and, if it is confirmed by full-scale production, some limitations of this kind of protein column will be overcome.

REFERENCES

1 E. Delee, L. Le Garrec, I. Jullien, S. Beranger, J. S. Pascal and H. Pinhas, *Chromatographia*, 24 (1987) 357–359.
2 L. Le Garrec, E. Delee, J. C. Pascal and I. Jullien, *J. Liq. Chromatogr.*, 10 (1987) 3015–3023.
3 E. Delee, I. Jullien, L. Le Garrec, A. Loupy, J. Sansoulet and A. Zaparucha, *J. Chromatogr.*, 450 (1988) 183–189.
4 J. Hermansson, K. Strom and R. Sandberg, *Chromatographia*, 24 (1987) 520–526.
5 G. Schill, I. W. Wainer and S. A. Barkan, *J. Chromatogr.*, 365 (1986) 73–88.
6 G. Schill, I. W. Wainer and S. A. Barkan, *J. Liq. Chromatogr.*, 9 (1986) 641–666.
7 R. Däppen, H. Arm and V. R. Meyer, *J. Chromatogr.*, 373 (1986) 1–20.
8 H. T. Karnes and M. A. Sarkar, *Pharm. Res.*, 4 (1987) 285–292.
9 W. Lindner, *Chromatographia*, 24 (1987) 97–107.
10 B. Testa, *Xenobiotica*, 16 (1986) 265–279.

Journal of Chromatography, 450 (1988) 199–210
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 698

CHROMATOGRAPHIC SEPARATION OF THE ENANTIOMERS OF 1,3-DITHIOLANE-1-OXIDES

WILLIAM H. PIRKLE* and BRUCE C. HAMPER*
School of Chemical Sciences, University of Illinois, Urbana, IL 61801 (U.S.A.)

SUMMARY

The enantiomers of 2,2-disubstituted 1,3-dithiolane-1-oxides (type **1** and **2**) are separable by direct high-performance liquid chromatography on commercially available chiral stationary phases derived from the N-3,5-dinitrobenzoyl derivatives of α-amino acids. The chromatographic data for a series of dithiolane oxides is presented. Two chiral recognition models are advanced to account for the structure–enantioselectivity relationships observed for these series. Using these models, absolute configurations of the enantiomers are assigned on the basis of chromatographic elution order. These assignments are consistent with those determined by independent chemical and spectroscopic means. Resolutions of the enantiomers are compared on nine different chiral stationary phases.

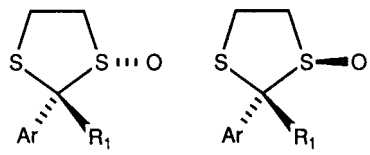
INTRODUCTION

In view of the importance of stereochemistry and particularly the absolute configuration of chiral compounds in biological systems, facile methods of determining enantiomeric purity and absolute configuration are highly desirable. Direct chromatography on a chiral stationary phase (CSP) offers the possibility of quickly and easily determining both enantiomeric purity and absolute configuration. For configurationally unknown compounds, the absolute configuration can be determined if the details of the chiral recognition process are known[1]. CSPs can also provide a means of preparatively resolving useful quantities of the enantiomers[2]. In the course of investigating the resolution of chiral sulfoxides on a series of CSPs, we became interested in the mechanisms by which the enantiomers of 2,2-disubstituted 1,3-dithiolane-1-oxides (type **1** and **2**) are chromatographically separated[3]. An understanding of the chiral recognition process allows estimation of the separability of a wide range of dithiolane oxides and assignments of their absolute configurations. Furthermore, advances in the understanding of enantioselective processes in this system can be extended to allow both rational design and employment of CSPs.

Several recently developed high-performance liquid chromatographic CSPs

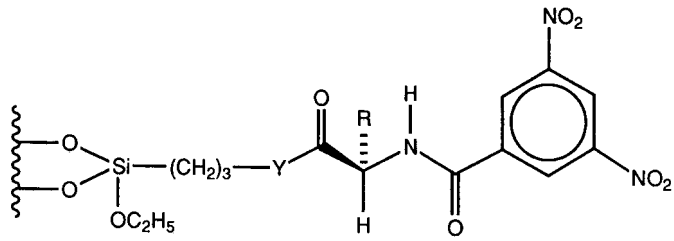
* Present address: Monsanto Agricultural Co., a Unit of Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167, U.S.A.

021-9673/88/$03.50



1 cis **2** trans

have been employed for the direct resolution of chiral sulfur compounds. Chiral sulfoxides and sulfoximines can be resolved by affinity chromatography utilizing bovine serum albumin immobilized on silica[4,5]. Wainer *et al.*[6] resolved sulfoximines, sulfinamides and sulfilimines on a cellulose-based CSP. Preparative resolution of up to 100 mg of a sulfoxide has been achieved on a column (50 × 2 cm I.D.) containing a cellulose tribenzoate derived CSP[7]. Sulfoxides and sulfoximines have also been resolved on CSP **3a**[8] which, in addition, can be used to resolve related selenoxides[9] and phosphine oxides[10].



3 Y = $NH_3^+O^-$
4 Y = NH

R
a phenyl
b isobutyl
c benzyl
d isopropyl
e β-naphthyl

CSPs **3** and **4**, derived from N-3,5-dinitrobenzoyl amino acids, are capable of resolving a wide variety of analytes[11]. These CSPs contain an electron-deficient aromatic ring, an amide hydrogen, a carboxylate (**3**) or amide group (**4**) and steric subunits which are capable of stereospecific interactions with complementary groups. Depending on which combination of interaction sites is utilized, two chiral recognition models, **5** and **7**, can be advanced to account for the resolution of a variety of analytes (Fig. 1)[12]. While alternative combinations are possible, even these two allow the categorization of many seemingly unrelated compounds into two groups, each resolvable on the CSPs. This categorization gives some insight into the scope of these CSPs, often allows one to predict whether an analyte will be resolved, and, in such cases, in what order the enantiomers may be expected to elute.

Chiral recognition model **5** invokes the 3,5-dinitrobenzoyl (DNB) substituent of the CSP as a π-acceptor site, the benzamide hydrogen as an acidic site and the amide

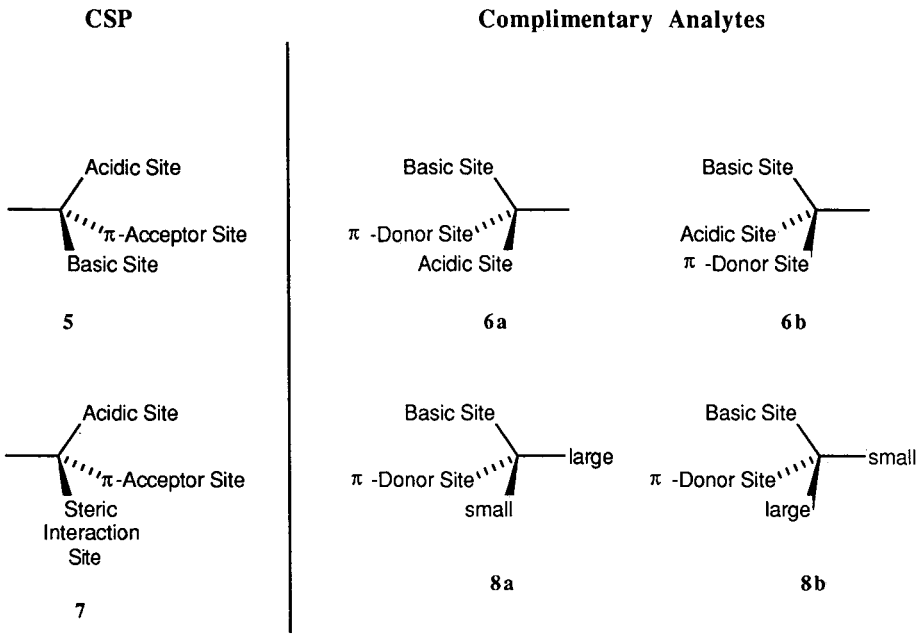


Fig. 1. Chiral recognition models invoked for CSPs **3** and **4** to account for the resolution of complimentary analytes of general structure **6** and **8**. For HPLC on the (*R*)-CSPs **3** and **4** at room temperature, analytes **6a** and **8a** will form the more stable diastereomeric adsorbates and will be retained more strongly than are their enantiomers.

(or carboxylate) carbonyl as the basic site. To interact with the CSP via this mechanism, the analyte must contain a π-donor site, a basic site for association with the benzamide hydrogen and an acidic site to hydrogen bond to the basic carbonyl oxygen, and these sites must be stereochemically disposed such that all of the interactions with the CSP can occur simultaneously. The analyte enantiomer which, on a time average basis, best maintains these simultaneous interactions with the CSP will be most retained. Using this general depiction, **6a** would be expected to afford three simultaneous bonding interactions with the CSP while both are in low energy conformations, and, at room temperature, would be preferentially retained. For **6b** to undergo these simultaneous bonding interactions, either it or the CSP would have to assume higher energy conformations. Accordingly, **6b** would be chromatographically less retained and would elute first. The second chiral recognition model, **7**, is analogous to **5** in that the CSP employs a π-acceptor site (DNB substituent) and an acidic site (benzamide hydrogen) to afford two-point binding to the analyte. However, the third interaction is repulsive rather than attractive in nature. The relative stability of the two diastereomeric adsorbates depends on juxtaposition of either a sterically small or large group of the bound analyte enantiomer towards the CSP. Owing to differential steric interaction, analyte **8a** will form a more stable diastereomeric adsorbate with the CSP. Since the small group is directed toward the CSP, this allows closer approach of the attractive interaction sites. Conversely, **8b** cannot approach the CSP so closely owing to unfavorable steric interaction with the large group. Conformational change to lessen this steric interaction would increase the energy of the adsorbate relative to the other diastereomeric adsorbate.

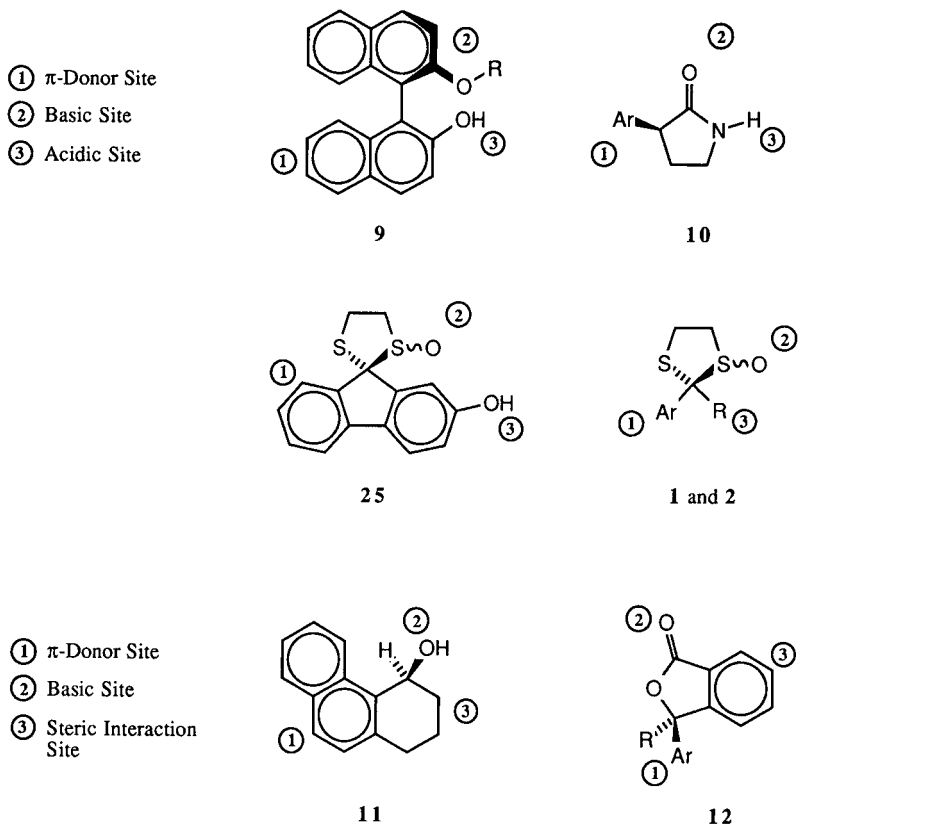


Fig. 2. Solutes which contain a π-donor site (1), a basic site (2), and an acidic site (3) and are resolved on CSPs **3** and **4** include compounds **9**, **10**, and spirosulfoxide **25** (top). Resolvable analytes which contain a π-donor site (1), a basic site (2), and a steric interaction site (3) include compounds **11**, **12**, and sulfoxides of type **1** and **2**.

The resolution of more than twenty analogous bi-β-naphthols, **9**, has been rationalized in terms of chiral recognition model **5** (Fig. 2)[13]. While one aromatic ring serves as a π-base, the oxygen on the second ring serves as a basic site and the phenolic hydrogen on the first ring serves as an acidic site. The relevant interaction sites are labelled 1, 2, and 3 respectively. Similarly, α-aryl-γ-lactams, **10**, appear to resolve by this mechanism[3]. Compounds which are thought to resolve by mechanism **7** include a number of cyclic alcohols[14], **11**, and phthalides[15], **12**. These compounds contain aromatic rings for π–π interaction with the CSP and either a hydroxyl or carbonyl oxygen to serve as a basic site. The stability of the two possible diastereomeric adsorbates is determined by the relative steric repulsion between the analyte and the CSP encountered in each adsorbate.

For studying chiral recognition processes, the cyclic sulfoxides **1** and **2** have advantages over acyclic sulfoxides in that the ring system limits the number of possible conformations. This significantly reduces the number of possible arrangements for association with the CSP and simplifies evaluation of the structure-

enantioselectivity relationships. While diastereomerically pure sulfoxides such as **1** or **2** have been prepared[16,17], to our knowledge none of these dithiolanes has previously been resolved or assigned absolute configurations*. A series of racemic dithiolane oxides of type **1** and **2** were prepared and found to be resolvable on CSPs **3** and **4**. The chromatographic structure–enantioselectivity relationships noted are consistent with the two chiral recognition models presently employed to assign absolute configurations to these configurationally unknown compounds.

EXPERIMENTAL

General

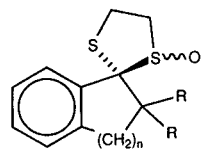
Analytical liquid chromatography was performed using an Altex 100 or 110A pump, a Valco 7000 p.s.i. or Altex 210 injector equipped with 10-μl or 20-μl sample loop, respectively, and an Altex Model 152 dual-wavelength (254 nm and 280 nm) detector or a Beckman 165 variable-wavelength detector. Chromatography columns containing CSP **3a,b** or **4a,b** were obtained from Regis and Baker. The covalently bound α-amino acid derived CSP **4c,d,e** were obtained by *in situ* modification of a commercially packed aminopropyl silanized silica column[19]. Preparation of ionically bound α-amino acid CSP **3c,d** followed the previously reported procedure[14]. Preparative separations (100 mg to 5 g) were performed by medium-pressure chromatography (MPLC) using Ventron 58-μm large-pore silica gel dry-packed into 30 in. × 1 in. I.D. columns. The cyanopropyl bonded phase for preparative separations was prepared from this silica using standard methods[20].

Melting points were taken on a Buchi apparatus and are uncorrected. IR spectra were recorded on a Beckman IR-12 or Nicolet FT-IR 7199 spectrometer and the adsorptions expressed in wavenumbers. ^{1}H NMR spectra were obtained on a Nicolet NTC (360 MHz), Varian HR-220 (220 MHz), EM-390 (90 MHz), or XL-200 (200MHz). All resonances are recorded as ppm relative to internal standard tetramethylsilane. Electron-impact mass spectra were recorded on a Varian MAT CH-5-DF spectrometer (low resolution) or a Varian MAT 311A or 731 (high resolution). Microanalyses were performed by Mr. J. Nemeth and co-workers at the University of Illinois

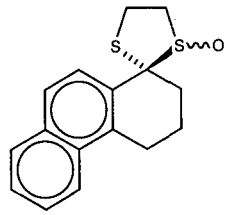
The 1,3-dithiolanes required for preparation of sulfoxides **1** and **2** were prepared from the corresponding ketones by standard methods[21]. Compounds **1a**, **2a**, and **18** have been previously prepared[16,17]. New compounds were fully characterized by melting point, NMR and IR spectroscopy, and either elemental analysis (within 0.40% of expected values for C, H, and S) or molecular formula determination by high-resolution mass spectrometry. Characterization data for the compounds listed in Tables I–III will be presented elsewhere. However, data is provided herein for the compounds to be presented only in this paper.

General synthesis of 1,3-dithiolane-1-oxides. The 1,3-dithiolane oxides were dissolved in dichloromethane, the solution was cooled in an ice-water bath and treated portionwise with an equimolar amount of 85% MCPBA. After addition was complete, the mixture was stirred for 15 min and washed twice with 10% sodium hydrogen-

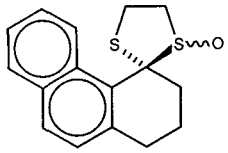
* In the case of 1,3-dithiane-1-oxide, the parent compound has been resolved by separation of diastereomeric derivatives prepared from (+)-camphor[18].



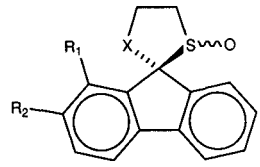
13-15



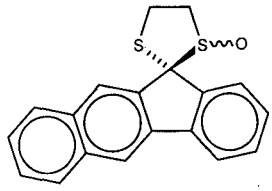
16



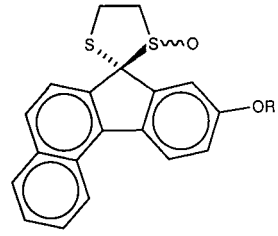
17



18-22



23



24-25

carbonate. The organic layer was dried with magnesium sulphate and the solvent removed to afford a mixture of diastereomers (in the case of symmetrical ketones, only one monosulfoxide, such as **18** or **22**, is obtained). Separation was achieved by MPLC on either a silica or cyanopropyl column using mixtures of 2-propanol in hexanes as the eluent.

*Cis- and trans-2-phenyl-2-methyl-1,3-dithiolane-1-oxide (***1b** *and* **2b***).* Oxidation of 2-phenyl-2-methyl-1,3-dithiolane afforded a mixture of the *cis* and *trans* diastereomers (9:91) as determined by ^{1}H NMR of the crude reaction mixture. Separation by MPLC (cyanopropyl silica, 5% 2-propanol in hexanes) afforded *trans*-**2b** (75%) which was recrystallized from hexanes: m.p. 57–58°C; IR (KBr) 3055, 2980 and 2922 (C–H, str.), 1055 (S–O), 771 and 698 (C–H, bend) cm^{-1}; ^{1}H NMR (C^2HCl_3) δ 2.05 (s,3H), 2.70 (m,1H), 3.20 (m,2H), 3.72 (m,1H), 7.36 (m,3H), 7.69 (dd,2H), MS (70 eV), *m/e* (relative intensity) 212 (100,M$^+$), 195 (27), 194 (36), 137 (19), 136 (46), 121 (18), 103 (31), 92 (33). Recrystallization from hexanes afforded *cis*-**1b** (9.6%) as a white crystalline solid: m.p. 71.5–72°C; IR (KBr) 1052 (S–O); ^{1}H NMR (C^2HCl_3) δ 1.95 (s,3H), 2.90 (ddd,1H), 3.32 (m,3H), 7.36 (m,3H), 7.65 (m,2H); MS (70 eV), *m/e* (relative intensity) 212 (100,M$^+$), 196 (19), 136 (41), 121 (12), 103 (25), 92 (22).

*Cis- and trans-2-phenyl-2-ethyl-1,3-dithiolane-1-oxide (***1c** *and* **2c***).* A mixture of

diastereomers obtained by oxidation using the above method was separated by MPLC (cyanopropyl silica, 5% 2-propanol in hexanes) to afford *trans*-**2c** (1.9 g, 59%) and *cis*-**1c** (0.42 g, 13%). The *trans*-**2c** isomer was obtained as a white solid: m.p. 63–64°C; IR (KBr) 1050 (S–O) cm^{-1}; ^1H NMR (C^2HCl_3) δ 0.87 (t,3H), 2.47 (q,2H), 2.64 (ddd,1H), 3.13 (ddd,1H), 3.71 (ddd,1H), 7.33 (t,1H), 7.40 (t,2H), 7.76 (d,2H); MS (70 eV) *m/e* (relative intensity) 226 (100), 181(25), 150 (31), 117 (96), 92 (18). The *cis*-**1c** isomer was obtained as a white solid: m.p. 56–58°C; IR (KBr) 1060 (S–O) cm^{-1}; ^1H NMR (C^2HCl_3) δ 0.91 (t,3H), 2.01 (dq,1H), 2.61 dq,1H), 2.69 (ddd,1H), 3.12 (ddd,1H), 3.32 (m,2H), 7.38 (t,1H), 7.45 (t,2H), 7.66 (d,2H); MS (70 eV), *m/e* (relative intensity) 208 (14), 150 (22), 121 (82), 117 (100), 91 (15), 77 (24).

*Cis- and trans-2-phenyl-2-isopropyl-1,3-dithiolane-1-oxide (***1d** *and* **2d***).* To a solution of 2-phenyl-2-isopropyl-1,3-dithiolane (4.37 g, 19.5 mmol) in 25 ml of glacial acetic acid was added 2.15 ml (21.5 mmol) of 30% hydrogen peroxide. After 2 h, the reaction mixture was diluted with 50 ml of water and extracted thrice with dichloromethane. The combined extracts were washed twice with 1 *N* sodium hydroxide, dried and the solvent removed to afford an oily solid consisting of a mixture of the *cis* and *trans* diastereomers (35:65). Fractional recrystallization from benzene–hexanes afforded 2.3 g (50%) of *trans*-**2d**: m.p. 127–128°C; IR (KBr) 1058 (S–O); ^1H NMR (C^2HCl_3) δ 0.92 (d,3H), 1.08 (d,3H), 2.65 (ddd,1H), 2.77 (dq,1H), 3.05 (ddd,1H), 3.13 (ddd,1H), 3.55 (ddd,1H), 7.34 (m,3H), 7.63 (d,2H); MS (70 eV), *m/e* (relative intensity) 240 (38,M^+), 181 (10), 164 (20), 131 (100), 121 (14), 117 (21), 91 (7.3). The *cis* diastereomer was obtained by concentration of the benzene–hexanes mother liquors and recrystallization to afford 0.90 g (20%) of *cis*-**1d**: m.p. 68–71°C; IR (KBr) 1060 (S–O) cm^{-1}; ^1H NMR (C^2HCl_3) δ 0.88 (d,3H), 1.25 (d,3H), 2.43 (ddd,1H), 2.78 (dq, 1H), 3.00 (m,2H), 3.31 (ddd,1H), 7.40 (d,1H), 7.46 (t,2H), 7.78 (d,2H); MS (70 eV), *m/e* (relative intensity) 240 (21,M^+), 181 (36), 164 (22), 131 (100), 121 (44), 117 (36), 91 (16).

*Cis- and trans-spiro-1,3-dithiolane-2,1'-(2',2'-dimethyltetralin)-1-oxide (***15a** *and* **15b***).* Oxidation by the usual method afforded a 55:45 mixture of the *cis*-**15a** and *trans*-**15b** diastereomers as determined by analytical HPLC (cyanopropyl silica, 5% 2-propanol in hexanes). Separation by MPLC afforded 0.18 g (53%) of *cis*-**15a**: m.p. 99.5–100.5°C; IR (KBr) 1098 and 1048 (S–O) cm^{-1}; ^1H NMR (C^2HCl_3) δ 1.06 (s,3H), 1.26 (s,3H), 1.77 (ddd,1H), 2.34 (ddd,1H), 2.94 (dd,2H), 3.22 (ddd,1H), 3.44 (m,2H), 3.86 (ddd,H), 7.11 (d,1H), 7.24 (m,2H), 7.70 (d,1H); MS (70 eV), *m/e* (relative intensity) 266 (30,M^+), 174 (14), 158 (100), 143 (43), 129 (8), 118 (8), 108 (13). The *trans*-**15b** was obtained as a white crystalline solid: m.p. 115–156°C; IR (KBr) 1085 and 1045 (S–O) cm^{-1}; ^1H NMR (C^2HCl_3) δ 1.44 (s,3H), 1.49 (s,3H), 1.66(ddd, 1H), 2.25 (ddd,1H), 2.85 (ddd,1H), 3.11 (ddd,1H), 3.79 (dd,2H), 4.10 (m,2H), 6.53 (d,1H), 7.21 (m,2H), 7.27 (d,1H); MS (70 eV), *m/e* (relative intensity) 266 (35,M^+), 158 (100),143 (28).

*Cis- and trans-spiro-1,3-dithiolane-2,9'-(2'-methoxy)-9'H-fluorene-1-oxide (***21a** *and* **21b***).* The diastereomers were obtained in the usual manner (nearly 1:1 mixture) and separated by MPLC (cyanopropyl silica, 5% 2-propanol in hexanes). Recrystallization from hexanes–benzene afforded *cis*-**21a** as a white, crystalline solid: m.p. 147.5–148°C; IR ($CHCl_3$) 1095, 1060 and 1040 (S–O) cm^{-1}; ^1H NMR ($C_6{}^2H_6$)δ 2.68 (m,2H), 2.94 (ddd,1H), 3.34 (s,3H), 4.05 (ddd,1H), 6.82–7.27 (m,6H), 7.70 (d,1H); MS (70 eV), *m/e* (relative intensity) 302 (52,M^+), 226 (100),210 (29), 78 (9), 76 (10). An

analytical sample of *trans*-**21b** was obtained by recrystallization from hexane–benzene; m.p. 138–139°C; IR ($CHCl_3$) 1096, 106 and 1045 (S–O) cm^{-1}; MS (70 eV), *m/e* (relative intensity) 302 (77,M^+), 226 (100), 210 (91), 195 (12), 183 (23), 139 (28), 27 (29).

RESULTS AND DISCUSSION

By comparing the separation factors, α, with the structure of the dithiolane oxides, structure–enantioselectivity relationships can be determined which provide insight into the nature of the chiral recognition processes (Table I). Since unsymmetrically substituted 1,3-dithiolane-1-oxides contain two stereocenters, there are two diastereomers, the *cis* **1** and *trans* **2** isomers, or a total of four possible stereoisomers. *Cis* and *trans* denotes the disposition of the sulfinyl oxygen with respect to the substituent on the adjacent stereogenic center having the highest Cahn–Ingold–Prelog priority. Chromatography on a CSP can, in principle, separate all of these and, for an isomeric mixture of the diastereomers **1i** and **2i**, this is in fact realized (Fig. 3). The enantiomers of diastereomer **1a** are separated quite well (α = 2.13), while those of the other isomer, **2i** are just barely resolved (α = 1.08). The assignment of which pairs of chromatographic peaks stem from which diastereomer was achieved by separation of the diastereomers and assignment of their structure by NMR. A survey of the data in Table I shows that the separation factors, α, of the enantiomers of the *cis* diastereomers **1** are significantly larger than are those of the enantiomers of the *trans* diastereomers **2** in a majority of cases. The relative configuration of the sulfoxide oxygen to the aromatic ring, Ar, plays a decisive role in determining the magnitude of enantioselectivity observed on CSP **3a**.

TABLE I

CHROMATOGRAPHIC SEPARATION OF THE STEREOISOMERS OF 2,2-DISUBSTITUTED 1,3-DITHIOLANE-1-OXIDES ON CSP **3a**

NS indicates an α value of less than 1.04. Chromatographic data were obtained with an analytical column (250 × 4.6 mm I.D.) containing (*R*)-CSP **3a**. Detection, UV 254 nm; flow-rate, 2 ml/min; mobile phase, 10% 2-propanol in hexanes. k' = Capacity factor.

Compound	*Ar*	*R*	*Cis* **1**		*Trans* **2**	
			k'	α	k'	α
a	Phenyl	H	10.4	1.05	8.0	NS
b	Phenyl	CH_3	10.3	NS	7.9	NS
c	Phenyl	C_2H_5	14.4*	1.09	10.6*	NS
d	Phenyl	$CH(CH_3)_2$	4.35**	1.16	4.0	NS
e	Phenyl	$C(CH_3)_3$	3.11*	1.08	6.37*	1.02
f	Phenyl	Cyclohexyl	5.7	1.35	1.8	NS
g	Phenyl	n-C_5H_{11}	10.1*	1.11	7.9*	NS
h	Phenyl	n-$C_{11}H_{23}$	9.2*	1.12	6.7*	NS
i	(6,7-Dimethyl-1-naphthyl)	Cyclohexyl	8.0	2.13	4.3	1.08
j	1-Naphthyl	H	23.1	1.13	11.4	1.10
k	2-Naphthyl	H	25.7	1.15	15.5	1.09
m	9-Anthryl	H	29.0	1.32	16.0	1.28

* Mobile phase, 2% 2-propanol in hexanes.
** Mobile phase, 5% 2-propanol in hexanes.

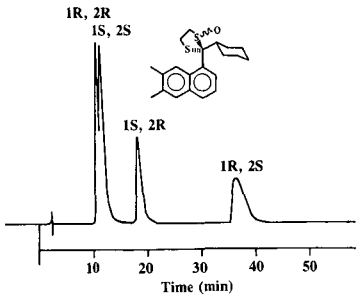


Fig. 3. Chromatographic separation of the four possible stereoisomers of **1i** and **2i** on (*R*)-CSP **3a**. Chromatographic conditions are given in Table I.

If we consider the most populated solution conformer of CSP **3a**, in which the average position of the methine hydrogen is approximately eclipsed with the dinitrobenzoyl carbonyl oxygen[22], the stationary phase can be considered as having two faces; the larger phenyl group protruding from one, and the sterically smaller carboxylate group protruding from the other. The functional subunits deemed necessary for the interactions essential for chiral recognition of the dithiolane oxide enantiomers include the π-acceptor site, the acidic site and a steric interaction site. Complementary interaction sites for **1** and **2** include a π-basic site, the basic sulfinyl oxygen, and a steric group (labelled as 1, 2 and 3, respectively, in Fig. 2). The enantiomeric analyte which has these groups in the appropriate geometric arrangement for simultaneous interaction with the CSP will be preferentially retained.

For **1b–h**, in which the Ar substituent is phenyl, the degree of separation of the enantiomers can be related to the steric size of the R substituent. As steric size increases, α values increase from almost no separation for **1b** ($R_1 = CH_3$) to 1.35 for **1f** (R_1 = cyclohexyl). In terms of the selectivity observed, the effect of the alkyl substituents is cyclohexyl > isopropyl > *n*-alkyl > ethyl, *tert.*-butyl > methyl. This correlates well with the relative steric size of these groups with the exception of **1e** (R_1 = *tert.*-butyl), which affords enantioselectivity similar to **1c** (R_1 = ethyl). However **1e** is not as chromatographically retained as other members of this series and thus adsorption by the CSP may be more difficult owing to the steric bulk of the *tert.*-butyl group. If a compound cannot undergo the proper orientation with the CSP, chiral recognition can be diminished. For the 2-aryl monosubstituted compounds **1a**, **2a**, **1j**, **2j**, **1k**, **1m** and **2m**, the *cis* and *trans* isomers resolve almost equally well. As the π-basicity of the aromatic substituent is increased, the selectivity for the enantiomers is increased. Consequently, **1m** and **2m**, in which Ar is a 9-anthryl group, exhibit the largest α values. The proposed chiral recognition model, **7**, adequately rationalizes the observed structure–enantioselectivity relationships; for either an increase in the π-basicity of the aryl substituent or an increase in the size of the steric group of the analytes, an increase in α value is observed, indicating a greater difference in stability of the two diastereomeric adsorbates. Knowledge of these two trends allows the design of compounds which afford even greater separation factors on CSP **3a**. Thus, **1i**, which contains a 6,7-dimethyl-1-naphthyl group (π base) and a cyclohexyl group (sterically large group) affords the largest enantioselectivity ($\alpha = 2.13$) on **3a** for this series of compounds.

TABLE II

CHROMATOGRAPHIC RESOLUTION OF CHIRAL SPIRO-2-ALKYL-2-ARYL-1,3-DITHIOLANE-1-OXIDES ON CSP **3a**

Chromatographic conditions as in Table I.

Compound			*Cis* **a**		*Trans* **b**	
No.	*n*	*R*	*k'*	*α*	*k'*	*α*
13	1	H	6.2	1.10	3.8	NS
14	2	H	6.2	1.14	4.2	1.04
15	2	CH_3	4.0	1.04	11.2	1.24
16			18.8	1.40	9.47	1.08
17					4.66*	1.07

* Mobile phase, 20% 2-propanol in hexanes.

Spiro-2,2-disubstituted 1,3-dithiolane-1-oxides **13–25** are also resolvable on **3a**. The chromatographic resolution of spiro compounds derived from α-indanone, α-tetralone and their derivatives (Table II) is similar to **1** and **2**. These compounds contain an aromatic ring (π-base), a sulfinyl oxygen (basic site) and an alkyl substituent at C2 (steric group) for site-specific interaction with the CSP. As the steric bulk of the alkyl substituent increases from a methylene group, **14b**, to dimethyl, **15b**, the separation factor increases from 1.04 to 1.24. An increase in π-basicity improves the enantioselectivity from 1.04 for **14a** (phenyl substituent) to 1.40 for **16b** (naphthyl substituent). While in this series the resolution of the *cis* diastereomer is usually more facile, *trans* **15b** resolves with greater ease than *cis* **15a**, perhaps due to changes in the preferred conformation of the ring due to the steric bulk of the two methyl substituents. Based on chiral recognition model **7**, the CSP differentiates the enantiomers on the basis of the absolute configuration of the C2 stereogenic center of the dithiolane ring. The absolute configuration at sulfur simply determines the degree of enantioselectivity observed. Thus, (*R*)-**3a**, will preferentially retain compounds which have the *S* configuration at C2 and therefore, the retained *cis* isomer is 1*R*,2*S*, while the retained *trans* isomer is 1*S*,2*S*.

The enantiomers of spiro-2,2-diaryl-1,3-dithiolane-1-oxides, derived from fluorenone and its derivatives, are readily resolved on **3a** (Table III). For most of these compounds both the *cis* and *trans* isomers are resolved. However, assignments of absolute configurations are confounded by the presence of two aromatic substituents. Depending on which aromatic ring is considered to serve as a π-base for site-specific interaction with the CSP, opposite assignments of absolute configuration are reached for the retained enantiomer. However, for **20** and **25**, which contain a hydroxyl group on one aromatic ring, chiral recognition model **5** can be invoked to predict enantioselectivity and absolute configuration of the more retained analyte. The CSP utilizes the 3,5-dinitrobenzoyl group (DNB, π-acceptor), the DNB amide hydrogen (acidic site) and the carboxylate group (basic site) for site-specific interactions. Analytes **20** and **25** contain the appropriate complementary sites; an aromatic ring (π-donor), a sulfinyl oxygen (basic site) and a hydroxyl group (acidic site). Since the aromatic ring without the hydroxyl substituent must serve as a π-donor site for

TABLE III

CHROMATOGRAPHIC RESOLUTION OF CHIRAL SPIRO-2,2-DIARYL-1,3-DITHIOLANE-1-OXIDES ON CSP **3a**

Chromatographic conditions as in Table I.

Compound				*Cis* **a**		*Trans* **b**	
No.	*X*	R_1	R_2	*k'*	α	*k'*	α
18	S	H	H	4.26	1.21		
19	S	CH_3	H	3.21	1.26	6.79	1.21
20	S	H	OH	13.1*	1.40	10.5*	NS
21	S	H	OCH_3	6.37	1.24	7.76	1.23
22	CH_2	H	H	4.95	1.21		
23				32.0**	1.52	32.0**	1.21
24	R = CH_3			8.25*	1.59	11.3*	1.48
25	R = H			25.6*	1.13	19.3*	1.29

* Mobile phase, 20% 2-propanol in hexanes.
** Mobile phase, 5% 2-propanol in hexanes.

simultaneous three-point interaction with the CSP, three unambiguous bonding interactions are possible. By this model, the enantiomers of *cis* isomers **20a** and **25a** preferentially retained on (*R*)-**3a** are assigned the 1*S*,2*R* configuration, while the retained enantiomer of **25b** is assigned the 1*R*,2*R* configuration.

For the most part, all of the CSPs **3a–d** and **4a–e** provide some separation of the dithiolane oxides (Table IV). Subtle variations between the selectivities of these CSPs are not easy to rationalize since they presumably stem from slight differences in the conformational preferences and the consequential "fits" between the analytes and the CSP. It is not surprising, however, to find that the compounds which are proposed to

TABLE IV

COMPARISON OF THE SELECTIVITY OF CSPs **3a–d** AND **4a–e** FOR THE STEREOISOMERS OF DITHIOLANE OXIDES

*CSP**	*Separation factor* α							
	1f	**1i**	**1k**	**15b**	**16a**	**20a**	**24b**	**25a**
3a	1.35	2.13	1.15	1.24	1.40	1.40	1.48	1.13
3b	1.12	1.08	1.29	1.56	1.13	1.94	1.07	1.74
3c	1.04	1.22	1.17	1.46	NS	1.46	1.05	1.46
3d	1.11	1.23	1.09	1.37	1.07	1.28	NS	1.22
4a	1.41	1.99	1.29	1.26	1.50	1.29	1.44	1.18
4b	1.11		1.57	1.78	NS	1.90	1.08	1.71
4c	1.06	1.20	1.31	1.61	NS	1.33	NS	1.21
4d	1.08	1.14	1.11	1.64	1.05	1.28	1.05	1.20
4e	1.85		1.33	1.29	1.45	1.23	1.36	1.13

* All CSPs were prepared on 5-μm spherical silica and packed into columns (250 × 4.6 mm I.D.). Chromatographic conditions as in Table I.

resolve by chiral recognition model **5** involving two bonding and one steric interaction (**1f**, **1i**, **16a**, and **24b**) afford the larger separation factors on **3a**, **4a**, and **4e**, which have sterically large aryl groups to prevent association of the analyte with the "back" face of the CSP. The leucine-derived CSPs **3b** and **4b** provide improved separations for **1k**, **15b**, **20a**, and **25a**.

In summary, the structure–enantioselectivity relationships observed for the chromatographic resolution of 1,3-dithiolane-1-oxides can be rationalized in terms of chiral recognition models **5** and **7**. These models can be applied to other analytes which contain the proper spatial arrangement of complementary interaction sites. In addition, absolute configurations can be assigned based on chromatographic elution order. Such assignments are only reliable in cases where the chiral recognition processes involved are adequately represented by the chiral recognition model. In a series of compounds, absolute configurations of unknown members can be determined even if an inversion of elution order occurs within the series by "tracking of absolute configurations"[12]. Independent determination of the absolute configurations of some of the 1,3-dithiolane-1-oxides in this series is desirable so that the validity of the assignments of absolute configuration made on the basis of the models may be ascertained.

ACKNOWLEDGEMENTS

This work was partially supported by grants from the National Science Foundation and the Eli Lilly Company.

REFERENCES

1 W. H. Pirkle in S. Ahuja (Editor), *Chromatography and Separation Chemistry: Advances and Developments* (*ACS Symposium Series*, No. 297), American Chemical Society, Washington, DC, 1986, Ch. 6, p. 101.
2 W. H. Pirkle and B. C. Hamper, in B. A. Bidlingmeyer (Editor), *Preparative Liquid Chromatography* (*Journal of Chromatography Library*, Vol. 38), Elsevier, Amsterdam, 1987, Ch. 7, p. 235.
3 W. H. Pirkle, J. M. Finn, B. C. Hamper, J. Schreiner and J. R. Pribish, in E. Eliel and S. Otsuka (Editors), *Asymmetric Reactions and Processes in Chemistry*, *No. 185*, American Chemical Society, Washington, DC, 1982.
4 S. Allenmark, B. Bomgren, H. Boren and P. Lagerström, *Anal. Biochem.*, 136 (1984) 293.
5 S. Allenmark and B. Bomgren, *J. Chromatogr.*, 252 (1982) 297.
6 I. W. Wainer, M. C. Alembik and C. R. Johnson, *J. Chromatogr.*, 361 (1986) 374.
7 T.Kaneko, Y. Okamoto and K. Hatada, *J. Chem. Soc., Chem. Comm.*, (1987) 1511.
8 S. Allenmark, L. Nielsen and W. H. Pirkle, *Acta Chem. Scand. B*, 37 (1983) 325.
9 T. Shimizu and M. Kobayashi, *Bull. Chem. Soc. Jpn.*, 59 (1986) 2654.
10 A. Tambute, P. Gareil, M. Caude and R. Rosset, *J. Chromatogr.*, 363 (1986) 81.
11 W. H. Pirkle, J. M. Finn, J. L. Schreiner and B. C. Hamper, *J. Amer. Chem. Soc.*, 103 (1981) 3964.
12 W. H. Pirkle, M. H. Hyun, A. Tsipouras, B. C. Hamper and B. Banks, *J. Pharm. Bio. Anal.*, 2 (1984) 173.
13 W. H. Pirkle and J. L. Schreiner, *J. Org. Chem.*, 46 (1981) 4988.
14 W. H. Pirkle and J. M. Finn, *J. Org. Chem.*, 46 (1981) 2935.
15 W. H. Pirkle and T. J. Sowin, *J. Chromatogr.*, 387 (1987) 313.
16 F. A. Carey, O. D. Dailey and T. E. Fromuth, *Phosphorus and Sulfur*, 10 (1981) 163.
17 R. Kuhn and F. A. Neugebauer, *Chem. Ber.*, 94 (1961) 2629.
18 R. F. Bryan, F. A. Carey, O. D. Dailey, R. J. Maher and R. W. Miller, *J. Org. Chem.*, 43 (1978) 90.
19 R. B. Stipanovic, J. P. McCormick, E. O. Schlemper, B. C. Hamper, T. Shinmyozu and W. H. Pirkle, *J. Org. Chem.*, 51 (1986) 2500.
20 M. B. Evans, A. D. Dale and C. J. Little, *Chromatographia*, 13 (1980) 5.
21 T. W. Greene, *Protective Groups in Organic Synthesis*, Wiley, New York, 1981, p. 133.
22 W. H. Pirkle, D. W. House and J. M. Finn, *J. Chromatogr.*, 192 (1980) 143.

Journal of Chromatography, 450 (1988) 211–216
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 699

DIRECT ENANTIOMERIC RESOLUTION OF DISOPYRAMIDE AND ITS METABOLITE USING CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO STEREOSELECTIVE METABOLISM AND PHARMACOKINETICS OF RACEMIC DISOPYRAMIDE IN MAN

PASCAL LE CORRE*, DENIS GIBASSIER, PIERRE SADO and ROGER LE VERGE
Laboratoire de Pharmacie Galénique et Biopharmacie, Université de Rennes I, 2 Avenue du Professeur Léon Bernard, 35033 Rennes (France)

SUMMARY

A method for the simultaneous determination of disopyramide and mono-N-desisopropyldisopyramide enantiomers extracted from human plasma and urine is presented. Separation and quantitation were carried out using two columns coupled in series, and UV detection at 254 nm. First, the racemates of the two compounds were separated using a reversed-phase column, and then the enantiomers were separated using a stereoselective column packed with human α_1-acid glycoprotein. The mobile phase was 8 m*M* phosphate buffer, pH 6.20–2-propanol (92:8, v/v). The coefficients of variation (%) for the plasma daily determination were 6.7% for *R*(−)- and *S*(+)-disopyramide at drug levels of 1.5 μg/ml, and 8.5% and 7.7% for *R*(−)- and *S*(+)-mono-N-desisopropyldisopyramide, respectively, at drug levels of 0.375 μg/ml.

The method has allowed the study of stereoselective metabolism and pharmacokinetics of disopyramide after oral administration as a racemate.

INTRODUCTION

Disopyramide (DP) is a class IA antiarrythmic agent used clinically as a racemic mixture of the *S*(+) and *R*(−) enantiomers (Rythmodan®, Roussel; Norpace®, Searle). In humans, mono-N-desisopropyldisopyramide (MND) is the only known metabolite of DP[1,2] (Fig. 1). The plasma protein binding of DP enantiomers, which

Disopyramide Mono-N-Desisopropyldisopyramide

Fig. 1. Metabolic pathway of disopyramide in man. The asterisk indicates the chiral centre.

0021-9673/88/$03.50

involves essentially the α_1-acid glycoprotein (α_1-AGP)[3,4], is concentration dependent and stereoselective[5–8]. Numerous investigators have described the pharmacokinetics of racemic DP[9–12] and of its enantiomers, when administered separately[6,7]. In humans, the unbound species pharmacokinetics[6] of DP enantiomers, when administered separately, are stereoselective. To date, little information is available describing the pharmacokinetic behaviour of each enantiomer following administration of the racemate[7]. This results essentially from the difficulty in evaluating simultaneously both enantiomers after co-administration as a racemate.

The two main approaches to the stereospecific analysis of racemic drugs in biological samples are (a) the use of stereoselective liquid or gas chromatographic methods and (b) the use of mass spectrometric analysis following the administration of a pseudo-racemate, one of the enantiomers being labelled with a stable isotope[7]. The latter technique requires several steps: (a) the isolation of the compounds of interest from biological samples, for instance by collection of a fraction of the mobile phase following high-performance liquid chromatographic (HPLC) analysis; (b) the determination of the concentration of the mixture of labelled and unlabelled forms; and (c) the evaluation of the ratio of labelled to unlabelled compounds. The main drawbacks of the stable-isotope-labelling techniques are that they are time consuming and costly. Further, the lack of an *in vivo* isotopic effect must be demonstrated.

As described previously, a stereoselective analysis of enantiomers of DP[13] and other basic drugs[14] can be achieved using a column packed with human α_1-AGP. The development of a stereoselective HPLC assay of DP and MND enantiomers[15] has allowed the evaluation of the metabolism and pharmacokinetics of DP enantiomers when administered as racemic DP.

EXPERIMENTAL

Chemicals and reagents

DP and MND standards, as racemic bases, were supplied by Roussel UCLAF (Paris, France). All other chemicals were of analytical-reagent grade.

Apparatus

The HPLC system consisted of a Waters Model 6000 B pump (Waters Assoc., Milford, MA, U.S.A.), a Waters WISP 710 B automatic injector, a Waters Model 440 detector set at 254 nm and a Delsi Enica 21 integrator (Delsi, Suresnes, France). As the affinity constants of *R*(−)-DP and *S*(+)-MND for human α_1-AGP are similar, the separation of both DP and MND enantiomers required the previous separation of the two racemic species on a hydrophobic packed column[13]. Hence, two columns were coupled: a Supelcosil LC-8-DB (50 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) and a human α_1-AGP packed column (LKB EnantioPac, 100 × 4 mm I.D.) (LKB, Bromma, Sweden).

Extraction procedure

DP and MND were isolated from biological samples using the two step-extraction procedure shown in Fig. 2. As urine concentrations of DP and MND are generally higher than plasma concentrations, urine samples (10 μl) were diluted with drug-free plasma (240 μl) and then handled according to the procedure described for plasma.

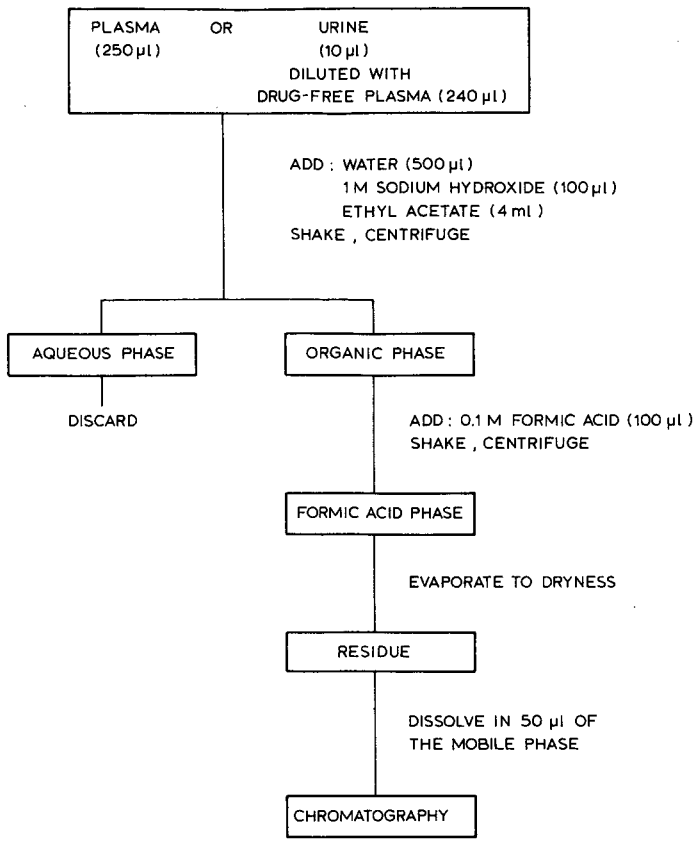


Fig. 2. Schematic outline of the sample preparation used in the determination of DP and MND enantiomers in plasma or urine.

Chromatographic conditions

Isocratic chromatography was carried out at a flow-rate of 0.3 ml/ml, the mobile phase being phosphate buffer (8 m*M*, pH 6.20)–2-propanol (92:8, v/v), with UV detection at 254 nm.

Study design

Six hospitalized patients, suffering from ventricular extrasystoles, were given racemic DP at a dose of 200 mg three times a day (at 8, 16 and 24 h). At the steady state, on day 4, venous blood was drawn prior to drug administration at 8 h and then at 9, 10, 11, 12, 14 and 16 h; 24-h urines were collected.

Pharmacokinetic analysis

Owing to the protocol, the plasma clearance of total DP enantiomers (*CL*) was evaluated relative to the systemic availability (*F*) according to

$$CL/F = \text{dose}/AUC$$

where AUC is the area under the total plasma concentration–time curve.

It has been reported that (a) the first-pass effect of DP is insignificant[11], (b) MND, the sole metabolite of DP, is not further biotransformed[1,2,12] and (c) both DP and MND are eliminated in the urine[12]. Hence, the extent of biotransformation (EB, %) of DP enantiomers was evaluated according to

$$EB = 100\ A_m/(A_p + A_m)$$

where A_p and A_m are the 24-h urinary cumulated amounts of parent drug and the stereochemically related metaolite, respectively.

Statistical analysis

The statistical significance of the stereoselective differences was estimated using Student's paired t-test.

RESULTS AND DISCUSSION

Chromatography

The specificity of the simultaneous determination of DP and MND enantiomers is illustrated by the chromatogram in Fig. 3. The separation factors (α) obtained for DP and MND enantiomers were 1.47 and 1.34, respectively. The resolution factors (R_s) of DP and MND enantiomers were 1.81 and 1.27, respectively.

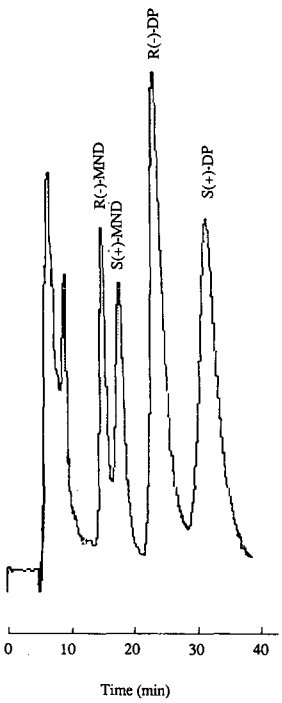


Fig. 3. Chromatogram of plasma extract containing $R(-)$-DP and $S(+)$-DP at 1 μg/ml and $R(-)$-MND and $S(+)$-MND at 0.5 μg/ml.

TABLE I

EXTRACTION YIELDS

Compound	*Concentration (μg/ml)*	*Yield (%)*	*Coefficient of variation (%)*	*n*	*p*
R(−)-DP	1	79.5	5.5	10	>0.05
S(+)-DP	1	79.9	5.7	10	
R(−)-MND	0.5	82.3	5.3	8	>0.05
S(+)-MND	0.5	82.1	6.6	8	

On account of the stereoselective α_1-AGP binding, the extraction yield of *R*(−)-DP and *S*(+)-DP and of *R*(−)-MND and *S*(+)-MND were compared. The results reported in Table I show that the extraction procedure is not stereoselective.

The linearity of the method was evaluated in the concentration range 0.5–4 μg/ml for DP enantiomers and 0.125–1 μg/ml for MND enantiomers. The coefficients of correlation were better than 0.999 for the four calibration graphs. The reproducibility was checked daily ($n = 12$) at 1.5 μg/ml for DP enantiomers and at 0.375 μg/ml for MND enantiomers. The coefficients of variation, 6.7% for *R*(−)-DP and *S*(+)-DP, 8.5% for *R*(−)-MND and 7.7% for *S*(+)-MND, indicate an acceptable reproducibility of the method.

Metabolism and pharmacokinetics

The mean extent of biotransformation of *S*(+)-DP (56.6%) was significantly higher than that of *R*(−)-RP (39.8%) ($p < 0.001$). The mean *S*(+)-DP/*R*(−)-DP metabolic ratio, *i.e.*, the stereoselective index, equal to 1.42, indicates that N-dealkylation of racemic DP is stereoselective. These results are consistent with data from our laboratory, which have pointed out stereoselectivity in DP metabolism, when *R*(−)-DP and *S*(+)-DP were administered separately to healthy volunteers; the stereoselective index was 1.55.

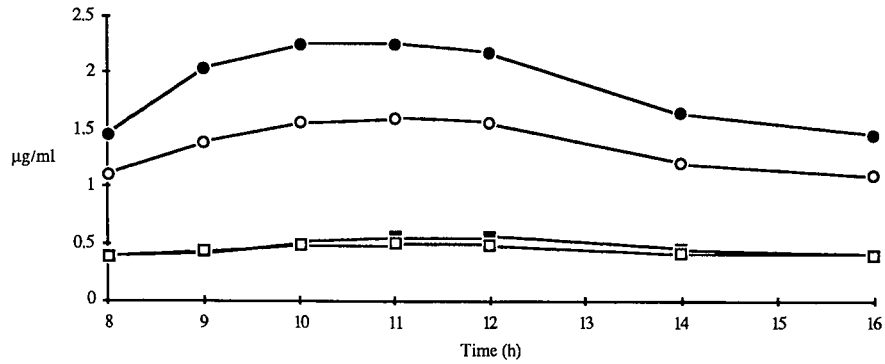


Fig. 4. Average total plasma concentrations of DP and MND enantiomers, at the steady state, following oral administration to patients ($n = 6$), of racemic DP at a dose of 200 mg three times daily. ● = *S*(+)-DP; ○ = *R*(−)-DP; ■ = *S*(+)-MND; □ = *R*(−)-MND.

The average total plasma concentrations of DP and MND enantiomers, at the steady state, are presented in Fig. 4.

When administered separately, the total plasma clearance of DP enantiomers is not stereoselective[6,7]. In this study, when the enantiomers are co-administered, the total clearances are significantly different: 114.6 and 157 ml/min for *S*(+)-DP and *R*(−)-DP, respectively ($p < 0.001$). These results, consistent with previous data obtained following a single parenteral administration of DP as a pseudo-racemate[7], suggest a stereoselective interaction between the DP enantiomers. A reciprocal displacement interaction between the DP enantiomers in plasma protein binding has been described, *S*(+)-DP being a more potent displacer than *R*(−)-DP[8]. Thus, in the presence of *S*(+)-DP, the unbound plasma fraction and consequently the total plasma clearance of *R*(−)-DP should be increased.

REFERENCES

1 C. S. Cook, A. Karim and P. Sollman, *Drug Metab. Dispos.*, 13 (1982) 116.
2 A. Karim, R. E. Ranney and S. Kraychy, *J. Pharm. Sci.*, 61 (1972) 888.
3 J. E. Bredesen, E. Pike and P. K. M. Lund, *Br. J. Clin. Pharmacol.*, 14 (1982) 673.
4 J. E. Bredesen and P. Kierulf, *Br. J. Clin. Pharmacol.*, 18 (1984) 779.
5 J. J. Lima, G. L. Jungbluth, T. Devine and L. W. Robertson, *Life Sci.*, 35 (1984) 835.
6 J. J. Lima, H. Boudoulas and B. J. Shields, *Drug. Metab. Dispos.*, 13 (1985) 572.
7 K. M. Giacomini, W. L. Nelson, R. A. Pershe, L. Valdivieso, K. Turner-Tamiyasu and T. F. Blaschke, *J. Pharmacokinet. Biopharm.*, 14 (1986) 335.
8 J. J. Lima, *Life Sci.*, 41 (1987) 2807.
9 M. Thibonnier, N. G. H. Holford, R. A. Upton, C. D. Blume and R. L. Williams, *J. Pharmacokinet. Biopharm.*, 12 (1984) 559.
10 P. H. Hinderling and E. R. Garrett, *J. Pharmacokinet. Biopharm.*, 4 (1976) 199.
11 J. J. Lima, D. B. Haughey and C. V. Leier, *J. Pharmacokinet. Biopharm.*, 12 (1984) 289.
12 A. Karim, *Angiology*, Suppl. I, Part 2 (1975) 85.
13 J. Hermansson, M. Eriksson and O. Nyquist, *J. Chromatogr.*, 336 (1984) 321.
14 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
15 P. Le Corre, D. Gibassier, P. Sado and R. Le Verge, *J. Chromatogr.*, 424 (1988) 424.

Journal of Chromatography, 450 (1988) 217–220
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 696

SELECTIVE EFFECT OF CLONAZEPAM AND (*S*)-UXEPAM ON THE BINDING OF WARFARIN ENANTIOMERS TO HUMAN SERUM ALBUMIN

ILONA FITOS* and MIKLÓS SIMONYI
Central Research Institute for Chemistry of The Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest (Hungary)

SUMMARY

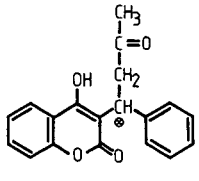
Both clonazepam and (*S*)-uxepam selectively increase the binding of (*S*)-warfarin to human serum albumin. By liquid affinity chromatography, improved resolution of *rac*-warfarin was achieved.

INTRODUCTION

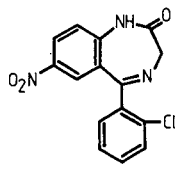
Binding studies performed by various methods have shown that serum proteins have different affinities for the enantiomers of several chiral ligands[1]. Liquid affinity chromatography on immobilized serum albumin is a sensitive technique to detect enantioselective binding and to perform resolution of racemates[2,3].

Warfarin is a chiral anticoagulant drug and its *S*-enantiomer has higher anticoagulant activity[4]. The binding affinities of warfarin enantiomers to human serum albumin (HSA) are slightly different[5,6], allowing a poor chromatographic resolution of *rac*-warfarin. Previously we found[7] stereoselective allosteric binding interactions in the binding of warfarin enantiomers and certain optically active 3-substituted 1,4-benzodiazepines to HSA, which were manifested by either mutually increased or decreased binding. The highest enhancements were observed with the *S*-enantiomers of both ligands. Increased stereoselective binding of these ligands resulted which can be utilized for improved chromatographic resolution of the racemates on an HSA-Sepharose column.

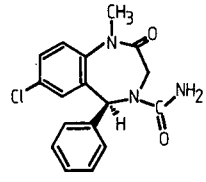
We present here the effects of clonazepam and the enantiomers of uxepam on the binding of warfarin enantiomers. Clonazepam is optically inactive, being an equimolar mixture of two conformers of opposite chirality, while uxepam has a centre of chirality in the C_5 position.



rac-warfarin



clonazepam



(S)-uxepam

0021-9673/88/$03.50

EXPERIMENTAL

Clonazepam and *rac*-warfarin were obtained from Sigma, [^{14}C]*rac*-warfarin from The Radiochemical Centre Amersham. *rac*-Warfarin was resolved into its enantiomers as described[8] and kindly donated by Dr. T. Larsson. It was applied as its sodium salt. Uxepam enantiomers were obtained from Chemical Works of Gedeon Richter (Budapest, Hungary).

About 10^{-4} *M* HSA (Miles Labs., Elkhart, IN, U.S.A.) was immobilized on CNBr-Sepharose 4B (Pharmacia). Elution was by Ringer buffer (pH 7.4) containing 0.01% sodium azide. Detection was by UV spectroscopy or radioactive liquid scintillation counting. Samples of 2–5 μg were applied.

RESULTS

Warfarin binds strongly[9] to HSA ($K \approx 1.8 \cdot 10^5\ M^{-1}$) and the affinities of the enantiomers are similar to each other. The direction of stereoselectivity can be opposite under slightly different experimental conditions. Fig. 1a shows the radiochromatogram of [^{14}C]*rac*-warfarin on a short HSA–Sepharose column and it is seen that no resolution occurred. When the eluent contained 10^{-4} *M* clonazepam (Fig. 1b) the enantiomers appeared in separate peaks. The assignment of the peaks were made with (*R*)- and (*S*)-warfarin. Table I shows the elution volumes of warfarin enantiomers obtained with buffer alone as well as with 10^{-4} *M* clonazepam in the buffer. The binding of (*S*)-warfarin is enhanced in the presence of clonazepam. This was confirmed as follows: on an HSA-Sepharose column (V_0 = 7 ml) the elution volume of clonazepam (12 ml) was practically unchanged in the presence of 10^{-4} *M* (*R*)-warfarin, while 10^{-4} *M* (*S*)-warfarin increased it (23 ml).

Fig. 2 shows the selective effects of uxepam enantiomers on the binding of *rac*-warfarin. It is the (*S*)-uxepam which brings about resolution. The binding of *rac*-uxepam to HSA, unlike the parent 4,5-dihydrodiazepam[10], is very weak and not

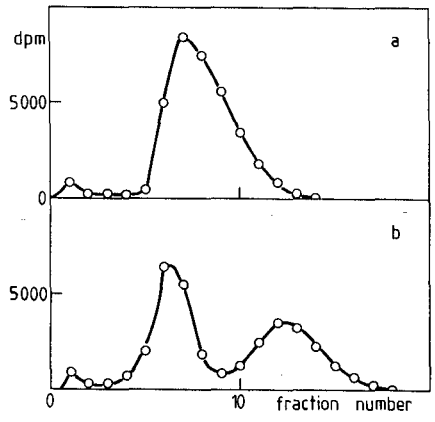


Fig. 1. Radiochromatogram of [^{14}C]*rac*-warfarin on a HSA–Sepharose column (V_0 = 3 ml, $V_{fraction}$ = 8 ml). Elution was by (a) buffer, (b) 10^{-4} *M* clonazepam.

TABLE I

EFFECT OF CLONAZEPAM ON THE ELUTION VOLUMES OF WARFARIN ENANTIOMERS ON A HSA-SEPHAROSE COLUMN

V_0 = 6 ml.

Sample	V_e *(ml)*	
	Eluent: buffer	*Eluent:* 10^{-4} *M clonazepam*
(*R*)-Warfarin	60	48
(*S*)-Warfarin	52	120

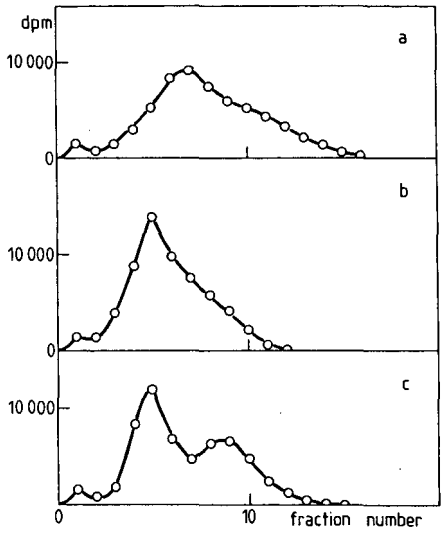


Fig. 2. Radiochromatogram of [^{14}C]*rac*-warfarin on a HSA–Sepharose column (V_0 = 4 ml, $V_{fraction}$ = 8 ml). Elution was by (a) buffer, (b) 10^{-4} *M* (*R*)-uxepam, (c) 10^{-4} *M* (*S*)-uxepam.

TABLE II

EFFECT OF (*R*)- AND (*S*)-WARFARIN ON THE ELUTION VOLUMES (IN ml) OF UXEPAM ENANTIOMERS

V_0 = 6 ml.

Sample	*Buffer*	10^{-4} *M (R)-Warfarin*	10^{-4} *M (S)-Warfarin*
(*R*)-Uxepam	8	8	8
(*S*)-Uxepam	8	8	12

stereoselective. In accordance with the above interaction, (*S*)-warfarin selectively increases the binding of (*S*)-uxepam (Table II).

DISCUSSION

Diazepam and *rac*-warfarin are generally considered[9,11] as markers of the two main binding sites on HSA. The binding interactions observed suggest that there are allosteric interactions between these binding sites. The manifestation of this phenomenon is strongly dependent on the configuration of both benzodiazepine and warfarin as well as on the structure of the benzodiazepine molecule. According to the above results the following conclusions can be drawn:

(1) It is (*S*)-warfarin which shows binding interactions with clonazepam and (*S*)-uxepam.

(2) In the clonazepam molecule it is the 2'-Cl substituent which is responsible for inducing the binding interaction, since desmethyldiazepam and warfarin enantiomers do not affect the binding of each other. The clonazepam molecule can exist in two chiral conformations. Analogously to diazepam and 3,3-dimethyl derivatives[12], and according to the difference in the circular dichroism (CD) spectra of the clonazepam–HSA complex[13], we suppose that the (*M*)-conformation is preferred by the binding process.

(3) In the (*S*)-uxepam molecule it is the 4-carbamoyl substituent which is responsible for inducing the binding interaction, since none of the enantiomers of 4,5-dihydrodiazepam and warfarin affects the binding of each other.

(4) Both clonazepam and (*S*)-uxepam have very low binding affinities to HSA, yet they are able to provoke binding interactions manifested in improved resolution of the strongly bound *rac*-warfarin. Liquid affinity chromatography is suitable to detect not only stereoselective binding[14] but even stereoselective binding interactions. Under favourable experimental conditions this can be utilized for chiral separations.

REFERENCES

1 M. Simonyi, I. Fitos and J. Visy, *Trends Pharm. Sci.*, 7 (1986) 112.
2 S. Allenmark, *Chem. Scr.*, 20 (1982) 5.
3 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
4 M. Simonyi, *Med. Res. Rev.*, 4 (1984) 359.
5 N. A. Brown, E. Jähnchen, W. E. Müller and U. Wollert, *Mol. Pharmacol.*, 13 (1977) 70.
6 C. Lagercrantz, T. Larsson and I. Denfors, *Compr. Biochem. Physiol.*, 69C (1981) 375.
7 I. Fitos, Zs. Tegyey, M. Simonyi, I. Sjöholm, T. Larsson and C. Lagercrantz, *Biochem. Pharmacol.*, 35 (1986) 263.
8 B. D. West, S. Price, C. H. Schroeder and K. P. Link, *J. Am. Chem. Soc.*, 83 (1961) 2676.
9 I. Sjöholm, B. Ekman, A. Kober, I. Ljungstadt-Pahlman, B. Seiving and T. Sjödin, *Mol. Pharmacol.*, 16 (1979) 767.
10 I. Fitos and M. Simonyi, *Experientia*, 39 (1983) 591.
11 G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, 12 (1976) 1052.
12 I. Fitos, M. Simonyi, Zs. Tegey, M. Kajtár and L. Ötvös, *Arch. Pharm. (Weinheim)*, 319 (1986) 744.
13 T. Sjödin, N. Roosdrop and I. Sjöholm, *Biochem. Pharmacol.*, 25 (1976) 2131.
14 I. Fitos, M. Simonyi, Zs. Tegyey, L. Ötvös, J. Kajtár and M. Kajtár, *J. Chromatogr.*, 259 (1983) 494.

Journal of Chromatography, 450 (1988) 221–232
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 722

DETERMINATION OF THE *S*(+)- AND *R*(−)-ENANTIOMERS OF BACLOFEN IN PLASMA AND URINE BY GAS CHROMATOGRAPHY USING A CHIRAL FUSED-SILICA CAPILLARY COLUMN AND AN ELECTRON-CAPTURE DETECTOR

A. SIOUFI*
Ciba-Geigy Laboratories, Biopharmaceutical Research Centre, P.O. Box 308, 92506 Rueil-Malmaison Cedex (France)
G. KAISER
Research and Development Department, Pharmaceutical Division, Ciba-Geigy Ltd., Basle (Switzerland)
and
F. LEROUX and J. P. DUBOIS
Ciba-Geigy Laboratories, Biopharmaceutical Research Centre, P.O. Box 308, 92506 Rueil-Malmaison Cedex (France)

SUMMARY

A sensitive and enantiospecific gas chromatographic method for the determination of the *S*(+)- and *R*(−)-enantiomers of baclofen (I and II) in plasma and urine has been developed and validated. The method is based on the complete resolution of the derivatized enantiomers on a chiral fused-silica capillary column. The hydrochloride salt of a (−)-fluoro analogue of baclofen (III · HCl) was used as the internal standard in plasma, the hydrochloride salt of a (+)-fluoro analogue of baclofen (IV · HCl) as the internal standard in urine. Rapid and convenient isolation of the compounds was achieved using reversed-phase Bond-Elut C_{18} columns. After elution, the compounds were converted into isobutyl esters and purified by base-specific solvent extraction. The isobutyl esters were then N-acylated with heptafluorobutyric anhydride The derivatives were quantitated after separation on the chiral column using electron-capture detection. The analysis of spiked plasma and urine samples demonstrated the good accuracy and precision of the method, with limits of quantitation of 25 nmol/l for I and II in plasma and of 2 μmol/l for I and II in urine. The method appears to be suitable for use in pharmacokinetic studies of the enantiomers in plasma and urine from animals and man after administration of the racemic baclofen.

INTRODUCTION

Baclofen is a centrally acting antispastic agent. The enantiomers of this compound (Fig. 1) differ in their pharmacodynamic and toxicological properties: the *R*(−)-enantiomer (II) is much more active but also more toxic than the *S*(+)-enantiomer (I)[1]. In addition, the "ineffective" isomer I antagonizes the action of the

0021-9673/88/$03.50

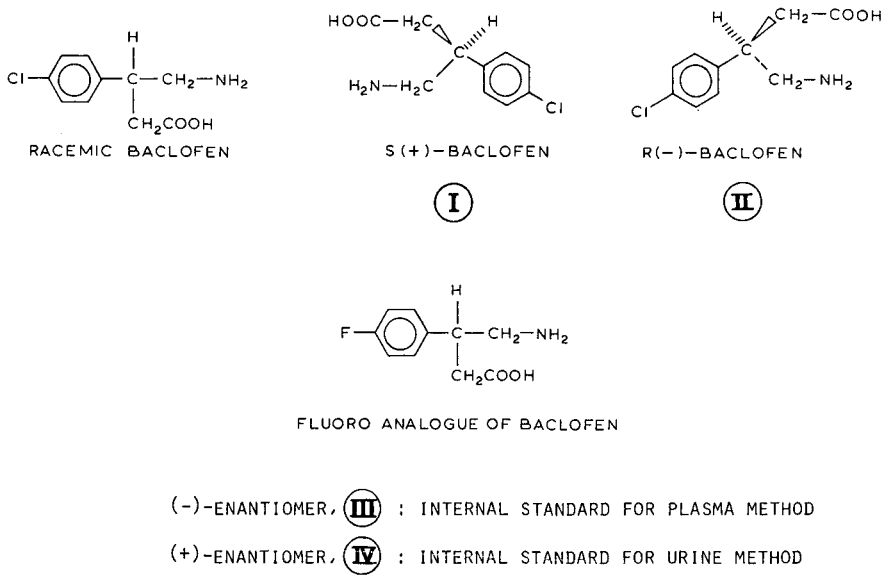


Fig. 1. Chemical structures of baclofen, I, II, III and IV. The absolute configuration of III and IV is unknown.

effective isomer II, so that II is also substantially more effective than racemic baclofen[2,3]. In spite of these interesting differences in the pharmacodynamics of I, II and racemic baclofen, the pharmacokinetics of the enantiomers have not yet been described due to the lack of analytical methods for their enantiospecific and sensitive determination. Between 1984 and 1987, several high-performance liquid chromatography (HPLC) methods for the separation of the enantiomers of baclofen were reported[4–7]. However, none of these methods has so far been extended to determinations in biological fluids.

In 1988, Spahn *et al.*[8] published an enantiospecific HPLC method which is suitable for the determination of I and II in biological material, but this method lacks sensitivity for the determination in plasma. Their paper also gives the first information on the urinary excretion in healthy volunteers.

Based on published gas chromatographic (GC) methods for racemic baclofen[9,10], we have now developed an enantiospecific GC procedure using a chiral capillary column. The method is less time consuming than the non-enantiospecific GC method described by Degen and Riess[9] and more sensitive than the method of Spahn *et al.*[8]. The present paper describes this method and its validation for the determination of I and II in plasma and urine.

EXPERIMENTAL

Chemicals and reagents

Racemic baclofen ($C_{10}H_{12}ClNO_2$; mol.wt. 213.66), the hydrochloric salts of the *S*(+)- and *R*(−)-enantiomers of baclofen, I · HCl and II · HCl, respectively

($C_{10}H_{12}ClNO_2$ · HCl; mol.wt. 250.12), and the two internal standards, the hydrochloride salts of the (−)- and (+)-fluoro analogue of baclofen, III · HCl and IV · HCl, respectively ($C_{10}H_{12}FNO_2$ · HCl; mol.wt. 233.67), were all supplied by Ciba-Geigy (Basle, Switzerland) (Fig. 1). III · HCl was used as the internal standard in the plasma method, IV · HCl in the urine method.

A 1-pmol amount of I or II is equivalent to 0.2501 ng of the hydrochloride salt of I or II or to 0.2137 ng of the free amino acid (I or II).

Borate buffer pH 10 was prepared by dissolving 24.6 g boric acid (No. 165; Merck, Damstadt, F.R.G.), 29.8 g potassium chloride (Merck No. 4936) and 14.1 g sodium hydroxide (Merck No. 5589) in 1 l of distilled water.

The solvents used for esterification were isobutanol (Merck No. 984) and acetyl chloride (Merck No. 31). For the acylation, we used heptafluorobutyric anhydride in glass ampoules of 1 ml (No. 63164; Pierce, Rockford, IL, U.S.A.). All other chemicals were of analytical grade.

Extraction columns and vacuum apparatus

Bond-Elut C_{18} columns with a capacity of 2.8 ml (607 303; Analytichem, Harbor City, CA, U.S.A.) were used for plasma and Bond-Elut C_{18} columns of 1 ml capacity (Analytichem, 607 101) were used for urine. Rapid sample processing was achieved in both cases with a vacuum manifold (No. 5-7030; Supelco, Bellefonte, PA, U.S.A.).

Chromatographic equipment

Gas chromatography was performed on an HP 5890 A chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with an electron-capture detector, an automatic sampler HP 7673 A and a gas chromatographic workstation HP 5895 A. Two types of chiral fused-silica capillary columns were tested: a 25 m × 0.25 mm I.D. column coated with Chirasil-Val III (Alltech, No. 13636; Applied Science, State College, PA, U.S.A.); a 25 m × 0.22 mm I.D. column coated with Chirasil-L-Val or Chirasil-D-Val, film thickness 0.12 μm (No. 7495 and No. 7496, respectively; Chrompack, Middelburg, The Netherlands).

Determination of the enantiomeric purity of I · HCl and II · HCl

The enantiomeric purity of I · HCl and II · HCl was determined by enantioselective GC of the N-heptafluorobutyryl isobutyl ester derivatives. These were prepared as described in the analytical procedure after evaporation of pure methanolic solutions of the compounds. Six samples containing 1000 pmol of one enantiomer were analyzed for the content of the other enantiomer using a calibration graph in the range 2–50 pmol. For the determination of I, a Chirasil-L-Val column was used, where this enantiomer was eluted before II. For the determination of II, a Chirasil-D-Val column was used, where the elution order was reversed.

Chromatographic performance

The chromatographic separation of the derivatives of I and II was characterized by the separation coefficient, α, and the resolution factor, R[11,12]

$$R = \frac{2\ (t_2 - t_1)}{1.699\ (w_{h_1} + w_{h_2})}$$

where t_1, t_2 = retention times of the enantiomers ($t_2 > t_1$), t_0 = retention time of a non-retained compound and w_h = peak width at half-height.

The retention time of the solvent (heptane) was considered as t_0. Assuming ideal Gaussian peaks, baseline separation is achieved for $R \geqslant 1.5$[12].

Extraction yield

The extraction yield of I and II and of the internal standards (III for plasma, IV for urine) from plasma and urine was determined by comparison with directly derivatized samples considering the detector response. The calculation was based on the average for six samples of each.

Stock solutions and working solutions

Stock solutions were prepared by dissolving all compounds in 0.1 *M* hydrochloric acid. Working solutions were obtained by dilution of the corresponding stock solution in distilled water. All solutions were stored at 4°C and were stable for at least 1 month.

The stock solutions were different for samples used for the calibration and method validation.

Preparation of spiked plasma samples

Aliquots of the working solutions of I · HCl and II · HCl were added together with a constant amount (850 pmol) of the internal standard III · HCl to 1 ml of plasma. The samples were shaken on a Vortex mixer for 15 s.

Calibration samples containing I · HCl and II · HCl in comparable amounts were prepared at six different concentrations between 20 and 800 nmol/l for each enantiomer. For method validation, the spiked samples contained I · HCl and II · HCl in comparable or different amounts between 20 and 835 nmol/l. Clinical samples were processed in the same way using water instead of the working solution of I · HCl and II · HCl.

Preparation of spiked urine samples

Calibration and validation samples containing equal amounts of the enantiomers I · HCl and II · HCl at six different concentrations were prepared by spiking urine with racemic baclofen. Validation samples containing different amounts of the enantiomers were prepared by spiking urine with I · HCl and II · HCl.

Aliquots of the working solutions were added with a constant amount (2.15 nmol) of the internal standard IV · HCl to 25 µl of urine. Distilled water was added to a total volume of 250 µl. Finally, 75 µl of blank urine were added. The samples were shaken on a Vortex mixer for 15 s. The amount of each compound added was related to the urine sample volume of 25 µl used to analyse clinical urine samples. Calibration samples were in the range corresponding to the concentrations of each enantiomer in urine, between 2.2 and 90 µmol/l. The concentrations of the validation samples covered the same concentration range. Clinical samples were processed in the same way using water instead of the working solutions of baclofen, I · HCl and II · HCl.

Extraction procedure for plasma

Extraction was performed with Bond-Elut C_{18} columns. The columns were positioned in the luer fittings of the vacuum manifold. Twelve columns can be used simultaneously; the columns were conditioned by washing with 2 × 3 ml of methanol and 2 × 3 ml of water. A vacuum of 25–50 cmHg was applied which was turned off as soon as the water had passed through, to prevent the columns from drying out. The plasma samples were applied to the columns and the biological matrix was sucked off by gently applying a vacuum to the manifold. Then each column was washed with 1 ml of water. The vacuum was maintained for 5.5 min until the columns were dry. The cover of the manifold was then removed and the stainless-steel needles of the column were wiped with a tissue to remove drops of the washing solution. Glass tubes of 10 ml were then positioned under each column and the columns were eluted with 2 × 500 µl of methanol by applying a vacuum to the manifold. The eluents were evaporated to dryness at 45°C under a stream of nitrogen.

Extraction procedure for urine

The extraction procedure for urine was that used for plasma except as follows: Bond-Elut C_{18} columns of 1 ml capacity were used and conditioned with 2 × 1 ml of methanol and 2 × 1 ml of water; after application of the sample, each column was washed with 100 µl of water, then the vacuum was maintained for 4 min until the columns were dry. The columns were eluted with 2 × 500 µl of methanol and the eluents evaporated to dryness at 45°C.

Derivatization

Esterification. The dry residue was dissolved in 0.5 ml hydrochloric acid in isobutanol (5 ml isobutanol and 0.25 ml acetyl chloride), heated at 100°C for 15 min and evaporated to dryness under nitrogen at 45°C.

Purification. The dry residue was dissolved in 5 ml of diethyl ether–dichloromethane (4:1, v/v) and the solution was extracted with 2 ml of 0.05 *M* sulphuric acid by shaking for 10 min. After a short period of centrifugation, the organic phase was discarded. The aqueous phase was made alkaline by addition of 2 ml of borate buffer pH 10 and extracted with 5 ml of hexane–acetone (50:1, v/v) by shaking for 10 min. After a short period of centrifugation, the organic phase was transferred to a 10-ml tube and evaporated to dryness under nitrogen at 45°C.

Acylation. A 100-µl volume of heptafluorobutyric anhydride and 500 µl of diethyl ether were added to the residue and allowed to react for 30 min at room temperature. Then the mixture was evaporated to dryness under nitrogen at 45°C. The dry residue was dissolved in 1 ml heptane and washed with 2 ml of 0.1 *M* sodium hydroxide by shaking for 10 min. After centrifugation, an aliquot of the organic phase was transferred to a conical vial of the automatic sampler; 2 µl were injected into the gas chromatograph.

Chromatographic conditions

Splitless injection was used with a splitless period of 1 min. The inlet pressure of the carrier gas, *i.e.*, helium, was 65 kPa for the Chirasil-Val-III column or 90 kPa for the Chirasil-L-Val or Chirasil-D-Val column. The septum purge was 3 ml/min and the flow-rate of the auxiliary gas (argon–methane, 90:10) for the detector was 60 ml/min. The injector temperature was 250°C and the detector temperature was 300°C.

After injection, the column was held at 80°C for 1 min, then heated at 30°C/min to 200°C.

To elute endogenous plasma or urine compounds, the Chirasil-L-Val or Chirasil-D-Val column was held at 200°C for 20 min. Using the Chirasil-Val III column, the temperature was held at 200°C for 9 min, then raised at 70°C/min to 240°C and held at this level for 3 min.

Quantitative evaluation

Quantitation was based on the peak height ratio, y, of the substance and the internal standard. Calibration graphs were obtained by plotting y *versus* the concentration, x, of I or II in the sample and calculating the regression line. This was done by weighted linear least-square regression analysis with a weighting factor of $1/x^2$.

RESULTS AND DISCUSSION

Enantiomeric purity

The analysis of pure samples of I · HCl and II · HCl revealed an optical purity of at least 99.8% for both enantiomers. The samples were derivatized in the usual manner as described. Thus, the high optical purity also demonstrated that no racemization occurred during the derivative formation.

Chromatographic purity and choice of the internal standard

Impurities were not detected when investigating the enantiomeric purity of I · HCl and II · HCl. When studying the determination in urine, the concentrations of the enantiomers were about 10 times higher than those for the determination in plasma. Under these conditions, there was an impurity peak with a retention time close to that of the III · HCl derivative used as the internal standard for the plasma method. This peak was present also in pure derivatized samples of racemic baclofen as well as of I · HCl and II · HCl. The peak height increased with increasing amounts of the compounds. The origin of this impurity is unknown. To avoid a possible interference with the internal standard, we used IV · HCl instead of III · HCl as the internal standard for the method in urine.

Choice of the analytical column

Baseline separation of the baclofen enantiomers (I and II) was achieved with each of the three chiral columns tested. The parameters characterizing the separation are listed in Table I.

The separation coefficient, α, and the resolution factor, R, gradually deteriorated with increasing number of injections. The values given in Table I were obtained using new columns.

In terms of the separation performance and the thermal stability, the Chirasil-L-Val or -D-Val column was found to be superior to the Chirasil-Val III column. The L- and D-Val columns are expected to exhibit an acceptable peak resolution even after more than 1000 injections of plasma or urine extracts.

All method validation data given in this paper were obtained using a Chirasil-L-Val column.

TABLE I

PARAMETERS CHARACTERIZING THE SEPARATION OF THE N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTER DERIVATIVES OF THE BACLOFEN ENANTIOMERS (I AND II) ON CHIRAL CAPILLARY COLUMNS

Chromatographic conditions and definitions of α and R as in Experimental.

Column	*Retention time (min)*		α	R
	I	*II*		
Chirasil-Val III	13.87	14.09	1.019	1.485
Chirasil-L-Val	11.71	11.96	1.024	1.998
Chirasil-D-Val	12.93	12.66	1.025	2.079

Extraction and derivatization

A solid–liquid extraction using Bond-Elut C_{18} columns (capacity 2.8 ml for plasma and 1 ml for urine) was found to produce the best extraction efficiency and throughput time. For the method in urine, the best results were achieved when the samples applied to the columns contained 100 μl of urine. Therefore the spiked (or clinical) urine samples of 25 μl were (will be) diluted in 75 μl of blank urine. The extraction yield for plasma was 85% for I, 89% for II and 65% for III. The extraction yield for urine was 83% for I, 82% for II and 89% for IV.

These values are comparable to those reported for adsorption on charcoal and XAD-2 resin of racemic baclofen[9] and markedly higher than those reported for ion-pair extraction[10]. In addition, the Bond-Elut method is less time consuming than the other two methods.

Different derivatives of baclofen were used in the GC methods reported for the racemic compound[9,10]. In a screening procedure, we have tested derivatives of I and II obtained by esterification with methanol, ethanol, propanol, pentafluoropropanol, isobutanol or *n*-butanol and by acylation with trifluoroacetic-, pentafluoropropionic- or heptafluorobutyric anhydride. The best results with respect to separation efficiency and sensitivity were achieved for the N-heptafluorobutyryl isobutyl ester derivatives. Therefore, these derivatives were chosen for the present method.

Gas chromatography of plasma and urine extracts

Typical chromatograms of extracts from blank plasma and plasma spiked with I · HCl, II · HCl and III · HCl are shown in Fig. 2. No interfering peaks derived from endogenous plasma components were observed at the expected retention times. The "background" peaks observed are negligible compared to the peaks obtained for a concentration of around 20 nmol/l of I · HCl and II · HCl.

Typical chromatograms of extracts from blank urine and urine spiked with racemic baclofen and IV · HCl are shown in Fig. 3. No interference peaks derived from endogenous urine components were observed at the expected retention times for I, II and IV.

Calibration graphs

Calibration graphs for I and II in plasma showed a linear response in the range 20–800 nmol/l. Typical parameters for the calibration graphs in plasma were

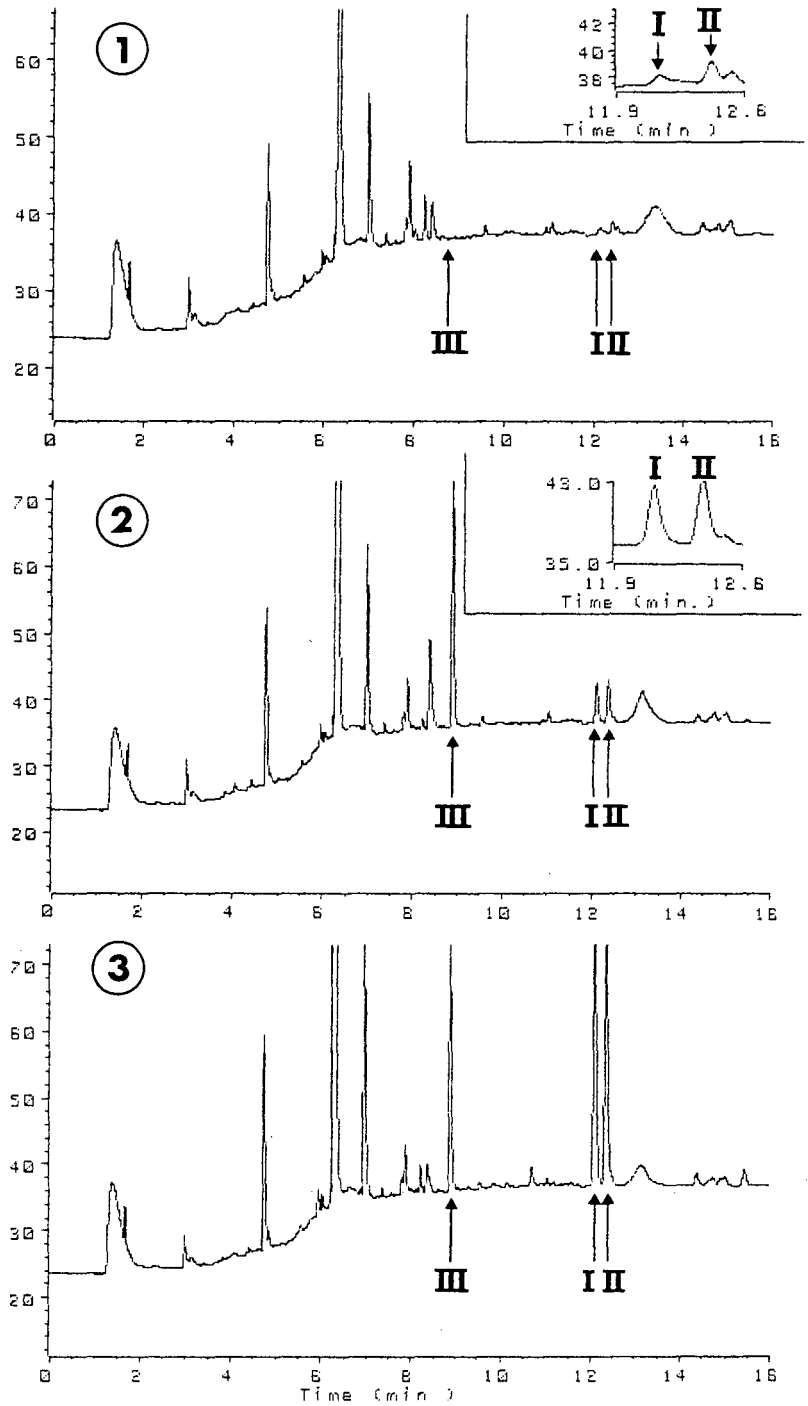


Fig. 2. Typical chromatograms of (1) a derivatized extract of a blank human plasma sample, (2) a derivatized extract of a spiked plasma sample containing 21.9 nmol/l of I · HCl, 20.4 nmol/l of II · HCl and 855.9 nmol/l of the internal standard (III · HCl), (3) a derivatized extract of a spiked plasma sample containing 784.7 nmol/l of I · HCl, 777.9 nmol/l of II · HCl and 855.9 nmol/l of the internal standard (III · HCl).

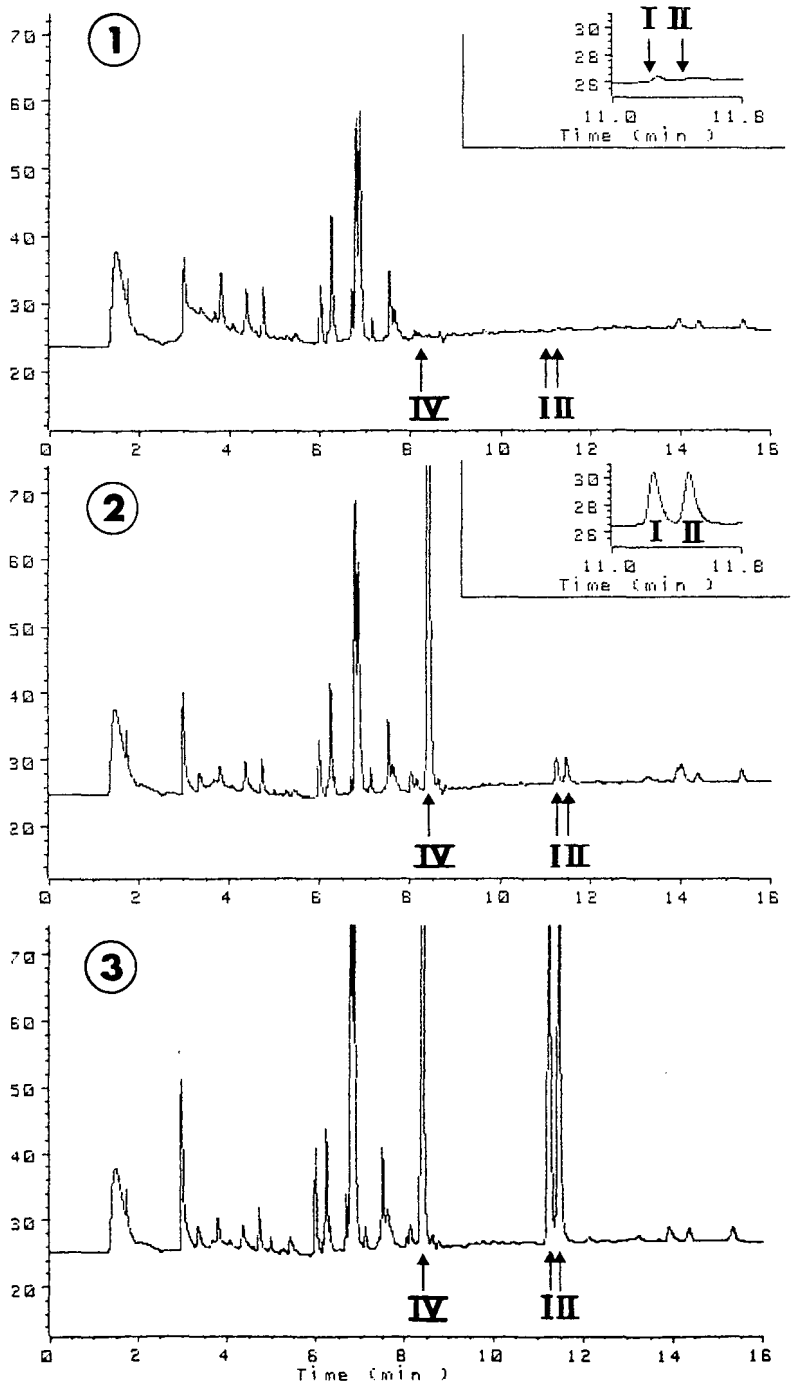


Fig. 3. Typical chromatograms of (1) a derivatized extract of a blank urine sample (25 μl), (2) a derivatized extract of a spiked urine sample containing 4.36 μmol/l of racemic baclofen (corresponding to 2.18 μmol/l of I · HCl and II · HCl) and 85.59 μmol/l of the internal standard (IV · HCl) and (3) a derivatized extract of a spiked urine sample containing 71.52 μmol/l of racemic baclofen (corresponding to 35.76 μmol/l of I · HCl and II · HCl) and 85.59 μmol/l of the internal standard (IV · HCl).

TABLE II

WITHIN-DAY PRECISION AND ACCURACY OF THE DETERMINATION OF I AND II IN SPIKED HUMAN PLASMA

Concentration of I added (nmol/l)	*n*	*Accuracy (%)**	*Concentration of II added (nmol/l)*	*n*	*Accuracy (%)**
20.9	5	102.5 (3.6)	20.0	5	93.8 (10.6)
41.7	6	101.6 (7.5)	40.1	6	105.5 (9.4)
130.3	6	96.7 (2.0)	139.1	6	100.3 (1.3)
260.7	6	100.4 (2.9)	278.3	6	100.1 (4.2)
521.4	6	97.2 (4.1)	500.9	6	106.3 (4.1)
834.2	6	96.9 (2.1)	834.8	6	98.1 (6.5)
Mean	35	99.1 (4.5)		35	100.9 (7.3)

* Mean of the individual recoveries; values in parentheses are coefficients of variation (%).

$y = 0.0019391\ x + 0.0067692$, $R = 0.9988$ for I and $y = 0.0018807\ x + 0.017155$, $R = 0.9987$ for II, where y denotes the peak-height ratio, x the concentration and R the coefficient of correlation.

Calibration graphs for I and II in urine showed a linear response in the range 2.2–90 μmol/l. Typical parameters were $y = 0.016898\ x + 0.009314$, $R = 0.9982$ for I and $y = 0.016707\ x + 0.009606$, $R = 0.9980$ for II.

Within-day accuracy and precision

Human plasma samples spiked with comparable amounts of I · HCl and II · HCl or human urine samples spiked with racemic baclofen at six different concentrations were analyzed six times on one day (Tables II and III). The overall accuracy for the determination of I and II in plasma and urine was characterized by mean recoveries ranging from 99.1 to 101.3% ($n = 35$ in plasma, $n = 36$ in urine). The precision of the method is shown by overall coefficients of variation between 4.5 and 7.3%.

TABLE III

WITHIN-DAY PRECISION AND ACCURACY OF THE DETERMINATION OF I AND II IN SPIKED HUMAN URINE

Concentration of each enantiomer added (μmol/l)	*n*	*Accuracy (%)**	
		I	*II*
2.29	6	100.3 (6.1)	99.7 (7.1)
6.08	6	106.7 (2.0)	105.1 (2.0)
16.98	6	106.5 (8.3)	106.1 (8.0)
44.70	6	97.1 (5.5)	98.1 (5.1)
53.64	6	100.9 (6.0)	102.1 (6.1)
87.60	6	96.6 (2.0)	96.6 (1.9)
Mean	36	101.3 (6.5)	101.3 (6.3)

* Mean of the individual recoveries; values in parentheses are coefficients of variation (%).

TABLE IV

DAY-TO-DAY PRECISION AND ACCURACY OF THE DETERMINATION OF I AND II IN SPIKED HUMAN PLASMA

Concentration of I added (nmol/l)	*n*	*Accuracy (%)**	*Concentration of II added (nmol/l)*	*n*	*Accuracy (%)**
20.9	5	92.9 (10.4)	20.0	5	97.9 (14.3)
41.7	5	95.4 (7.4)	40.1	5	98.7 (5.3)
130.3	5	100.3 (3.4)	139.1	5	104.0 (3.1)
260.7	5	94.7 (5.7)	278.3	5	97.7 (4.9)
521.4	5	96.3 (4.7)	500.9	5	99.6 (4.5)
834.2	5	95.5 (5.3)	834.8	5	98.5 (5.8)
Mean	30	95.8 (6.4)		30	99.4 (6.9)

* Mean of the individual recoveries; values in parentheses are coefficients of variation (%).

Day-to-day accuracy and precision

Human plasma samples spiked with comparable amounts of I · HCl and II · HCl or human urine samples spiked with racemic baclofen at six different concentrations were analyzed in duplicate on five consecutive days. All chromatograms were evaluated using the calibration graph obtained on day 1. The results are listed in Tables IV and V. The overall accuracy ($n = 30$) for I and II was 95.8 and 99.4%, respectively, in plasma and 104.2 and 104.3%, respectively, in urine. The overall coefficients of variation ranged from 6.4 to 7.6%. These results demonstrate the good accuracy and precision of the method in the concentration ranges tested. In addition, the day-to-day validation data are comparable to the within-day data.

Samples spiked with different amounts of I · HCl and II · HCl

Seven plasma and urine samples were spiked with the same amount of one enantiomer each at one of seven different concentration levels, and variable amounts of the other enantiomer were added. The concentration ranges were comparable to

TABLE V

DAY-TO-DAY PRECISION AND ACCURACY OF THE DETERMINATION OF I AND II IN SPIKED HUMAN URINE

Concentration of each enantiomer added (μmol/l)	*n*	*Accuracy (%)**	
		I	*II*
2.29	5	107.5 (8.7)	109.8 (9.2)
6.44	5	109.2 (4.1)	109.2 (4.6)
16.99	5	107.9 (3.1)	107.4 (3.0)
37.55	5	102.0 (3.5)	101.8 (4.0)
64.37	5	99.9 (6.6)	99.8 (6.3)
87.60	5	98.4 (9.9)	97.7 (10.5)
Mean	30	104.2 (7.1)	104.3 (7.6)

* Mean of the individual recoveries; values in parentheses are coefficients of variation (%).

those given in Tables II–V. A total of 49 plasma and 49 urine samples prepared in this way were analyzed to investigate the accuracy and precision of the method when measuring one enantiomer in the presence of markedly different amounts of the other one. In plasma, the mean accuracies for each concentration level of I and II were between 92.1 and 104.9% with coefficients of variation of 8.6% or less. In urine, the mean accuracies ranged from 91.0 to 109.2% with coefficients of variation of 9.5% or less. Thus, reliable results can also be obtained when the concentrations of the enantiomers in the same urine or plasma sample are quite different.

Limit of quantitation

The limit of quantitation (coefficient of variation $\leqslant 10\%$) was estimated from the results listed in Tables II–V. For both baclofen enantiomers, the limit of quantitation was 25 nmol/l in plasma and 2 μmol/l in urine. In urine, lower concentrations can be determined with comparable precision if the sample volume used is larger than 25 μl, *e.g.*, 50 or 100 μl. In plasma, lower concentrations can be determined with a reduced precision.

CONCLUSION

The capillary GC method described here permits the enantiospecific determination of *S*(+)- and *R*(−)-enantiomers of baclofen in plasma and urine with an high specificity and sensitivity. The method is suitable for investigation of the pharmacokinetics of the enantiomers and will be applied in biological media from animals and man dosed with racemic baclofen.

REFERENCES

1 H. R. Olpe, H. Demieville, V. Baltzer, W. L. Bencze, W. P. Koella, P. Wolf and H. L. Haas, *Eur. J. Pharmacol.*, 52 (1978) 133.
2 C. F. Terrence, M. Sax, G. H. Fromm, C.-H. Chang and C. S. Yoo, *Pharmacology*, 27 (1983) 85.
3 G. H. Fromm and C. F. Terrence, *Neurology*, 37 (1987) 1725.
4 R. P. Weatherby, R. D. Allan and G. A. R. Johnston, *J. Neurosci. Methods*, 10 (1984) 23.
5 K. G. Feitsma, J. Bosman, B. F. H. Drenth and R. A. de Zeeuw, *J. Chromatogr.*, 333 (1985) 59.
6 D. F. Smith and W. H. Pirkle, *Psychopharmacology*, 89 (1986) 392.
7 E. W. Wuis, E. W. J. Beneken Kolmer, L. E. C. van Beijsterveldt, R. C. M. Burgers, T. B. Vree and E. van Der Kleyn, *J. Chromatogr.*, 415 (1987) 419.
8 H. Spahn, D. Krauss and E. Mutschler, *Pharmacol. Res.*, 5 (1988) 107.
9 P. H. Degen and W. Riess, *J. Chromatogr.*, 117 (1976) 399.
10 G. Kochak and F. Honc, *J. Chromatogr.*, 310 (1984) 319.
11 B. Testa, *Xenobiotica*, 16 (1986) 265.
12 R. R. Freeman (Editor), *High Resolution Gas Chromatography*, Hewlett-Packard, Palo Alto, CA, 1981, p. 12.

Journal of Chromatography, 450 (1988) 233–240
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 707

SEPARATION OF THE STEREOISOMERS OF AN ALLENIC E-TYPE PROSTAGLANDIN

JOHN R. KERN*, DAVID M. LOKENSGARD and LAWRENCE V. MANES
Analytical and Environmental Research, Syntex Research, Syntex USA Inc., Palo Alto, CA 94304 (U.S.A.)
and
MASAAKI MATSUO and KOUJI NAKAMURA
Analytical Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd., Osaka (Japan)

SUMMARY

Enprostil (I) is a synthetic dehydro-prostaglandin E_2 containing a chiral allene moiety which is unresolved relative to the four remaining chiral centers. The relative configuration of the four remaining chiral centers is consistent with that of the naturally occurring E series of prostaglandins. Thus, enprostil exists as enantiomeric pairs of two allenic epimers. An analytical procedure has been developed that separates the four optical isomers present in enprostil. This procedure involves, first, the acid-catalyzed dehydration of enprostil to its corresponding prostaglandin A analogue followed by derivatization with β-naphthylsulfonyl-L-prolyl chloride. The resulting diastereomeric sulfonate esters are separated on an achiral silica gel high-performance liquid chromatographic column. This procedure has been applied to the analysis of both enprostil drug substance and enprostil formulated in a propylene carbonate solution from soft elastic gelatin capsules. An efficient procedure for the recovery of enprostil from the solution formulation is also described.

INTRODUCTION

Enprostil (methyl (±)-7-{(1*R**,2*R**,3*R**)-3-hydroxy-2-[(*E*)-(3*R**)-3-hydroxy-4-phenoxy-1-butenyl]-5-oxocyclopentyl}-4,5-heptadienoate) (Fig. 1) is a novel prostaglandin drug currently under development as a treatment for duodenal and gastric ulcers. It has been shown[1–8] to be a potent inhibitor of gastric acid secretion. Enprostil is formulated as a 125 μg/ml solution in propylene carbonate encased in a soft elastic gelatin capsule under the trademark Gardrin®. The product has been granted marketing approval in Mexico, New Zealand, France and Italy for use in treating gastric and duodenal ulcers.

Enprostil is a unique type of prostaglandin E_2 in that it contains a chiral allene moiety at the C-4–C-6 position which is unresolved with respect to the four remaining chiral centers. The synthesis of enprostil produces a racemic mixture of four stereoisomers (Fig. 1), consisting of a pair of diastereomers that are epimeric at the allene center, together with the corresponding enantiomers. The relative configuration of the four remaining chiral centers in enprostil is consistent with that of the naturally occurring E series of prostaglandins (PGEs).

0021-9673/88/$03.50

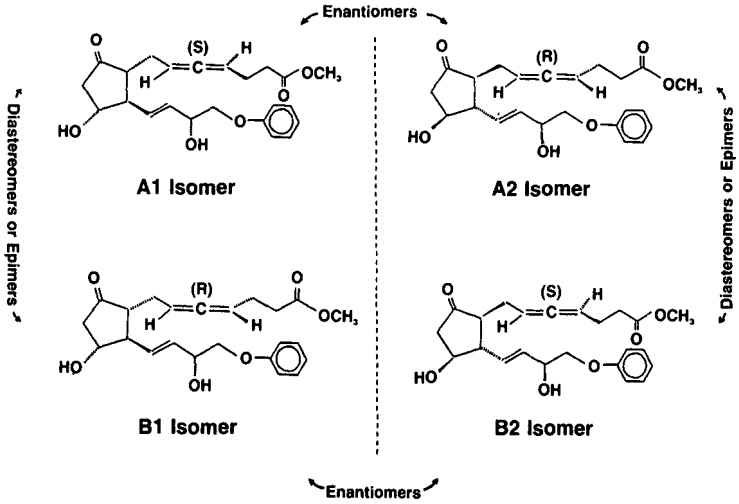


Fig. 1. Structures of the stereoisomers of enprostil (I).

Significant advances have been made in the area of chiral separations in recent years[9–13] in terms of development of novel chiral stationary phases, chiral derivatizing agents and chiral mobile phase additives. These analytical advances, when applied to the analysis of chiral drug substances as the raw material and in formulations, allow rapid and quantitative measurements of optical purity which may be required for both quality control and stability testing. When applied to the analysis of biological fluids, the ability to quantitate individual optical isomers enables one to define the enantioselective metabolism, if present, of a drug.

During the early development of enprostil it was observed that the diastereomers of enprostil separate readily with either reversed-phase or normal-phase high-performance liquid chromatography (HPLC)[14,15]. Diastereomer ratios were obtained for stressed stability samples of the drug substance and formulated drug, and deviations in the diastereomer ratios over a 2-year period were not observed. Using a non-chiral HPLC separation, Kenley *et al.*[16] addressed the relative reactivity of enprostil stereoisomers in soft elastic gelatin capsules by comparing degradation rates from mixtures composed of individual enprostil optical isomers. This work alluded to the absence of significant enantioselective degradation of enprostil in soft elastic gelatin capsules.

It is also of interest to follow the metabolic fate of each individual optical isomer of this racemic drug upon administration to patients. Therefore, an analytical procedure for the separation of the individual stereoisomers present in enprostil was developed and is described in this communication.

The use of chiral chromatography for the separation of the optical isomers of prostaglandins has met with little success, as evidenced by the paucity of publications appearing in the literature. Recently, Roston and Wijayaratne[17] reported the enantiomeric separation of the pharmacologically active form of misoprostil with a multi-column HPLC method. Misoprostil exists as a mixture of four isomers or, two enantiomeric pairs of diastereomers. This separation was achieved with the use of column switching, combining a reversed-phase HPLC diastereomer separation and

subsequent chiral separation of the enantiomer pairs of each diastereomer with an EnantioPak® HPLC column. Clarke *et al.*[18] reported a partial resolution of the diastereomeric derivatives of a benzidine-type prostaglandin using a Chiralcel® OT column. Armstrong *et al.*[19] reported that the structural isomers of prostaglandins A and B (PGAs and PGBs) could be separated on a β-cyclodextrin HPLC column. Snider[20] reported the separation of *cis* and *trans* isomers of several prostaglandins with β- and γ-cyclodextrin HPLC columns.

In this work, five commercially available chiral HPLC columns (Pirkle covalent D-naphthylalanine, Pirkle covalent 3,5-DNB-D-phenylglycine, Resolvosil® bovine serum albumin, EnantioPak α-1-acid-glycoprotein and Cyclobond® β-cyclodextrin) were investigated for their ability to separate the four individual optical isomers of enprostil without success. Therefore, the use of derivatization with chiral reagents was pursued as an alternative.

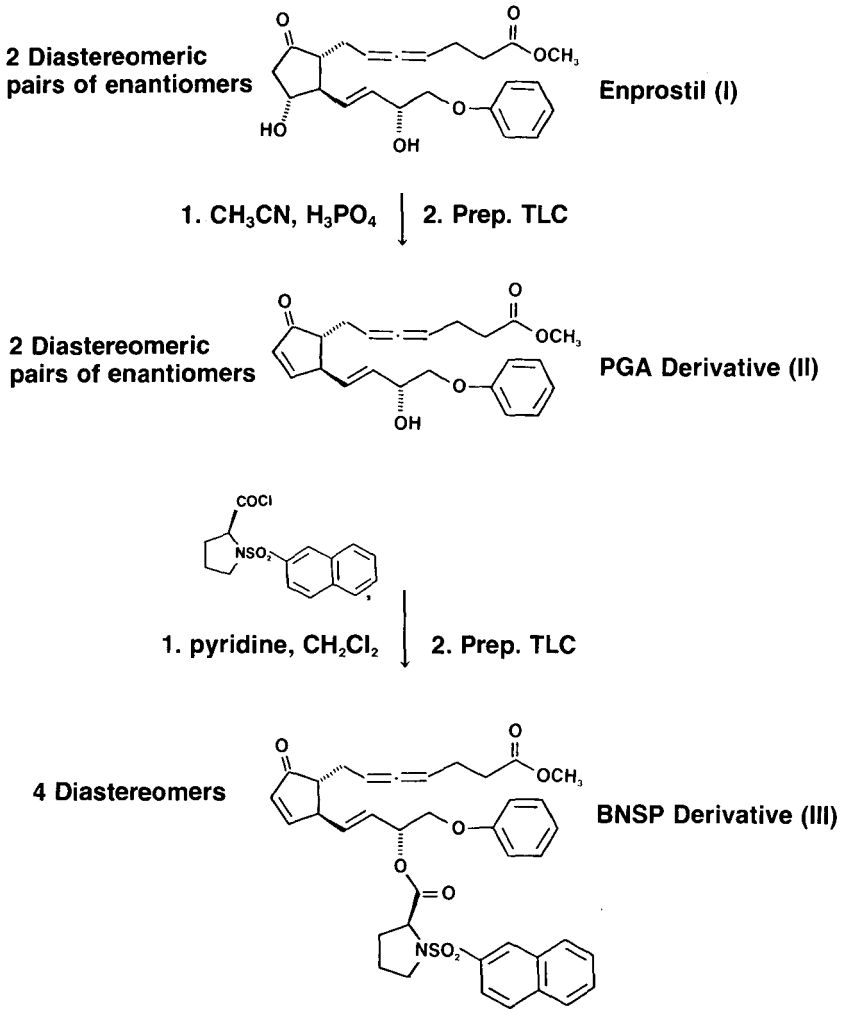


Fig. 2. Derivatization of enprostil for stereoisomer analysis by HPLC.

A method was developed which involves acid-catalyzed dehydration of enprostil to its corresponding PGA analogue (II) and subsequent derivatization of the 15-hydroxyl group with β-naphthylsulfonyl-L-prolyl chloride (BNSPC) (Fig. 2). The isolated β-naphthylsulfonyl-L-prolyl ester derivative (BNSP, III) is then chromatographed under normal-phase HPLC conditions leading to the facile separation of all four individual optical isomers of enprostil.

EXPERIMENTAL

Apparatus

HPLC was performed on a Spectra-Physics 8100XR (San Jose, CA, U.S.A.) chromatograph equipped with a Valco (Houston, TX, U.S.A.) 20-μl fixed-loop injector and a Kratos (Ramsey, NJ, U.S.A.) 737 UV detector set at 270 nm.

Materials

The prostaglandins were synthesized at Syntex Research, Palo Alto, CA, U.S.A.[1]. BNSPC was prepared according to the procedure of Shimizu *et al.*[21]. The optical purity of BNSPC was determined to be greater than 99.5% according to the procedure described in ref. 21. HPLC-grade solvents were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Phosphoric acid was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.) and pyridine from Aldrich (Milwaukee, WI, U.S.A.). The C_{18} Sep-Pak® was obtained from Waters (Milford, MA, U.S.A.). The TLC plates used were obtained from Analtech (Newark, NJ, U.S.A.).

All HPLC separations were carried out on a DuPont Zorbax® Sil (250 × 4.6 mm I.D.) (Wilmington, DE, U.S.A.) HPLC column. The mobile phase consisted of water-saturated dichloromethane. The water-saturated dichloromethane was degassed for only 2 min and was not stirred during the course of chromatography. The flow-rate was 2.0 ml/min, and the column temperature was kept constant at 40°C.

Isolation of enprostil from Gardrin captures

The contents of 50 Gardrin capsules were collected and transferred to a 500-ml round-bottomed flask. Fifty capsules will yield *ca.* 14 ml of propylene carbonate solution containing *ca.* 1.6 mg of enprostil. The propylene carbonate solution was then diluted with 15 volumes of HPLC-grade water (210 ml) and mixed well. A C_{18} Sep-Pak was prepared for use by passing 10 ml of methanol followed by 10 ml of HPLC-grade water through the cartridge. The diluted capsule contents were then passed through an apparatus as shown in Fig. 3 at a flow-rate of 2–4 ml/min. The cartridge was then rinsed with 20 ml of HPLC-grade water to remove residual propylene carbonate and the combined cartridge eluents were discarded. The cartridge was thoroughly dried by passing purified nitrogen through it at a flow-rate of *ca.* 50 ml/min for 30 min at room temperature. The enprostil is recovered by allowing 5.0 ml of acetonitrile to pass through the cartridge by gravity in the same flow direction used to process the diluted capsule contents. The collected eluent was transferred to a clean 75 × 12 mm test-tube and the recovered enprostil was evaporated to dryness under a stream of nitrogen.

Preparation of Enprostil PGA analogue (II)

Enprostil was converted into its PGA analogue by acid-catalyzed dehydration in

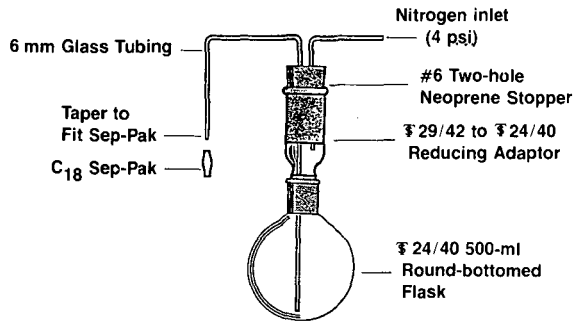


Fig. 3. Apparatus for isolation of enprostil from aqueous propylene carbonate solution.

acetonitrile, and the product was isolated by preparative thin-layer chromatography (TLC).

A sample of *ca.* 5 mg of enprostil drug substance or enprostil isolated from Gardrin capsules was placed in a 12 × 75 mm test-tube, and 1.0 ml of HPLC-grade acetonitrile containing 50 μl of 85% phosphoric acid was added. The reaction mixture was stoppered, mixed well, and allowed to stand at room temperature in the dark for 12–16 h. The volume of the reaction mixture was reduced to *ca.* 0.5 ml and the concentrated reaction mixture was applied to a TLC plate in a continuous narrow band 2 cm from the bottom of the plate using an appropriate streaking device. The TLC plate was developed (Analtech silica gel GF, 20 × 20 cm, 250 μm, water-saturated diethyl ether), and dried. The band at $R_F = 0.7$ was scraped from the plate and stirred with 5.0 ml of dichloromethane–methanol (10:1). The resulting slurry was filtered, rinsed and evaporated to dryness in a 10-ml round-bottomed flask.

*Preparation of PGA β-naphthylsulfonyl-*L*-prolyl ester (III)*

The 15-hydroxyl group of the enprostil PGA analogue was derivatized with BNSPC and the product was isolated by preparative TLC.

To the PGA analogue in a 10-ml round-bottomed flask was added 2.0 ml of dry dichloromethane, 20 mg of BNSPC and 20 μl of anhydrous pyridine. The flask was stoppered and the contents were stirred at room temperature for 30 min. The reaction mixture was then evaporated to dryness and the residue was dissolved in 0.5 ml of dichloromethane and applied to a TLC plate as described previously. The TLC plate was developed (Analtech silica gel GF, 20 × 20 cm, 250 μm, dichloromethane–ethyl acetate, 4:1) and the BNSP ester of the PGA analogue ($R_F = 0.6$) was isolated and recovered as previously described. The recovered BNSP ester was then diluted with dichloromethane to give a sample concentration of *ca.* 0.4 mg/ml. This solution was injected onto the column and afforded the chromatogram shown in Fig. 4. The isolated BNSP ester derivative was characterized by mass spectrometry, exhibiting M^+ at m/z 765 (electron impact mode).

RESULTS AND DISCUSSION

The need to derivatize enprostil in order to achieve separation of the individual optical isomers stemmed from the inability to separate the isomers by means of

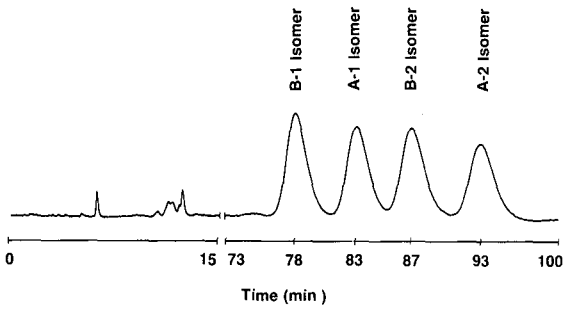


Fig. 4. Chromatographic separation of the BNSP ester of the PGA analogue of enprostil. Column, Zorbax Sil (250 × 4.6 mm I.D.); mobile phase, water-saturated dichloromethane; flow-rate, 2.0 ml/min; detector, 270 nm; temperature, 40°C.

"chiral" HPLC as discussed previously. A recent review[12] details the applications of chiral derivatizing agents. The two alcohol moieties of enprostil provide ready access for derivatization but, at the same time, create complications by the fact that both mono- and disubstituted products may be produced. This problem was circumvented by elimination of the C-11 hydroxyl group, which is β to the C-9 carbonyl function, by dehydration.

The conversion of PGE-type prostaglandins into their PGA forms has been reported to be very facile[22–25], catalyzed by acid, UV light or heat. Base-catalyzed dehydration of PGEs gives predominantly the PGB form while acid catalysis, in general, yields the PGA form with some PGB. The transformation of enprostil to its PGA analogue, without further rearrangement to the PGB analogue or to other degradation products, is best effected with the use of a mild acid catalyst. Several acid-catalyzed dehydration reactions using acetic acid, trifluoroacetic acid, phosphoric acid and sulfuric acid were performed at various acid concentrations and reaction times. The use of 5% phosphoric acid in acetonitrile and reaction times of 12–16 h, in the dark, at room temperature led to the desired PGA analogue with the least amount of by-product formation. The PGA analogue was isolated by preparative TLC in order to remove phosphoric acid or any traces of residual water which would interfere with the subsequent derivatization with BNSPC.

Enprostil, upon acid-catalyzed dehydration to its PGA analogue and subsequent derivatization with BNSPC, separates into four peaks (Fig. 4) under the chromatographic conditions described in the Experimental section. The identity of these components was determined by separately derivatizing each individual optical isomer of enprostil and chromatographing each as described. The order of elution of the four stereoisomers (see Fig. 4) was B1, A1, B2 and A2 at retention times of 78, 83, 87 and 93 min, respectively.

The precision of the chromatography was assessed by six replicate injections of the BNSP derivative of the PGA analogue of enprostil. The standard deviation for the area-normalized values for each peak was 0.3% indicating that the method is precise.

Conversion of enprostil into the PGB analogue (IV) offers another possible approach for chiral resolution via derivatization of the hydroxyl group at C-15. The resulting PGB analogue was derivatized with BNSPC and the PGB BNSP ester was isolated and chromatographed under the same conditions as described for the PGA

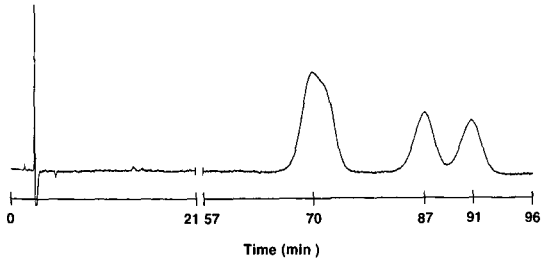
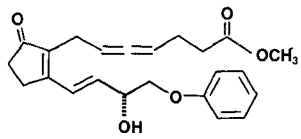


Fig. 5. Chromatographic separation of the BNSP ester of the PGB analogue of enprostil. HPLC conditions as in Fig. 4.

analysis. As can be seen from Fig. 5, the PGB BNSP ester does not provice baseline resolution of the four optical isomers of enprosil. Also, the presence of any PGB analogue from the acid-catalyzed dehydration of enprostil to PGA would interfere with the chromatographic analysis. The PGB analogue is successfully eliminated, however, by preparative TLC purification at both the dehydration and BNSPC derivatization steps.



IV

It is of interest to note that superior resolution was observed with a Zorbax Sil HPLC column when water-saturated organic mobile phase was used or a small (*ca.* 0.1%) amount of water was added to the mobile phase. Apparently, the addition of a small amount of water moderately deactivates the silica gel and the efficiency is enhanced. Thus, a solvent system consisting of water-saturated dichloromethane offers the best resolution. However, a gradual decrease in the resolution and retention times of the four optical isomers of enprostil is observed after 1–2 days of continual chromatography using this mobile phase. The column can be reactivated by flushing with anhydrous tetrahydrofuran for a period of 2 h.

ACKNOWLEDGEMENTS

The authors thank Dr. Kelvin Chan for obtaining the mass spectrum, Douglass L. Wren, E. Galeazzi and Gary F. Cooper for the synthetic preparation of the enprostil stereoisomers and Dr. Taiyin Yang for her assistance with the manuscript.

REFERENCES

1 H. Carpio, G. F. Cooper, J. A. Edwards, J. A. Mendez, J. M. Muchowski, A. P. Roszkiwski, A. R. Van Horn and D. Wren, *Prostaglandins*, 33 (1987) 169.
2 M. M. Cohen, D. R. McCreedy, L. Clark and H. Sevelius, *Grastroenterology*, 88 (1985) 382.
3 V. Mahachai, K. Walker, H. Sevelius and A. B. R. Thompson, *Gastroenterology*, 89 (1985) 555.
4 A. P. Roszkowski, G. L. Garay, S. Baker, M. Schuller and H. Carter, *J. Pharm. Exp. Ther.*, 239(2) (1986) 382.

5 A. P. Roszkowski, G. L. Garay, S. Baker, M. Schuler, J. A. Edwards, D. Wren and A. R. Van Horn, *Pharmacologist*, 23 (1981) 121.
6 G. R. Davis, J. H. Walsh, C. H. Santa Ana, S. G. Morawski and J. S. Fordtran, *Gastroenterology*, 86 (1984) 1058.
7 L. D. Waterbury, J. M. Mahoney and G. L. Garay, *Gastroenterology*, 86 (1984) 1294.
8 L. Carling, P. Unger, C. Almstrom, J. Cronstedt, P. Ekstrom, S. Hagg and B. Hansson, *Scan. J. Gastroenterology*, 22 (1987) 325.
9 S. Allemark, *J. Biochem. Biophys. Meth.*, 9 (1984) 1.
10 D. W. Armstrong, *J. Liq. Chromatogr.*, 7 (1984) 353.
11 W. H. Pirkle and J. Finn, *Asym. Syn.*, 1 (1983) 87.
12 R. W. Souter, *Chromatographic Separation of Stereoisomers*, CRC Press, Boca Raton, FL, 1985.
13 W. Lindner and C. Pettersson, in I. Wainer (Editor), *Liquid Chromatography in Pharmaceutical Development: An Introduction*, Aster, Springfield, OR, 1985, p. 63.
14 R. A. Kenley, K. M. Lee and T. R. Mahony, *J. Liq. Chromatogr.*, 9 (1986) 3577.
15 J. Kern, G. Otsuki and T. Yang, Syntex Internal communication, 1985.
16 R. A. Kenley, J. S. Fleitman and I. W. Partridge, *J. Pharm. Sci.*, 75 (1986) 842.
17 D. A. Roston and R. Wijayaratne, *Anal. Chem.*, 60 (1988) 950.
18 C. P. Clarke, B. G. Snider and P. B. Bowman, *J. Chromatogr.*, 408 (1987) 275.
19 D. W. Armstrong, W. DeMond, A. Alak, W. L. Hinze, T. E. Diehl and K. H. Bui, *Anal. Chem.*, 57 (1985) 234.
20 B. G. Snider, *J. Chromatogr.*, 351 (1986) 548.
21 R. Shimizu, T. Kakimoto, K. Ishii, Y. Fujimoto, H. Nishi and N. Tsumagari, *J. Chromatogr.*, 357 (1986) 119.
22 J. E. Pike, F. H. Lincoln and W. P. Schneider, *J. Org. Chem.*, 34 (1969) 3552.
23 D. C. Monkhouse, L. Van Campen and A. J. Aguair, *J. Pharm. Sci.*, 62(4) (1973) 576.
24 N. H. Anderson, *J. Lipid Res.*, 10 (1969) 320.
25 S. K. Perera and L. R. Fedor, *J. Am. Chem. Soc.*, 101 (1979) 7390.

Journal of Chromatography, 450 (1988) 241–252
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 746

APPLICABILITY OF FORCED-FLOW PLANAR CHROMATOGRAPHIC METHODS FOR THE SEPARATION OF ENANTIOMERS ON CHIRALPLATE®

SZABOLCS NYIREDY, KARIN DALLENBACH-TOELKE and OTTO STICHER*
Swiss Federal Institute of Technology (ETH) Zurich, Department of Pharmacy, CH-8092 Zürich (Switzerland)

SUMMARY

The separation of enantiomers using forced-flow planar chromatographic techniques such as overpressured layer chromatography and various rotation planar chromatographic methods such as ultra-microchamber rotation planar chromatography and microchamber rotation planar chromatography is reported for the first time. For circular and linear overpressured layer chromatography and ultra-microchamber rotation planar chromatography, the thin-layer chromatographic (TLC) mobile phase system from chromatography in an unsaturated chamber could be applied directly. In overpressured layer chromatography, a prerun had to be made before starting the separation in order to eliminate the disturbing zone. To achieve better migration of the α-front on the Chiralplate® in ultra-microchamber rotation planar chromatography an area of 10–20 cm^2 had to be wetted with a component of the mobile phase in which the substances did not migrate. The TLC mobile phase used in a saturated chamber exhibits similar properties when transferred to microchamber rotation planar chromatography. Employing forced-flow planar chromatographic techniques as many as 80 samples can be separated on a single plate. In all instances the separations were better and faster with the forced-flow methods than those achieved with TLC with the same mobile phase.

INTRODUCTION

Over the last decade, numerous gas chromatographic[1–3] and high-performance liquid chromatographic[4–7] methods have been developed. Thin-layer chromatographic (TLC) separations of enantiomers without derivatization were introduced by Günther[8] in 1983. Günther *et al.*[9–13] later described a chiral phase made by treating an octadecyl-modified silica TLC plate with a solution of copper acetate followed by a solution of (2*S*,4*R*,2′*RS*)-4-hydroxy-1-(2-hydroxydodecyl)proline. Using the commercially available Chiralplate®, Günther[14] and Günther and Schickedanz[15] recently separated several types of amino acids (proteinogenic, non-proteinogenic, N-alkyl and halogenic) and dipeptides. Brinkman and Kamminga[16] found that a 5-cm development distance in an unsaturated system was sufficient for satisfactory resolution in most cases.

0021-9673/88/$03.50

Forced-flow planar chromatographic methods, such as overpressured layer chromatography[17–19] and the various types of analytical rotation planar chromatographic[20,21] techniques, have the advantage of achieving better separation with the selected optimum mobile phase velocity. This advantage should also be apparent during the separation of enantiomers, especially when the separation is insufficient in densitometric determinations.

From the special preparation of the Chiralplate, two main questions arose for the transfer of the mobile phase from TLC to the various forced-flow techniques: can be mobile phase be transferred directly to forced-flow techniques or are modifications necessary?; and will better resolution and shorter separation times be achieved using the forced flow techniques?

In this paper, we report the applicability of overpressured layer chromatography, ultra-microchamber rotation planar chromatography, and microchamber rotation planar chromatography for the separation of amino acids in the linear and circular development modes. In all experiments, D,L-alanine was used to study the applicability of overpressured layer chromatography and ultra-microchamber rotation planar chromatography and for the transfer of the mobile phase to these methods from an unsaturated TLC system. The transfer of the mobile phase from the saturated TLC system to microchamber rotation planar chromatography is shown with D,L-α-methylserine as an example.

EXPERIMENTAL

Materials and instrumentation

Separations were carried out using Chiralplate obtained from Macherey, Nagel & Co. (Düren, F.R.G.). D,L-Alanine was obtained from Merck (Darmstadt, F.R.G.) and D,L-α-methylserine from Sigma (St. Louis, MO, U.S.A.). All reagents used were of analytical-reagent grade. The plates were either sprayed with 0.3% ninhydrin in *n*-butanol or dipped into 0.3% ninhydrin in acetone.

For all overpressured layer chromatography separations, a Chrompress 10 overpressured layer chromatograph (Labor MIM, Budapest, Hungary) was used at a 10-bar overpressure. To eliminate the height difference between the support and the glass plate, a silicone sheet was placed around the plate to protect the water cushion (Fig. 1a).

The rotation planar chromatographic methods were carried out in a Rotachrom® Model P rotation planar chromatograph (Petazon, Zug, Switzerland). The speed of rotation varied between 600 and 1500 rpm. The ultra-microchamber rotation planar chromatography is almost a closed system, the layer being placed in a special co-rotating aluminium support on crepe rubber (see Fig. 1b). For microchamber rotation planar chromatography separations the layer was put directly in the support on a silicone plate, and on top of the plate two silicone rings with different diameters were used to define the small vapour space (see Fig. 1c). For both separation techniques, a modified perfusor pump (Braun, Melsungen, F.R.G.) was used.

Densitograms were taken either with a Shimadzu 920 scanner (Shimadzu, Kyoto, Japan) or with a Camag TLC scanner II, coupled with an HP computer 9000/216 (Camag, Muttenz, Switzerland).

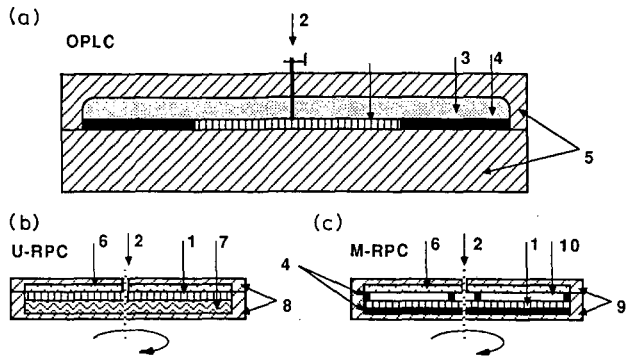


Fig. 1. Schematic diagram of the overpressured layer chromatography and rotation planar chromatography chambers. 1 = Layer; 2 = solvent inlet; 3 = cushion system; 4 = silicone; 5 = pressurized chamber; 6 = glass cover-plate; 7 = crepe rubber; 8 = ultra-microchamber; 9 = microchamber; 10 = vapour room. M-RPC = Microchamber rotation planar chromatography; OPLC = overpressured layer chromatography; U-RPC = ultra-microchamber rotation planar chromatography.

Preparation of the plates

For circular overpressured layer chromatography separations, preparation of the plates was not necessary (see Fig. 2a). For the one-directional linear overpressured layer chromatography separations, all four sides of the plates were impregnated with an Impress polymer suspension (Labor MIM). Two channels for the solvent inlet and outlet were scratched out at an 18-cm distance (Fig. 2b). For the two-directional linear overpressured layer chromatography separations, only two sides of the plate were impregnated; one channel was scratched out in the middle of the plate (see Fig. 2c). Two filter-papers were placed at the ends of the plate to ensure overrunning.

For the rotation planar chromatographic separations, the corners of the plates were removed with a special glass cutting instrument (Ciba Geigy, Central Analytik, Basle, Switzerland) in order to fit the plates into the rotating chamber. For circular ultra-microchamber rotation planar chromatography and microchamber rotation planar chromatography separations, no modifications were necessary (Fig. 2d); for linear separations, the mobile phase velocity was linearized by scraping lines in the layer (see Fig. 2e).

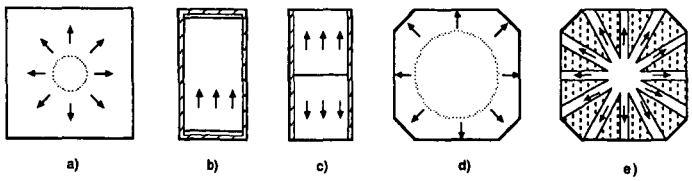


Fig. 2. Preparation of the plates and migration of the mobile phases in circular and linear overpressured layer chromatography and rotation planar chromatography (the arrows indicate the direction of mobile phase migration). (a) Circular overpressured layer chromatography; (b) linear, one-directional overpressured layer chromatography; (c) linear, two-directional overpressured layer chromatography; (d) circular ultra-microchamber rotation planar chromatography and microchamber rotation planar chromatography; (e) linear ultra-microchamber rotation planar chromatography and microchamber rotation planar chromatography.

For linear overpressured layer chromatography separations, samples were applied in the form of points with a 100- and 200-nl Pt–Ir capillary (Camag) at various distances from the inlet channel, so that sixteen samples could be applied on a 20 × 10 cm plate at an 18-cm separation distance, or 32 samples in the two-directional development mode. For linear microchamber rotation planar chromatography and ultra-microchamber rotation planar chromatography, sixteen samples were applied 3.5 cm from the centre of the plate. For various circular forced-flow planar chromatographic methods, 48 samples were applied on a 5-cm or 78 samples on a 10.5-cm diameter circle around the centre of the plate.

RESULTS AND DISCUSSION

Study of the behaviour of the Chiralplate in TLC

For the successful transfer of the mobile phase to the different forced-flow techniques, the specific properties of the Chiralplate had to be determined.

During our studies of the chromatographic behaviour of the Chiralplate in a normal TLC tank, we observed that the properties of these plates are similar to those of normal-phase plates; for example, the R_F values of the compounds may be increased with water or reduced with hexane. The behaviour of the amino acids in different solvents also had to be studied, because in overpressured layer chromatography a prerun is usually necessary[22]. For such a prerun, a solvent should be chosen in which the compounds to be separated do not migrate, and it should be a component of the mobile phase. The amino acids studied did not migrate with many neat solvents, *e.g.*, dichloromethane, chloroform, diethyl ether, acetone and acetonitrile; the last two are components of the solvent mixtures commonly used for their separation[14].

A certain type of gradient was observed with the mobile phases, *e.g.*, during the development a broad blue zone migrates below the front, which we call the "active zone". The compounds are, in most instances, separated on this zone. This "active zone" seems to be necessary for the successful separation of the enantiomers, because when the zone is eliminated by prewashing the plate with the same mobile phase and after drying and sample application, separation is almost impossible on the plate. A typical mobile phase mixture is acetone–methanol–water (10:2:2), where the blue zone is very pronounced. In Fig. 3, the densitogram of a developed plate with this mobile phase, the densitogram of an undeveloped plate as a control and the resulting curve are shown. This experiment illustrates that a certain gradient type of the "active zone" is formed during the development of the plate.

Circular overpressured layer chromatography separations

The applicability of overpressured layer chromatography was studied for the separation of D,L-alanine with acetone–methanol–water (10:2:2) as the mobile phase. Using this solvent, the two enantiomers applied as small points on a Chiralplate can be separated visually in an unsaturated TLC tank, but densitometrically the two compounds are virtually unseparated.

From our earlier work on the overpressured layer chromatography separation of polar compounds[23], the circular separation mode was studied first because preparation of the plate is not necessary and the separation can be started without an inlet pressure. Moreover, the effect of the disturbing zone[22] is not as evident as in the linear

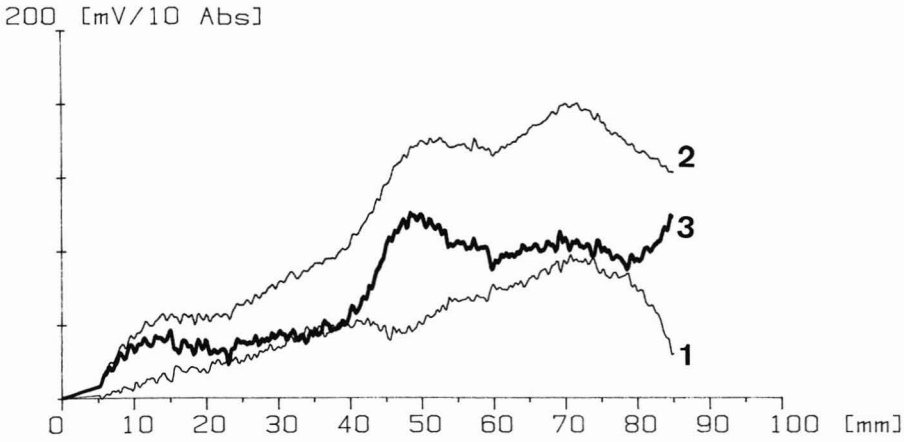


Fig. 3. The "active zone" of the Chiralplate. 1 = Densitogram of control plate without development after detection with ninhydrin reagent; 2 = densitogram of developed plate [acetone–methanol–water (10:2:2)] after detection; 3 = resultant curve.

development mode, as the volume of the stationary phase is not constant. Therefore, by selection of the mobile phase velocity the disturbing zone may be located as such that it does not distort the separation.

The first experiment was carried out so that 48 samples were applied in the form of points on a 5-cm diameter circle around the centre of the plate. The α-front migrated within 20 min over the 10-cm separation distance. The disturbing zone was observed in the lower R_F-range. The migration of the α-front was totally irregular, because with this mobile phase and velocity, the wettability of the plate was not satisfactory. To increase the wettability of the stationary phase with the α-front, acetone was selected as a coadjuvant. This solvent is a component of the mobile phase and had been shown in

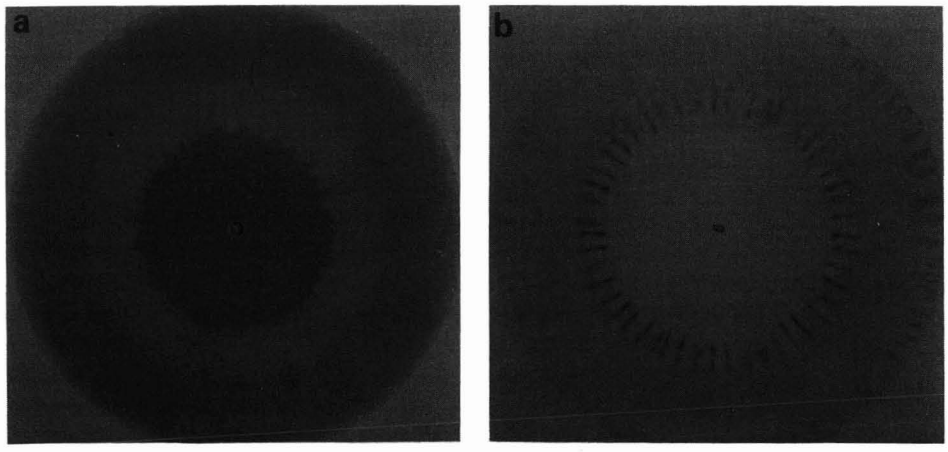


Fig. 4. The disturbing zone in circular overpressured layer chromatography (for conditions, see text). (a) The disturbing zone, photograph taken directly after development; (b) the effect of the disturbing zone after detection with reagent.

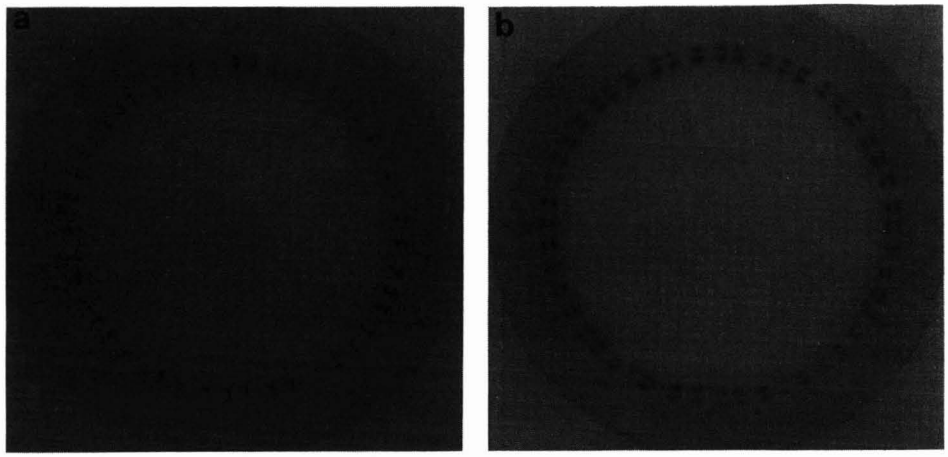


Fig. 5. Circular overpressured layer chromatography separation of D,L-alanine (for conditions, see text). (a) Diameter of sample application circle = 5 cm, separation time = 7 min; (b) diameter of sample application circle = 5 cm, separation time = 50 min.

TLC experiments to cause no migration of D- and L-alanine. In all of the following experiments, a small amount of acetone (about 15 cm^2 of the stationary phase was wetted with this solvent) was injected before initiating the separation with the mobile phase.

In the following experiments, higher mobile phase velocities were used. In Fig. 4, the effect of the disturbing zone is shown at two different mobile phase velocities. In Fig. 4a, development at 5 min is shown. The disturbing zone, which is the inner irregular zone (totally wetted zone[19]), was photographed directly after the plate had been taken out of the overpressured layer chromatography chamber. The effect of the disturbing zone on the separation at 3 min can be seen in Fig. 4b following application

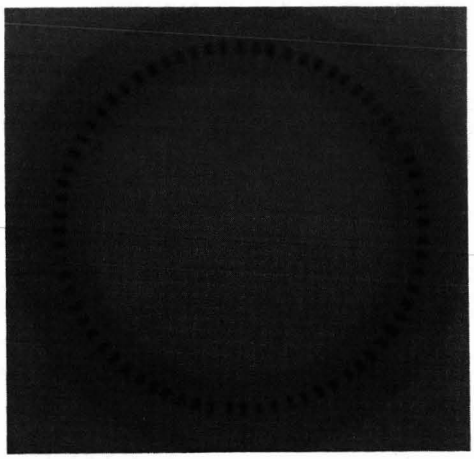


Fig. 6. Circular overpressured layer chromatography separation of D,L-alanine (for conditions, see text). Diameter of sample application circle = 10.5 cm, separation time = 50 min.

of ninhydrin reagent. The disturbance made it impossible to determine the extent of separation.

Subsequently, the mobile phase velocity was decreased successively so that the negative effect of the disturbing zone could be eliminated. Of the various mobile phase velocities tested, two are shown in Fig. 5. In Fig. 5a, the separation is shown at a 7-min development time and in Fig. 5b at 50 min. The separation was significantly better at a slower mobile phase velocity (separation time 50 min), although for qualitative analysis the fast separation (7 min) was sufficient. For a quantitative determination with a minimum resolution of 1.2, a separation time of 60 min was required when 48 samples were applied on a 5-cm circle.

Owing to the expense of the Chiralplate, as many samples as possible should be analysed on a single plate. We tested a larger application diameter of 10.5 cm with different mobile phase velocities. Considering the resolution and the separation times, the best results are shown in Fig. 6, where 78 samples were separated in 60 min.

Linear overpressured layer chromatography separations

Because in linear overpressured layer chromatography the disturbing zone is more prominent than in the circular development mode, the only general method for eliminating this effect is the prerun, which pushes the adsorbed air or gas completely out of the plate.

The linear overpressured layer chromatography separation could be carried out either as a one-directional development with a separation distance of 17 cm or as a two-directional development with a separation distance of 9 cm. With the one-directional development, the prerun was carried out such that the solvent, in which the compounds to be separated did not migrate, was pumped through the plate and out of the solvent outlet until no more bubbles were formed, then the actual separation with the mobile phase was started. Five experiments were made on 20 × 10 cm plates, and the mobile phase velocity was varied between 0.1 and 0.5 cm/min at an inlet pressure of 1 bar. The distance between the inlet channel and the sample application was also varied between 1 and 3 cm. The ΔR_F values between the two enantiomers were larger when the distance between the solvent inlet and the start was shorter, but the shape of the compound zones was more compact at the 3-cm distance. The lower the mobile phase velocity, the larger was the ΔR_F value, but in all instances tailing was observed.

From the results of circular and one-dimensional linear overpressured layer chromatography, it could be presumed that a shorter separation distance would be sufficient. Therefore, the two-directional overpressured layer chromatography was employed, which allowed the separation of twice as many samples. For these experiments, the solvent of the prerun was absorbed with small sheets of paper at the end of the plates. Again, the mobile phase velocity and the distance of the sample application were tested in the same range. The chromatogram obtained with a 1-cm distance from the inlet channel and a mobile phase velocity of 0.22 cm/min is shown in Fig. 7a. The separation was complete within 45 min. Fig. 7b shows a plate developed with the same velocity but a slight overrun was made. On the top of this plate, the samples were applied 5 cm from the inlet channel (similar to the circular overpressured layer chromatography experiment) and on the bottom located 1 cm from the inlet channel. The latter gave a better separation, but the spots were less compact.

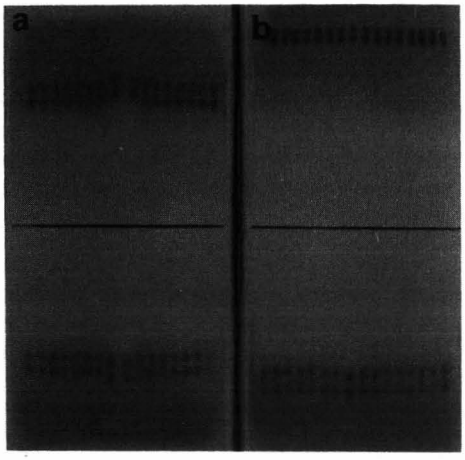


Fig. 7. Two-directional linear overpressured layer chromatography separation of D,L-alanine (for conditions, see text). (a) Sample application distance 1 cm; separation time 45 min; (b) sample application distance on top 5 cm, on bottom 1 cm, separation time 60 min.

Ultra-microchamber rotation planar chromatography separations

Ultra-microchamber rotation planar chromatography is a forced-flow planar chromatographic technique in which the eluent flow is accelerated by centrifugal force in an unsaturated chromatographic system, because the vapour space is almost eliminated. The circular ultra-microchamber rotation planar chromatography separation of D,L-alanine was started with a high mobile phase velocity at a high rotational speed (1500 rpm). The result is depicted in Fig. 8a directly after the plate was taken out of the ultra-microchamber and in Fig. 8b after detection with the ninhydrin reagent. The migration of the α-front was totally irregular and the wettability of the stationary

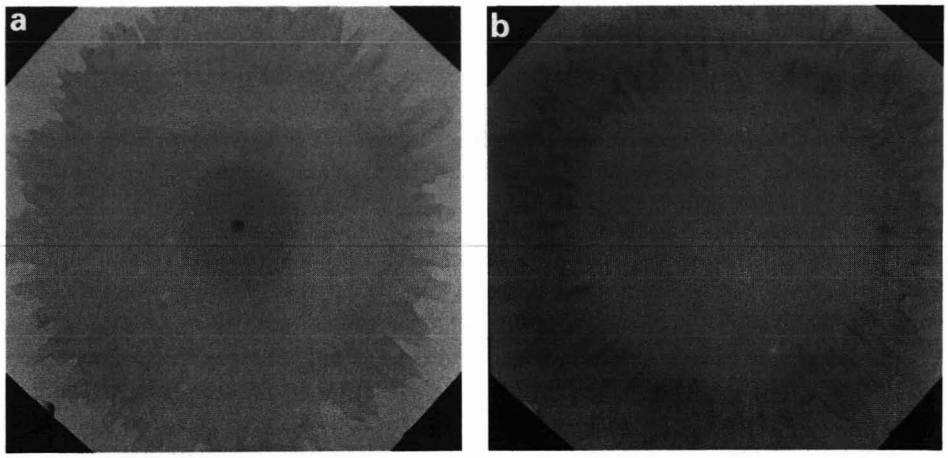


Fig. 8. Migration of the α-front on Chiralplate in circular ultra-microchamber rotation planar chromatography without pre-wetting (for conditions, see text). (a) Photograph of the plate taken directly after development; (b) after detection with the ninhydrin reagent.

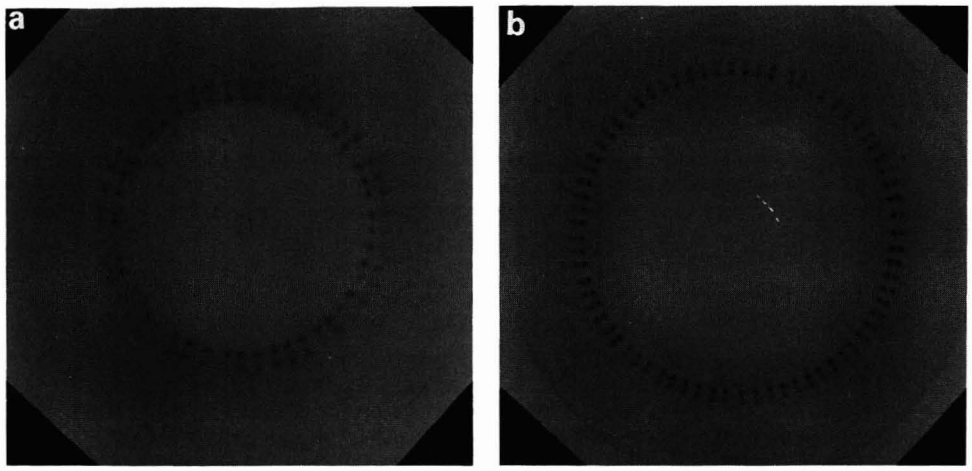


Fig. 9. Ultra-microchamber rotation planar chromatography separation of D,L-alanine (for conditions, see text). (a) Diameter of sample application circle = 5 cm, separation time 45 min; (b) diameter of sample application circle = 10.5 cm, separation time 43 min.

phase with this mobile phase was not sufficient, so a similar result was obtained as with the surface effect published in 1986[24].

To increase the wettability of the layer with the mobile phase, a solvent was first injected to wet approximately 15 cm^2 of the layer, then the mobile phase was applied at a rotational speed of 700 rpm. In Fig. 9, two chromatograms for the separation of D,L-alanine are depicted. Fig. 9a shows the separation where the samples were applied on the smaller circle of 5 cm diameter and Fig. 9b on the larger circle of 10.5 cm diameter. In both instances, the separations were initiated at a higher mobile phase velocity until the blue "active zone" had reached the applied substances. The mobile phase velocity was then decreased to 10 ml/h. The separation was finished in 45 and 43 min, respectively. In both instances, densitometrically a very good separation was obtained. In Fig. 9a with the smaller radius, although the resolution (R_s = 1.2) is better, the substance zones are more diffuse. Also, the compounds are located at the beginning of the "active zone", and this zone is not as pronounced as in the chromatogram obtained with the large radius. The reason for the latter is the longer development at a high initial mobile phase velocity to concentrate the "active zone".

Microchamber rotation planar chromatography

Many separations of the various types of amino acids are made in saturated systems. These enantiomers cannot be separated by either overpressured layer chromatography or ultra-microchamber rotation planar chromatography. At present microchamber rotation planar chromatography is the only saturated forced-flow planar chromatographic system. In microchamber rotation planar chromatography, the small, defined vapour space (see Fig. 1) is saturated within a few seconds, so generally the mobile phase may be transferred from the saturated TLC system to microchamber rotation planar chromatography[21] without any modification.

The same negative effects due to the wettability of the plate were observed as in ultra-microchamber rotation planar chromatography (see Fig. 8). In microchamber

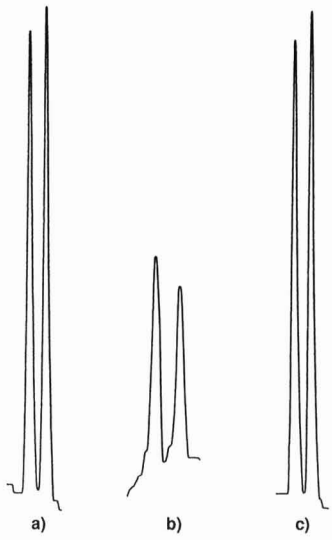


Fig. 10. Densitograms of the separation of D,L-α-methylserine by different methods (for conditions, see text). (a) Circular microchamber rotation planar chromatography separation; separation distance 7.5 cm, separation time 42 min; (b) linear TLC separation; separation distance 13 cm, separation time 95 min; (c) linear microchamber rotation planar chromatography separation; separation distance 6.5 cm, separation time 40 min.

rotation planar chromatography, no pre-wetting of the plate is possible, because this would change the composition of the gas phase in the vapour space. To get the mobile phase into the layer more regularly, a second silicone ring (with a smaller diameter than the circle for the sample application) was placed around the centre. This allowed for better wetting of the stationary phase. Enlargement of the radius of the ring also increased the flow-rate.

A circular microchamber rotation planar chromatography separation of D,L-α-methylserine gave good resolution (Fig. 10a). Compared with the TLC separation (Fig. 10b), the microchamber rotation planar chromatography technique resulted in better peak shapes with shorter development times. With linear microchamber rotation planar chromatography (channels had to be scraped out of the plate to linearize the mobile phase velocity), the same resolution could be achieved (Fig. 10c). This separation technique is only useful when the sample application in the form of bands is of importance. However, in this case fewer samples can be applied.

CONCLUSION

For the application of TLC mobile phases to the various types of forced-flow planar chromatographic techniques, the following observations can be made:

(i) The TLC mobile phase may be transferred from the unsaturated TLC chamber to overpressured layer chromatography and ultra-microchamber rotation planar chromatography without any modification.

(ii) For circular overpressured layer chromatography, pre-wetting of the plate is necessary, so that approximately 15 cm^2 are covered with solvent. The solvent used for

this purpose has to be chosen so that the compounds to be separated do not migrate, and the solvent should be a component of the mobile phase.

(iii) For linear overpressured layer chromatography, a prerun has to be made in order to eliminate the effect of the disturbing zone. The solvent used for this prerun has to be chosen so that the compounds to be separated do not migrate, and it should be a component of the mobile phase.

(iv) Owing the special properties of the Chiralplate, pre-wetting of the plates is necessary for circular and linear ultra-microchamber rotation planar chromatography as for circular overpressured layer chromatography.

(v) For circular and linear microchamber rotation planar chromatography, a special ring was introduced around the centre between the quartz glass and the layer, which allowed for more regular migration of the α-front.

(vi) Comparing circular overpressured layer chromatography with ultra-microchamber rotation planar chromatography, ultra-microchamber rotation planar chromatography gave more compact zones.

(vii) Comparing linear overpressured layer chromatography with ultra-microchamber rotation planar chromatography and microchamber rotation planar chromatography, overpressured layer chromatography has the advantage that more samples can be applied on a single plate with the same resolution as in ultra-microchamber rotation planar chromatography and microchamber rotation planar chromatography.

(viii) For qualitative analysis with all three methods, very rapid separations could be obtained, generally within 10 min.

(ix) For quantitative determinations with all three methods, longer separation times (generally between 45 and 60 min) are needed to achieve an even better resolution for the densitometric evaluation compared with development in a TLC chamber.

(x) The quality of the separation depends not only on the separation distance, but also on the location of the "active zone".

(xi) Depending on the separation problem and method, between 50 and 80 samples may be applied on a single 20 $\times$ 20 cm Chiralplate.

The experiments show that after the basic properties of the Chiralplate have been studied and adapted to the various forced-flow planar chromatographic techniques, these methods may be applied successfully for the routine separation of enantiomers. The use of these forced-flow techniques is especially indicated when the separation in a TLC tank is insufficient and more samples per plate need to be separated.

ACKNOWLEDGEMENTS

The authors thank K. Günther and R. Rausch for valuable discussions. They are grateful for a gift of Chiralplate from Macherey, Nagel & Co., Düren, FRG. Financial support from the Swiss KWF Commission is gratefully acknowledged.

REFERENCES

1 H. Frank, G. J. Nicholson and E. Bayer, *Angew. Chem.*, 90 (1978) 396.
2 R. H. Liu and W. W. Ku, *J. Chromatogr.*, 271 (1983) 309.
3 W. A. König, E. Steinbach and K. Ernst, *J. Chromatogr.*, 301 (1984) 129.
4 R. Audebert, *J. Liq. Chromatogr.*, 2 (1979) 1063.

5 W. H. Pirkle, D. W. House and J. M. Finn, *J. Chromatogr.*, 192 (1980) 143.
6 G. Blaschke, *Angew. Chem.*, 92 (1980) 14.
7 V. A. Davankov, A. S. Bochkov and Yu. P. Belov, *J. Chromatogr.*, 218 (1981) 547.
8 K. Günther, J. Martens and M. Schickedanz, *DE Pat.*, P 3328348.6 (1983).
9 K. Günther, J. Martens and M. Schickedanz, *Angew. Chem., Int. Ed. Engl.*, 23 (1984) 506.
10 K. Günther, J. Martens and M. Schickedanz, *Angew. Chem., Int. Ed. Engl.*, 25 (1986) 278.
11 K. Günther, J. Martens and M. Schickedanz, *Naturwissenschaften*, 72 (1985) 149.
12 K. Günther, J. Martens and M. Schickedanz, *Arch. Pharm. (Weinheim)*, 396 (1986) 461 and 572.
13 K. Günther, J. Martens and M. Schickedanz, *Fresenius Z. Anal. Chem.*, 322 (1985) 513.
14 K. Günther, *GIT Suppl. Chromatogr.*, 3 (1986) 6.
15 K. Günther and M. Schickedanz, *GIT Suppl. Chromatogr.*, 3 (1987) 27.
16 U. A. Th. Brinkman and D. Kamminga, *J. Chromatogr.*, 330 (1985) 375.
17 E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.*, 174 (1980) 75.
18 E. Mincsovics, E. Tyihák and H. Kalász, *J. Chromatogr.*, 191 (1980) 293.
19 E. Tyihák and E. Mincsovics, *J. Planar Chromatogr.*, 1 (1988) 6.
20 Sz. Nyiredy, S. Y. Mészáros, K. Dallenbach-Tölke, K. Nyiredy-Mikita and O. Sticher, *J. Planar Chromatogr.*, 1 (1988) 54.
21 Sz. Nyiredy, K. Dallenbach-Toelke and O. Sticher, in I. D. Wilson (Editor), *Recent Advances in Thin-Layer Chromatography*, Plenum Press, London, 1988, p. 45.
22 Sz. Nyiredy, S. Y. Mészáros, K. Dallenbach-Toelke, K. Nyiredy-Mikita and O. Sticher, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 352.
23 K. Dallenbach-Toelke, Sz. Nyiredy, B. Meier and O. Sticher, *J. Chromatogr.*, 365 (1986) 63.
24 Sz. Nyiredy, S. Y. Mészáros, K. Nyiredy-Mikita, K. Dallenbach-Toelke and O. Sticher, *GIT Suppl. Chromatogr.*, 3 (1986) 51.

1st International Symposium on Separation of Chiral Molecules, Paris, May 31–June 2, 1988

END OF SYMPOSIUM PAPERS

Journal of Chromatography, 450 (1988) 255–269
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 752

CHIRAL RESOLUTION OF A SERIES OF 3-THIENYLCYCLOHEXYLGLYCOLIC ACIDS BY LIQUID OR SUBCRITICAL FLUID CHROMATOGRAPHY

A MECHANISTIC STUDY

P. MACAUDIÈRE*, M. CAUDE and R. ROSSET
Laboratoire de Chimie Analytique, École Supérieure de Physique et Chimie Industrielles de Paris, 10 Rue Vauquelin, 75231 Paris Cedex 05 (France)
and
A. TAMBUTÉ
Direction des Recherches et Études Techniques, Centre d'Études du Bouchet, B.P. 3, Le Bouchet, 91710 Vert-le-Petit (France)
(First received April 11th, 1988; revised manuscript received June 27th, 1988)

SUMMARY

The chiral resolution of 3-thienylcyclohexylglycolic acid on a β-cyclodextrin-bonded chiral stationary phase (CSP) is described. A complete study was made of the influence of the organic solvent (nature and content) and phosphate buffer (pH and concentration) on retention, selectivity, efficiency and resolution and the effects of small structural changes to the solute on selectivity. The results allow a better understanding of retention and chiral recognition mechanisms. A model consistent with the fact that high retentions are observed in methanol in conjunction with the separation of enantiomers is proposed; it involves the degree of ionization of the solute in the mobile phase and in the liquid stationary phase. The separation of the methyl ester of 3-thienylcyclohexylglycolic acid cannot be achieved on Cyclobond-I CSP but is successful on ChiralCel OB CSP under both liquid and subcritical fluid chromatographic conditions. A comparison of these two analytical techniques is presented. 3-Thienylcyclohexylglycolic acid and its methyl ester are important reactants for the synthesis of new potent anticholinergic drugs.

INTRODUCTION

It is well known that different enantiomers of drugs often give different pharmaceutical effects and that the use of pure isomers can greatly improve the therapeutic effect of such drugs. This explains why there is increasing interest in chiral separations, especially in liquid chromatography, which is the most sensitive technique for the determination of the optical purity of enantiomeric compounds. It is now possible to analyse a wide range of drugs as a large number of chiral stationary phases (CSPs) are commercially available or easily accessible by synthesis.

0021-9673/88/$03.50

TCGA MTCGA

Fig. 1. Structures of test solutes, (±)-3-thienylcyclohexylglycolic acid (TCGA) and its (±)-methyl ester (MTCGA).

Recently, we were involved in the development of new potent anticholinergic drugs containing a chiral centre and derived from 3-thienylcyclohexylglycolic acid (TCGA). Tambuté and Collet[1] reported the chemical separation of TCGA isomers by mean of diastereomeric salts starting from ephedrine. It was important for us to possess a reliable means of controlling the enantiomeric purity of TCGA and of its methyl ester (MTCGA) (Fig. 1) before using them for the synthesis of anticholinergic drugs.

In this paper, the separation of TCGA enantiomers on a β-cyclodextrin-bonded CSP (Cyclobond-I) is described. A study of the influence of various parameters such as pH, the nature and content of the organic component in mobile phase and the phosphate buffer concentration was conducted in order (a) to improve the chiral separation and (b) to understand retention and chiral recognition mechanisms better. For this last purpose many structurally related acids were also studied, in addition to another chiral stationary phase (the acetylated form of the β-cyclodextrin-bonded CSP).

Unfortunately, it was impossible to resolve the methyl ester of TCGA on these stationary phases. However, a good separation was observed on ChiralCel OB CSP in both the liquid and subcritical fluid chromatographic (LC and SubFC) modes. This work represents a continuation of our systematic investigation of the use of chiral stationary phases in SubFC[2–5].

EXPERIMENTAL

Apparatus

For LC, a modular liquid chromatograph (Gilson, Villiers-le-Bel, France) was used. The standard operating conditions were flow-rate 1 ml/min and temperature 25°C. For SubFC, the apparatus has been described previously[6]. Carbon dioxide, kept in a container with an eductor tube, was passed into a Model 303 pump (Gilson) through an ethanol cooling bath.

The pump head (10SC) was cooled in order to improve the pump efficiency. The inlet adaptor and cooling jacket were laboratory-made. Polar modifiers were added by use of a second Gilson pump and mixed with carbon dioxide through a Gilson mixer (Model 802). A constant-temperature water-bath provided temperature control for the column.

A polychrom 9060 diode-array detector (Varian, Palo Alto, CA, U.S.A.) set at 215 nm was used without modification. The pressure was monitored by a manual back-pressure regulator (Tescom, Model 26-1700; GEC Composants, Asnières, France) connected in-line after the detector and maintained at 45°C by a water-bath.

All results were recorded with a Shimadzu CR 3A integrator (Touzart et Matignon, Vitry-sur-Seine, France). The standard operating conditions were average pressure 120 bar, temperature 25°C and average carbon dioxide flow-rate 2 ml/min at 0°C.

Chiral stationary phases

Two types of columns were used: (1) cyclodextrin-bonded columns (Cyclobond CSP) (Advanced Separation Technologies, Prolabo, Paris, France), particle size 5 μm (25 cm × 4.6 mm I.D.); we used more specifically the β-cyclodextrin-bonded phase (Cyclobond-I) and its acetylated form; and (2) cellulose tribenzoate coated on macroporous silica gel (ChiralCel OB) (Daicel Chemical Industries, Sochibo, Velizy-Villacoublay, France), particle size 10 μm (25 cm × 4.6 mm I.D.).

Mobile phase, reagents and solutes

Carbon dioxide was of N45-grade (99.995% pure) (Air Liquide, Alphagaz, Paris, France). Methanol, 2-propanol and acetronitrile of chromatographic grade were obtained from Merck (Darmstadt, F.R.G.). Phosphoric acid was purchased from Fluka (Interchim, Paris, France) and sodium hydrogenphosphate and dihydrogenphosphate from Merck. Water (deionized) was doubly distilled with a Buchi Fontavapor 285 apparatus before use. pH values were measured by means of a Minisis 8000 (Tacussel, Prolabo, Paris, France). A type TB/H glass electrode and a type CB reference electrode were employed. pK_a values were determined by titration of methanol–water containing 10^{-2} *M* TCGA solutions with standardized sodium hydroxide solution (1 *M*).

Glycolic acids were kindly provided by Professor M. Robba (Directeur de l'UER des Sciences Pharmaceutiques, Laboratoire de Chimie Thérapeutique, Université de Caen, France). Other test solutes (such as glycolic esters) were synthesized in our laboratory according to previously described procedures[1]; we then had available racemic and pure enantiomeric forms of TCGA.

RESULTS AND DISCUSSION

Separation of TCGA on Cyclobond-I CSP

Among various commercially available CPSs, Cyclobond-I CSP was selected because of its ability to resolve some structurally related compounds such as cyclohexylphenylglycolic acid (CHPGA) or cyclohexylphenylacetic acid[7,8]. Preliminary results, obtained with the mobile phase conditions found to be optimal by Feitsma *et al.*[8] for the resolution of CHPGA [aqueous sodium phosphate buffer (pH 4.3, 0.1 *M*)–acetonitrile (40:60, v/v)], were unsatisfactory for TCGA enantiomers. The mobile phase composition was then investigated in order to optimize the separation. The influence of the organic modifier (nature and content) and phosphate buffer (pH and concentration) on retention, selectivity and resolution was successively studied.

Nature of the organic modifier

Methanol was used instead of acetonitrile while keeping constant all other analytical parameters. A comparison of the chromatograms is presented in Fig. 2. As expected, the retention of TCGA is increased because methanol shows less interaction with the β-cyclodextrin (β-CD) cavity than does acetonitrile. As chiral recognition on

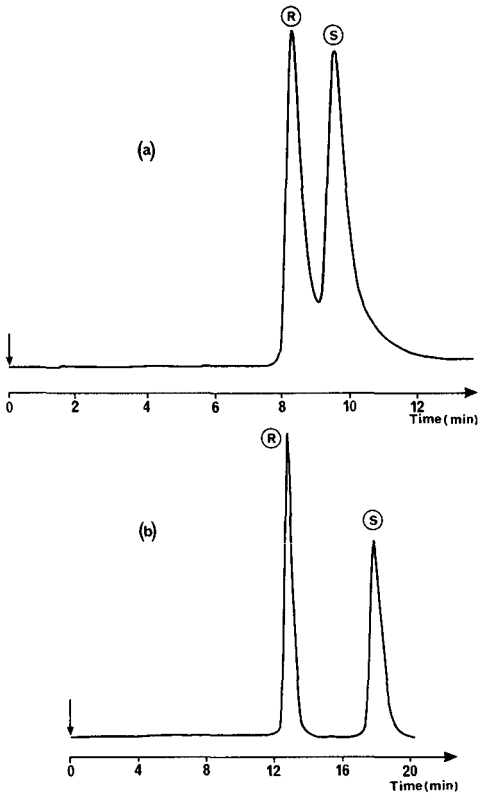


Fig. 2. Comparison of chromatograms obtained with (a) acetonitrile and (b) methanol as organic solvent in mobile phase. Operating conditions: CSP, Cyclobond-I; solute, TCGA; mobile phase, (a) acetonitrile or (b) methanol–phosphate buffer (pH 4.3, 0.1 *M*) (70:30, v/v); flow-rate, 1 ml/min; temperature, 25°C; detection, 205 nm.

cyclodextrin-bonded phases depends on the formation of inclusion complexes between a hydrophobic moiety of the solute and the hydrophobic cavity of the CD molecule[9,10], the selectivity, α, increases from 1.21 for acetonitrile to 1.54 for methanol. Further evidence that inclusion is favoured when methanol is used instead of acetonitrile is that the retention of the last-eluted enantiomer, k'_2, which gives the strongest interactions with the chiral cavity, is much more affected by the change in solvent nature than is the other antipode: $k_1'^{CH_3OH}/k_1'^{CH_3CN} = 1.04$ and $k_2'^{CH_3OH}/k_2'^{CH_3CN} = 1.30$.

A large baseline resolution is obtained for methanol within 18 min. However, the efficiency remains poor ($h \approx 17$), probably owing to slow dissociation of the diastereomeric complexes formed during the chiral recognition (strong interactions between the acidic group of TCGA and secondary hydroxyl groups of the cyclodextrin).

Methanol content in mobile phase

The methanol content in the mixture was increased in order to decrease the

retention of TCGA enantiomers. As expected, the capacity factors began to decrease (Fig. 3a) as the hydrophobic character of mobile phase was increased at high alcohol content. However, in contrast to what is commonly observed on cyclodextrin-bonded CSPs, the retention shows a minimum. At the same time, the selectivity, α, remains roughly constant (Fig. 3b). This phenomenon is surprising because, if the number of solute–CD cavity interactions is lowered, then the retention and the selectivity should decrease uniformly. A tentative explanation is proposed under Discussion.

Maximum efficiency is also observed (Fig. 3c) at a methanol content of 95% in the mobile phase ($h \approx 10$, which can be considered to be satisfactory). The large decrease in efficiency at higher alcohol contents may be attributed to the increase in strong interactions between secondary hydroxyl groups and the acidic moiety of TCGA (see additional comments under Discussion).

Finally, optimum resolution per unit time (Fig. 3d) is observed with phosphate buffer (pH 4.3, 0.1 *M*)–methanol (10:90, v/v) as the mobile phase. The resolution is then 4.9 for an analysis time of 11 min.

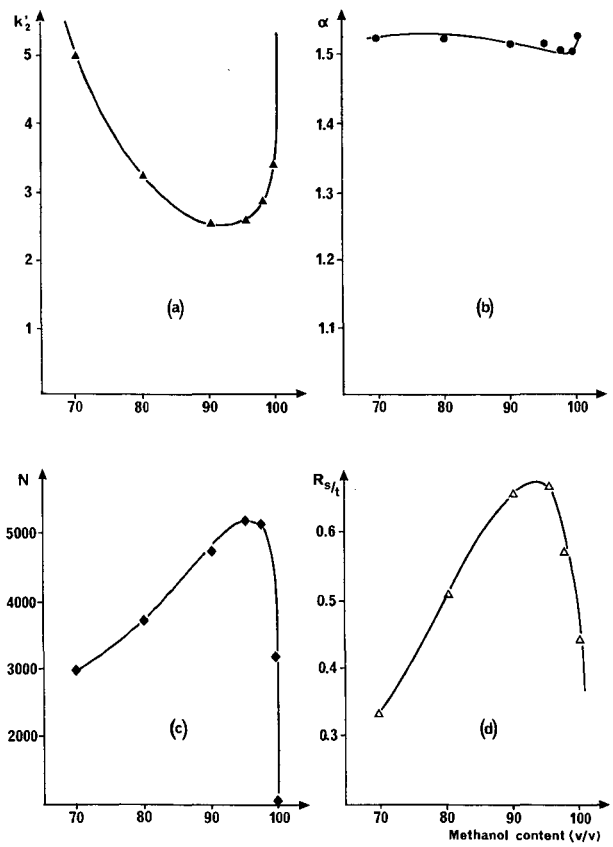


Fig. 3. Influence of the methanol content in the mobile phase on (a) retention of the last eluted enantiomer, k'_2, (b) selectivity, α, (c) efficiency, N, and (d) resolution per unit time, R_s/t, in the separation of TCGA enantiomers on Cyclobond-I CSP. Other operating conditions as in Fig. 2b.

Phosphate buffer pH and concentration

As the solute is a weak acid, the pH of the mobile phase is one of the major parameters for governing retention, selectivity and efficiency. The pH values given in the figures are those of the aqueous part of the mobile phase and were measured before mixing with methanol.

The influence of pH is clearly demonstrated in Fig. 4. The retention of TCGA decreases with increasing mobile phase pH. Three chromatograms are presented in Fig. 5, obtained at (a) pH 4.3, (b) pH 5 and (c) pH 5.8 with 70% methanol in the mobile

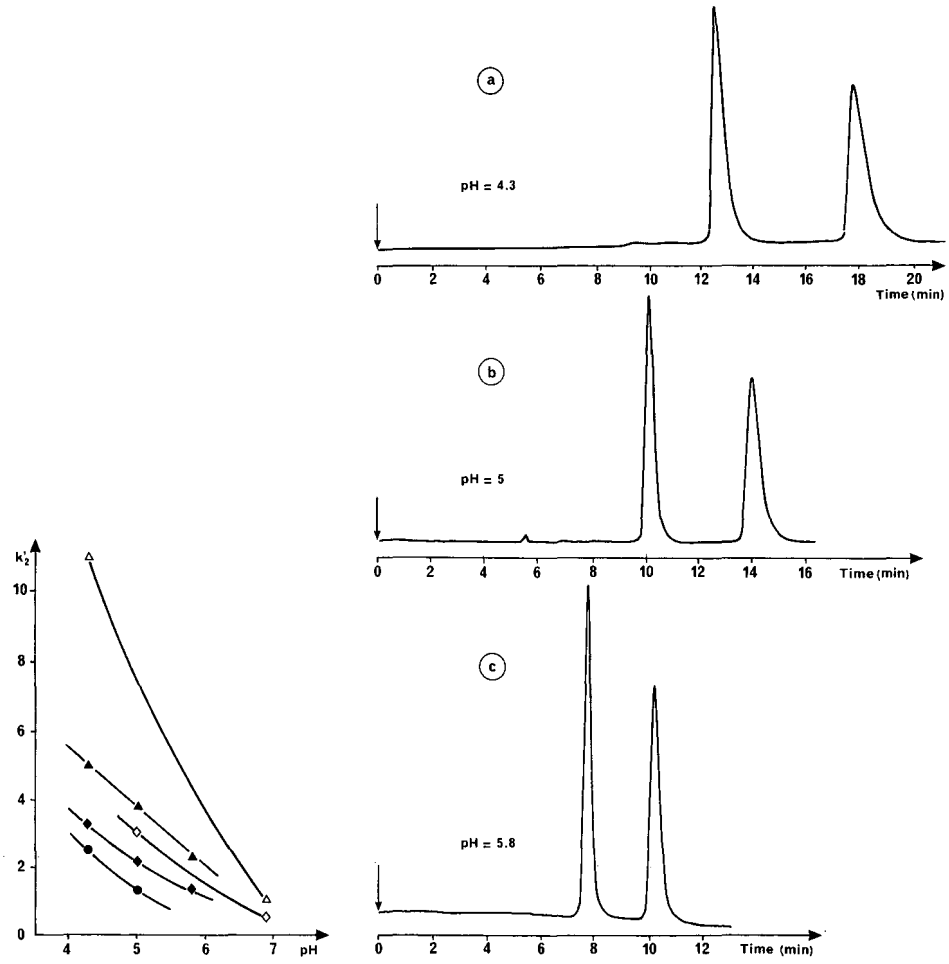


Fig. 4. Influence of the initial pH of phosphate buffer on the capacity factor of the last-eluted enantiomer of TCGA, k'_2. Operating conditions as in Fig. 2b, except for the methanol content in the mobile phase and the phosphate buffer concentration: (△) 70%, 0.025 *M*; (▲) 70%, 0.1 *M*; (◇) 80%, 0.025 *M*; (◆) 80%, 0.1 *M*; (●) 90%, 0.1 *M*.

Fig. 5. Comparison of chromatograms obtained at (a) pH 4.3, (b) pH 5 and (c) pH 5.8. Other operating conditions as in Fig. 2b.

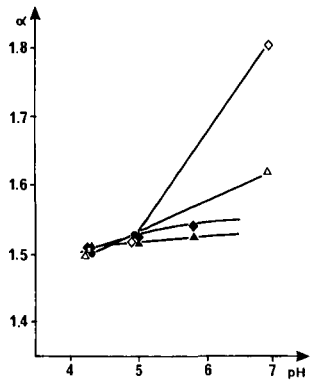


Fig. 6. Influence of the initial pH of phosphate buffer on the selectivity, α, of the separation of the enantiomers of TCGA. Operating conditions and symbols as in Fig. 4.

phase. Again, a decrease in retention is not achieved to the detriment of selectivity (Fig. 6), which increases slightly with increasing pH; in fact, at pH 4.3 and for a buffer concentration of 0.1 *M*, $\alpha = 1.52$ and $k'_2 = 5.01$, whereas at pH 5.8, $\alpha = 1.55$ and $k'_2 = 2.36$. The evolution of selectivity is more pronounced at a concentration of 0.025 *M* as α increases from 1.54 at pH 5 to 1.82 at pH 6.9 (80% methanol in the mobile phase). However, there is some uncertainty in this last selectivity value because the retention time of the first-eluted enantiomer is close to that of an unretained solute; imprecision in the determination of the column dead volume can induce substantial artificial effects on the selectivity calculation.

The effect of the phosphate buffer on resolution was studied. Two experiments were conducted with two mobile phases consisting of (1) methanol–water and (2) methanol–phosphate buffer mixtures; the concentration of buffer was kept constant in the mobile phase (0.002 *M*), whatever the methanol content, and the initial pH was maintained at 5 before the addition of methanol. Results are presented in Fig. 7. Minimum retention is observed with mobile phases (1) and (2) with *ca.* 98% methanol, but the presence of buffer salts decreases the retention consideration (Fig. 7a). Moreover, the efficiency is dramatically increased with mobile phase (2), as shown in Fig. 7b. Finally, the selectivity of the separation is favoured in the presence of buffer salts (Fig. 7c): whereas a maximum is observed with methanol–phosphate buffer mixtures ($\alpha = 1.61$), a minimum occurs with methanol–water mixtures ($\alpha = 1.30$).

The influence of the buffer concentration in the aqueous part of the mobile phase was also studied (Fig. 8). As expected, the retention is lower at high buffer concentrations. At the same time, a small decrease in selectivity is observed.

DISCUSSION

We have demonstrated that retention is governed by (a) methanol content, (b) the pH of the mobile phase and (c) buffer concentration. Uncommon phenomena are observed, such as a minimum in the curve of retention *versus* methanol content and the resolution of TCGA enantiomers in pure methanol.

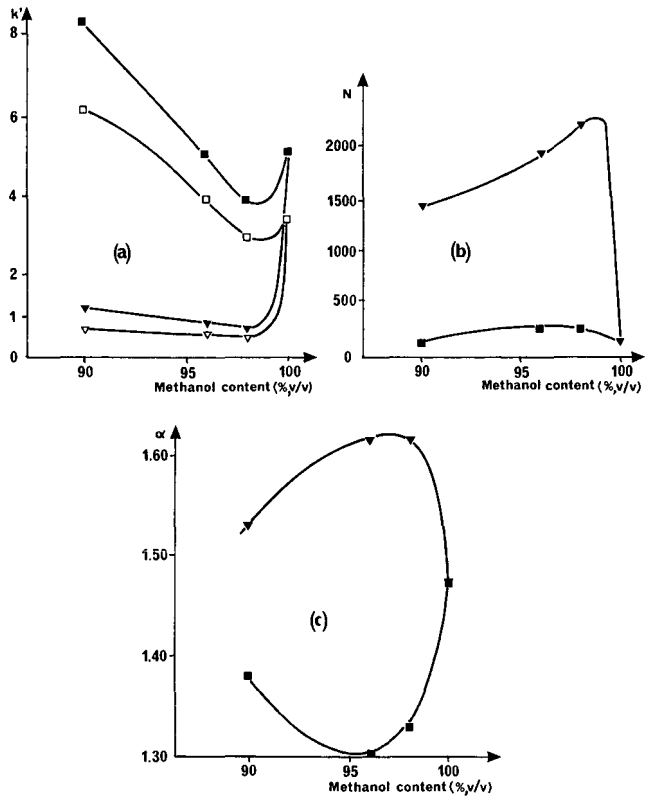


Fig. 7. Comparison of results obtained with methanol–water (pH 5.6) (■) and methanol–phosphate buffer (▼) (pH 5, 0.002 *M* in mobile phase) mixtures as mobile phase. Influence on (a) retention, *k'*, (b) efficiency, *N*, and (c) selectivity, α. Operating conditions as in Fig. 2b except for flow-rate, 2 ml/min. Symbols in (a): (□) and (▽), capacity factors of the first-eluted enantiomer; (■) and (▼), capacity factors of the last-eluted enantiomer.

Similar curves were described by Armstrong and co-workers[11,12] but no selectivity was observed in methanol[12]. The increase in retention was explained by the low solubility of the amino acid (tryptophan) in methanol. In the present work this explanation is not satisfactory as TCGA is very soluble in methanol whereas its solubility is low in water (10^{-3} mol l^{-1}).

Nevertheless, the chromatographic behaviour of TCGA appears to be better reflected by its degree of ionization in the mobile phase. This is supported by the fact that, at a constant methanol content, the retention decreases with increasing pH (Figs. 4 and 5); as the anionic form (denoted $TCGA^-$) is less hydrophobic than its corresponding molecular form, it is consequently much less retained. The ratio $|TCGA^-|/|TCGA|$ is obviously related to the difference pH − pK_a by the equation

$$\frac{|TCGA^-|}{|TCGA|} = 10^{(pH - pK_a)} \quad (1)$$

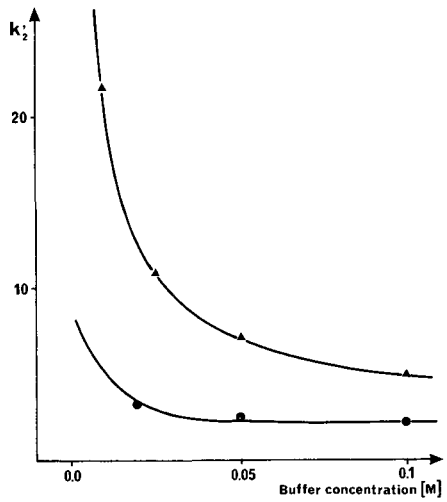


Fig. 8. Variation of the capacity factor of the last-eluted enantiomer of TCGA, k'_2, with the concentration of the phosphate buffer before its mixture with methanol. Operating conditions as in Fig. 2b; (▲) 70% methanol, pH 4.3; (●) 80% methanol, pH 5.

where | | represents species activities in solution.

The difference pH − pK_a for each mobile phase composition was determined by measuring (a) the pK_a* of TCGA in a methanol–water mixture and (b) the pH* of the same composition of methanol–phosphate buffer (initial pH 4.3, 5 or 5.8 and initial concentration 0.1 *M*) mixture. The asterisk indicates that we did not calibrate the pH meter before each measurement. However, the fact that we are interested only in the difference pH − pK_a overcomes all the problems related to the shift of the origin of the pH scale with water–methanol mixtures.

The results are presented in Fig. 9. The difference pH − pK_a and the degree of ionization of TCGA increase not only with the initial pH of the aqueous part of mobile phase but also with the dilution of the buffer in methanol. This was expected as the variations of the pK_a of acids between two solvents depend mainly on the charges of anionic species in solution[13]; consequently, variations of the buffer pH will be different from that of the TCGA pK_a.

Qualitative explanations can then be proposed. We must assume, in a classical manner, that the stationary liquid phase in contact with the CSP is enriched in methanol owing to the hydrophobic character of the CSP[10]. As the solubility of phosphate buffer salts is low in methanol, then, locally, the pH conditions are different from those of the mobile phase. Our proposed model is in two parts, as follows.

(1) For a low methanol content, the pH is still fixed by the buffer in the liquid stationary phase owing to the presence of water; TCGA is then mainly eluted in its anionic form. For instance, the ratio |TCGA$^-$|/|TCGA| varies in the mobile phase between 15.8 at pH 4.3 and more than 1000 at pH 5.8 (from eqn. 1 and Fig. 9, methanol content 70%). A similar evolution may occur in the liquid stationary phase; this will explain the decrease in retention times at high pH.

(2) For higher methanol contents, the liquid stationary phase can be assimilated to pure methanol. The solute (weak acid of pK_a 4.1 in water and 8.9 in methanol) is

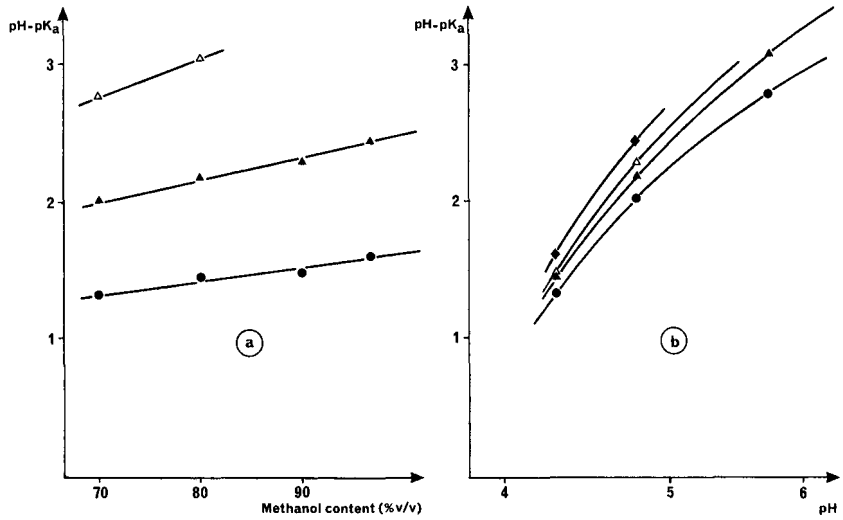


Fig. 9. Influence of (a) methanol content and (b) pH on the difference pH − pK_a representing the degree of ionization of TCGA in the mobile phase. Symbols: (a) 0.1 *M* phosphate buffer, (⬬) pH 4.3, (▲) pH 5 and (△) pH 5.8; (b) methanol content, (●) 70%, (▲) 80%, (△) 90% and (◆) 95% (0.1 *M* phosphate buffer).

eluted mainly in its molecular form[14,15]. There is then a competition between two opposing mechanisms: (a) the decrease in retention due to high alcohol content and high solubility in the mobile phase, and (b) the increase in retention due to enhancement of interactions between the acidic moiety of TCGA and secondary hydroxyl groups of the cyclodextrin cavity; this mechanism seems to become predominant at high contents.

Minimum retention is observed between cases (1) and (2). A similar explanation can be proposed for methanol–water mixtures. All the chromatographic results are in good agreement with these explanations. At high alcohol contents or in methanol–water mixtures, strong acid–hydroxyl group interactions are responsible for the low efficiencies and the low selectivities (the predominance of one interaction decreases the selectivity as discrimination between the enantiomers occurs by means of weak interactions).

Finally, the separation of TCGA enantiomers in methanol supports the hypothesis of a chiral mechanism in two steps: (1) interactions with the secondary hydroxyl groups of the rim of the CD cavity followed by (2) inclusion complex formation. For a methanol mobile phase, the chiral cavity is totally masked for solutes giving weak interactions such as TCGA methyl ester, which consequently remains unretained and unresolved (Table I, solute 2). In contrast, acids (such as TCGA) may interact with the cavity and therefore may be included and resolved. This two-step mechanism is also evidenced by replacing the Cyclobond-I CSP with its acetylated form; with the mobile phase found to be optimum on Cyclobond-I, TCGA is unretained ($k' = 0.3$) and unresolved ($\alpha = 1.00$) as it does not "see" the chiral cavity. With a mobile phase giving higher capacity factor values [phosphate buffer (pH 4.3, 0.1 *M*)–methanol (40:60, v/v)], a low selectivity is observed ($\alpha = 1.07$, $k'_2 = 2.88$); inclusion by hydrophobic effects may then become the dominant interaction at this higher water content.

Influence of the nature of the solute

If interactions with the CD rim and inclusion complex formation are essential conditions for optical resolution on Cyclobond-I CSP, structural requirements are also necessary. Small structural changes in the solute or CSP induce drastic effects on the selectivity. Various related compounds were evaluated and the results are given in Tables I and II.

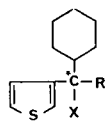
Both the acidic and the alcohol groups play a major role; a total loss of selectivity is observed for the methyl ester of TCGA ($\alpha = 1.00$, Table I, solute 2) and low values are obtained for the thienylcyclohexylacetic acid ($\alpha = 1.17$, solute 3) and for the diol solute ($\alpha = 1.09$, solute 4).

The results in Table II indicate that the cyclohexyl group appears to be preferentially included, presumably as it is most hydrophobic moiety close to the chiral centre. A smaller group (cyclopentenyl) gives lower selectivity and retention (Table II, solute 5). When a methylene group is intercalated between the chiral centre and the cyclohexyl moiety, both the retention and the selectivity decrease considerably (Table II, solute 6); if the thienyl group had formed a strong inclusion complex with the cavity, only the selectivity should have been affected by this structural change owing to modifications of the steric hindrance. In fact, the addition of a methylene group induces a "free rotation"[4] of the solute that results in a total loss of selectivity. The lack of a cyclic aliphatic group prevents any chiral recognition; we cannot separate enantiomers containing alkyl chains (no inclusion) or those with a second aromatic substituent (the symmetry of the two substituents is probably responsible for the loss of selectivity). Finally, cyclohexylphenylglycolic acid (Table II, solute 7) and the *p*-chloro derivative (solute 8) show a lower selectivity and retention than TCGA; this can be attributed to an increase in steric hindrance in the vicinity of the chiral centre, preventing the complete "tight fit" between the solute and the cavity[9].

TABLE I

INFLUENCE OF THE NATURE OF THE SOLUTE ON SELECTIVITY, α, AND CAPACITY FACTOR OF THE LAST-ELUTED ENANTIOMER, k'_2, ON CYCLOBOND-I CSP

Operating conditions as in Fig. 2b except for mobile phase, methanol–phosphate buffer (pH 5, 0.1 *M*) (95:5, v/v).

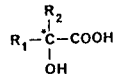


Solute	*No.*	α	k'_2
R = CO_2H X = OH	1	1.54	1.4
R = CO_2CH_3 X = OH	2	Unresolved	Unretained
R = CO_2H X = H	3	1.17	1.0
R = CH_2OH X = OH	4	1.09	0.4

TABLE II

INFLUENCE OF THE NATURE OF THE HYDROPHOBIC MOIETY OF SOLUTES ON SELECTIVITY, α, AND CAPACITY FACTOR OF THE LAST ELUTED ENANTIOMER, k'_2, ON CYCLOBOND-I CSP

Operating conditions as in Table I.



R_1	R_2	*No.*	α	k'_2
3-Thienyl	Cyclohexyl	1	1.54	1.4
3-Thienyl	Cyclopentyl	5	1.32	0.6
3-Thienyl	$-CH_2$-cyclohexyl	6	1.00	0.2
3-Thienyl	$-C_2H_5$		Unresolved	Unretained
3-Thienyl	$-CH(CH_3)_2$		Unresolved	Unretained
3-Thienyl	$-CH(CH_3)-C_2H_5$		Unresolved	Unretained
3-Thienyl	$-CH_2-(C_3H_5)$		Unresolved	Unretained
3-Thienyl	$-n-C_4H_9$		Unresolved	Unretained
3-Thienyl	*p*-Chlorophenyl		Unresolved	Unretained
3-Thienyl	*p*-Methoxyphenyl		Unresolved	Unretained
3-Thienyl	$-CH_2$-(*p*-methylphenyl)		Unresolved	Unretained
Phenyl	Cyclohehyl	7	1.34	1.2
p-Chlorophenyl	Cyclohexyl	8	1.22	1.0

Chiral recognition model

From the experimental data, a chiral recognition model can be advanced for the resolution of 3-thienylglycolic acids on Cyclobond-I CSP. It involves two steps: (1) interaction with the secondary hydroxyl groups of the CD cavity, followed by (2) inclusion complex formation.

The chromatographic results are influenced by various parameters, such as (a) the "tight fit" between the solute and the cavity (better inclusion of the cyclohexyl moiety), (b) the possibility of giving two additional strong interactions with the cyclodextrin rim and (c) the degree of ionization of the solute in the liquid stationary phase.

This mechanism is not basically different from that proposed by Armstrong and co-workers[9,11,12,16,17]; it is an adaptation of the proposed model for mobile phases containing high contents of organic solvent and for ionizable solutes. A similar two-step model was proposed by Wainer *et al.*[18] for the resolution of enantiomeric alcohols on ChiralCel OB CSP.

Chiral resolution of the methyl ester of TCGA

The methyl ester of TCGA remained unresolved on Cyclobond-I CSP. Among various other chiral stationary phases, ChiralCel OB, which is a benzoyl derivative of cellulose coated on silica gel, was tested. This column was used under SubFC conditions[3–5] with various carbon dioxide–2-propanol mixtures. The results are shown in Fig. 10; in spite of the good selectivity, the resolution remained low owing to the poor column efficiency.

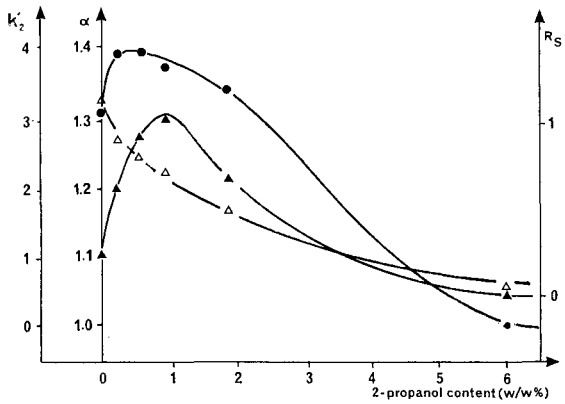


Fig. 10. Influence of 2-propanol content on the stereoselectivity, α, (●), the retention, k'_2 (△), and the resolution, R_s (▲), of MTCGA enantiomers on ChiralCel OB CSP in SubFC. Operating conditions: mobile phase, carbon dioxide–2-propanol; flow-rate, 5 ml/min at 0°C; temperature, 25°C; average column pressure, 120 Bar; detection, 215 nm.

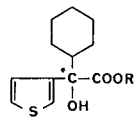
The selectivity shows a maximum when the modifier content in the mobile phase is increased. An explanation can be proposed: (a) at high alcohol content there is no interaction between the solute and the chiral moieties (cavities?), resulting in no selectivity, and (b) at low alcohol content stronger CSP–solute interactions are observed; however, both stereoselective and non-stereoselective interactions are favoured, resulting in a decrease in selectivity.

Optimum selectivity is observed with a 2-propanol content of 0.5% (w/w). However, owing to the low efficiency at low alcohol contents, maximum resolution is obtained with a 2-propanol content of 1% (w/w).

TABLE III

CHROMATOGRAPHIC SEPARATION OF TCGA ESTER DERIVATIVES ON CHIRALCEL OB CSP IN SubFC

Operating conditions as in Fig. 11a.



R	α	k'_2	*Modifier (%, w/w)*
$-CH_3$	1.40	2.5	2-Propanol (0.50)
$-C_2H_5$	1.12*	1.7	2-Propanol (0.40)
$-CH(CH_3)_2$	1.00	1.3	2-Propanol (0.40)
$-CH_2$-(3,5-dinitrophenyl)	1.17	16.9	2-Propanol (6.90)
$-CH_2$-(9-anthryl)	1.62	14.7	Methanol (12.0)

* Calculated value obtained by successive injections of (+)- and (−)-forms.

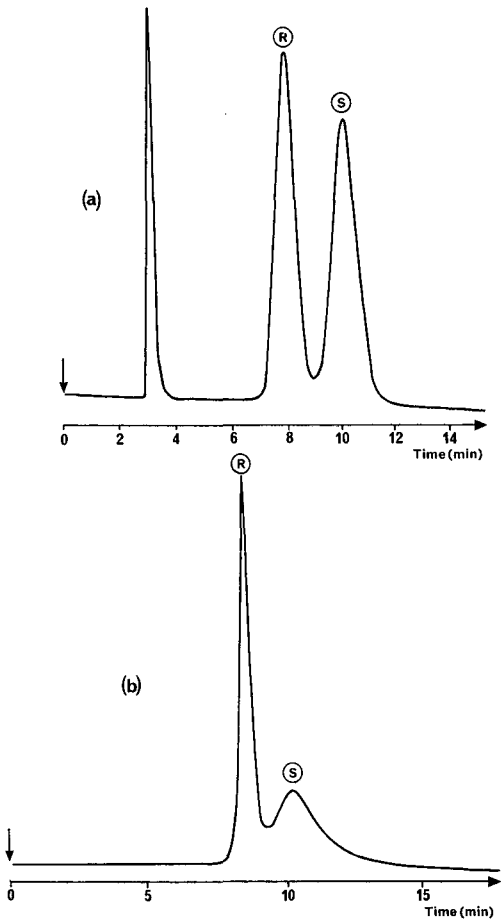


Fig. 11. Comparison of the separation of MTCGA on ChiralCel OB CSP using LC and SubFC modes. Operating conditions: (a) SubFC, as in Fig. 10 except for mobile phase carbon dioxide–2-propanol (99.5:0.5, w/w) and flow-rate 1.2 ml/min at 0°C; (b) LC, mobile phase hexane–ethanol (85:15, v/v), flow-rate 0.5 ml/min, temperature 25°C, detection 215 nm.

A comparison of LC and SubFC chromatograms is presented in Fig. 11; the superiority of SubFC over LC for the determination of the enantiomeric purity of MTCGA is evident with this type of stationary phase, as reported previously[5].

Other solutes were tested and the results are given in Table III. It is important to note that no resolution is observed for the ethyl ester analysed as a racemic mixture. However, if the (+)- and (−)-antipodes are injected separately, a selectivity of 1.12 can be calculated, in addition to a theoretical resolution of 0.8. This may be the consequence of the formation of stable diastereomeric dimers [(+)/(−)] in the mobile phase. No other explanation consistent with the fact that the methyl ester is correctly separated can be advocated.

CONCLUSION

The study of the separation of 3-thienylcyclohexylglycolic acid on Cyclobond-I CSP allows a better understanding of retention and chiral recognition mechanisms with this type of CSP. The influence of secondary hydroxyl groups of the cyclodextrin rim was clearly established, in addition to the influence of solute functional moieties on selectivity. A new approach to retention phenomena is proposed, based on the methanol content in the mobile phase and on the degree of ionization of the solute in the liquid stationary phase. This approach permits an explanation of why chiral separations can be effective in pure methanol.

The superiority of SubFC over LC was demonstrated on ChiralCel OB for the resolution of the methyl ester of TCGA; only SubFC provides a reliable means of controlling the optical purity of the solute.

ACKNOWLEDGEMENTS

The authors thank Dr. A. Jardy for helpful discussions of pH determination in non-aqueous media. They are also grateful for support from Prolabo and Advanced Separation Technologies, who are thanked for a gift of Cyclobond CSPs.

REFERENCES

1 A. Tambuté and A. Collet, *Bull. Soc. Chim. Fr.*, 2 (1984) 77.
2 P. Mourier, E. Eliot, A. Tambuté, M. Caude and R. Rosset, *Anal. Chem.*, 57 (1985) 2819.
3 P. Macaudière, A. Tambuté, M. Caude, R. Rosset, M. A. Alembik and I. W. Wainer, *J. Chromatogr.*, 371 (1986) 177.
4 P. Macaudière, M. Caude, R. Rosset and A. Tambuté, *J. Chromatogr.*, 405 (1987) 135.
5 P. Macaudière, M. Caude, R. Rosset and A. Tambuté, in *Proceedings of the International Symposium on Chiral Separations, Guildford, September 3–4, 1987*, in press.
6 P. Mourier, M. Caude and R. Rosset, *Analusis*, 13 (1985) 299.
7 K. G. Feitsma, J. Bosman, B. F. H. Drenth and R. A. de Zeeuw, *J. Chromatogr.*, 333 (1985) 59.
8 K. G. Feitsma, B. F. H. Drenth and R. A. de Zeeuw, *J. Chromatogr.*, 387 (1987) 447.
9 D. W. Armstrong, W. Demond and B. P. Czech, *Anal. Chem.*, 57 (1985) 481.
10 D. W. Armstrong, T. J. Ward, R. D. Armstrong and T. E. Beesley, *Science*, 232 (1986) 1132.
11 S. M. Han and D. W. Armstrong, *J. Chromatogr.*, 389 (1987) 256.
12 D. W. Armstrong, X. Yang, S. M. Han and R. A. Menges, *Anal. Chem.*, 59 (1987) 2594.
13 G. Charlot, *Cours de Chimie Analytique Générale: Solutions Aqueuses et Non Aqueuses*, Masson, Paris, 2nd ed., 1978.
14 M. C. Hennion, C. Picard, C. Combellas, M. Caude and R. Rosset, *J. Chromatogr.*, 210 (1981) 211.
15 R. Rosset, D. Bauer and J. Desbarres, *Chimie Analytique des Solutions et Microinformatique*, Masson, Paris, 1979.
16 R. Rosset, M. Caude and A. Jardy, *Manuel Pratique de Chromatographie en Phase Liquide*, Masson, Paris, 2nd ed., 1982, p. 214
17 T. J. Ward and D. Armstrong, in M. Zief and L. J. Crane (Editors), *Chromatographic Chiral Separations*, Marcel Dekker, New York, 1988, p. 131.
18 I. W. Wainer, R. M. Stiffin and T. Shibata, *J. Chromatogr.*, 411 (1987) 139.

Journal of Chromatography, 450 (1988) 271–276
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 799

CHROMATOGRAPHIC RESOLUTION OF DIPEPTIDE ENANTIOMERS AND DIASTEREOMERS ON CHIRAL STATIONARY PHASES FROM POLY-(L-LEUCINE) OR POLY(L-PHENYLALANINE)

CHUICHI HIRAYAMA*, HIROTAKA IHARA and KATSUNORI TANAKA
Department of Applied Chemistry, Faculty of Engineering, Kumamoto University, Kurokami 2-39-1, Kumamoto 860 (Japan)
(First received January 22nd, 1988; revised manuscript received April 29th, 1988)

SUMMARY

Two types of chiral stationary phase, polymer-bonded and non-bonded, containing poly(L-leucine) and poly(L-phenylalanine) were prepared. These stationary phases gave higher enantioselectivity for the optical resolution of various derivatives of leucylphenylalanine methyl ester by liquid chromatography than did poly(L-alanine) and poly(L-glutamate).

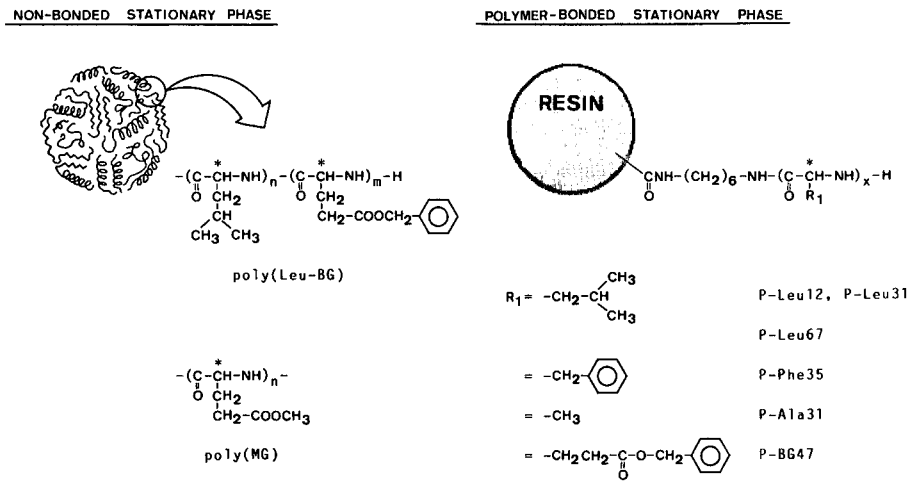
INTRODUCTION

Optical resolution by liquid chromatography has been achieved on various chiral stationary phases having helical polymers. For example, on poly(triphenylmethyl methacrylate) prepared by asymmetric polymerization, which has an helical structure, racemates of binaphthyl derivatives[1], tröger bases[1] and *trans*-disubstituted cyclic compounds have been resolved[2]. Similarly, helical polymers from cellulose triphenylcarbamate derivatives have been used to resolve chiral isomers of tröger bases and a cobalt complex[3]. Poly(α-amino acid) also produces helical forms. Recently, the resolution of chiral hydantoins from amino acids on poly(N^5-benzyl-L-glutamine) bound to polystyrene resin was reported[4].

On the other hand, it is known that the enantioselectivity in micellar hydrolysis of esters of amino acids is critically dependent on the chemical structure of the substrate and catalyst. For example, we observed that esters from leucine and phenylalanine gave higher selectivity than those of alanine and valine in the hydrolysis catalyzed by histidine derivatives[5]. In addition, Okahata[6] reported that membranes containing poly(L-glutamic acid) show enantioselective permeabilitty of α-amino acids when in the α-helix state. Selectivity is not obtained in the random coil state.

On the basis of these observations, we prepared chiral stationary phases, polymer-bonded (I) and non-bonded (II), from poly(L-leucine) and poly(L-phenylalanine). These stationary phases gave higher enantioselectivity than those of poly(L-alanine) and poly(L-glutamate). The chiral stationary phases used in this study are schematically represented below.

0021-9673/88/$03.50



EXPERIMENTAL

Polymer-bonded chiral stationary phase

Porous spherical beads with the average diameter of 15–25 μm were prepared by suspension polymerization of 70 mol% of methyl acrylate and 30 mol% divinylbenzene (containing 40 mol% of ethylvinylbenzene). The beads were packed in a stainless-steel column and the gel permeation chromatographic (GPC) behaviours were examined in tetrahydrofuran. The exclusion molecular weights, M_{lim}, of the beads were about $1.1 \cdot 10^5$ (polystyrene standard). The beads were partially aminolyzed by 1,6-diaminohexane in decahydronaphthalene. The content of the amino group introduced on the beads were adjusted to 0.5–1.0 mequiv./g according to the reaction conditions. N-carboxyanhydrides (NCA) of L-leucine (Leu), L-phenylalanine (Phe), L-alanine (Ala) and γ-benzyl-L-glutamate (BG) (prepared by Mitsui Toatsu Chemicals) were polymerized by the aminated beads as an initiator. The content of the poly(α-amino acid) grafted in the beads could be adjusted from 12 to 67% (w/w) by the ratio of the beads to NCA in the polymerization process. The chiral beads prepared are abbreviated P-Leu11, P-Leu31, P-Leu67, P-Phe35, P-Ala31 and P-BG47, where the number in the abbreviations stands for the content (%, w/w) of poly(α-amino acid).

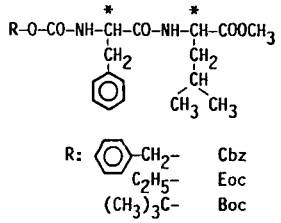
Non-bonded chiral stationary phase

NCA of L-leucine and γ-benzyl-L-glutamate was copolymerized by triethylamine. The copolymer obtained was dissolved in dichloromethane and converted into spheres by our previous technique (the suspension and evaporation method[7]). For comparison, spherical beads from poly(γ-methyl-L-glutamate) were prepared. These chiral beads are abbreviated poly(Leu-BG) and poly(MG), respectively.

Chiral dipeptides

As a model sample for optical resolution, enantiomers and diastereomers of leucylphenylalanine derivatives were synthesized by a conventional coupling method

by Mr. Matsuyama of our laboratory. The chemical structures of the dipeptides are given below.



Chromatography

The spherical polyamino acids beads were packed in a stainless-steel column (15 cm × 0.4 cm I.D.). The chromatographic properties were examined at a flow-rate of 0.5 ml/min and 40°C, employing a Waters Assoc. 6000-p.s.i. pump (Model 510) controlled by a Type 680 automated gradient controller and a LC spectrophotometer (Model 481).

RESULTS AND DISCUSSION

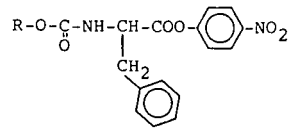
Table I shows the capacity factor, k', and the separation factor, α, for the enantiomers of the methyl ester of N-protected leucylphenylalanine in liquid chromatography using P-Leu31, P-Phe35, P-Ala31 and P-BG47 columns. The eluent was hexane–chloroform (9:1). The capacity factor was calculated as $(t - t_0)/t_0$, where t is the retention time and t_0 is the dead time determined with 1,3,5-tri-*tert.*-butylbenzene as a non-retained sample. As expected, the chiral stationary phase containing poly(L-leucine) and poly(L-phenylalanine) gave higher separation coefficients with values of

TABLE I

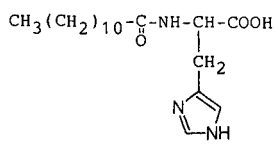
CHROMATOGRAPHIC RESOLUTION OF N-PROTECTED DERIVATIVES OF LEUCYLPHENYLA NINE METHYL ESTER BY VARIOUS TYPE I STATIONARY PHASE

The number of each stationary phase represents the content of poly(L-amino acid). Eluent: 1 hexane–chlorof (90:10). Temperature: 40°C.

Dipeptide		*Stationary phase*							
R	*Chirality*	*P-Leu31*		*P-Phe35*		*P-Ala35*		*P-BG47*	
		k'	α	k'	α	k'	α	k'	α
Cbz	L-L	9.10	1.59	10.83	1.37	10.03	1.01	9.14	1.02
	D-D	14.51		14.84		10.14		9.33	
Boc	L-L	2.63	1.60	2.08	1.31	2.90	1.04	2.18	1.03
	D-D	4.22		2.73		3.02		2.24	
Eoc	L-L	5.69	1.74	5.18	1.42	6.39	1.01	5.97	1.03
	D-D	9.90		7.33		6.32		6.14	



1: R = benzyl
= ethyl
= tert.-butyl



2

1.59–1.74 and 1.31–1.42, respectively. On the contrary, those of poly(L-alanine) and poly(γ-benzyl-L-glutamate) gave less selectivity ($\alpha < 1.04$).

In general, it is known that the hydrogen bonding among peptides plays a significant role in the appearance of enantioselectivity. For example, the structural effect of the N-protecting groups (R) of chiral substrate 1 was examined in the hydrolysis reaction catalyzed by N-acylhistidine (2)[5]. When R is a bulky, *tert.*-butyl group which prevents the hydrogen bonding interaction between the substrate and catalyst by steric hindrance, the degree of enantioselectivity is lowered remarkably. On the contrary, no significant lowering of selectivity, α, in the Boc derivative was observed in this study (Table I). The large k' value of the Cbz derivative compared with those of the Boc and Eoc derivatives is attributable to π–π interactions between the sample and the stationary phase based on the divinylbenzene in the polymer support. An aprotic solvent is used as an eluent so as not to prevent the hydrogen bonding interaction. Therefore, it is estimated that the recognition site is on the amide bond between the leucine and phenylalanine rather than that of N-protected group.

Table II shows the relationship between the leucine content of the polymer and the values of k' and α. Although k' (related to the retention time) increased with increasing content of leucine, the selectivity, α, did not necessarily increase. The selectivity of P-Leu67 is remarkable low. Okamoto and Hatada[8] proposed that an in-

TABLE II

CHROMATOGRAPHIC RESOLUTION OF N-PROTECTED DERIVATIVES OF LEUCYLPHENYLALANINE METHYL ESTER BY CHIRAL POLYMER BEADS WITH VARIOUS CONTENTS OF L-LEUCINE

Eluent: hexane–chloroform (90:10). Temperature: 40°C.

Dipeptide		*Stationary phase*					
R	*Chirality*	*P-Leu12*		*P-Leu31*		*P-Leu67*	
		k'	α	k'	α	k'	α
Cbz	L-L	0.93	1.45	9.10	1.59	13.51	1.30
	D-D	1.35		14.51		17.56	
Boc	L-L	0.28	1.39	2.63	1.60	3.10	1.43
	D-D	0.39		4.22		4.43	
Eoc	L-L	0.64	1.58	5.69	74	8.95	1.30
	D-D	1.01		9.90		.68	

TABLE III

CHROMATOGRAPHIC RESOLUTION OF N-PROTECTED DERIVATIVES OF LEUCYLPHENYLALANINE METHYL ESTERS BY TYPE II STATIONARY PHASE

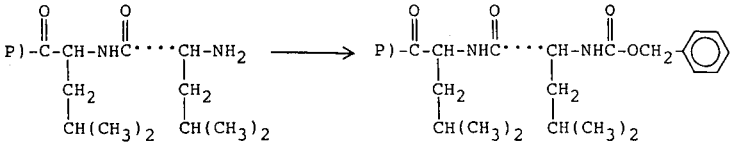
Eluent: hexane–chloroform (90:10). Temperature: 40°C.

Dipeptide		*Stationary phase*			
R	*Chirality*	*poly(Leu-BG)*		*poly(MG)*	
		k'	α	*k'*	α
Cbz	L-L	1.67	1.08	7.36	1.05
	D-D	1.55		7.73	
	L-D	1.97	1.27	7.22	1.07
	D-L	1.82	1.08	7.22	1.00
Boc	L-L	0.58	1.07	2.36	1.02
	D-D	0.54		2.31	
Eoc	L-L	1.05	1.10	4.34	1.02
	D-D	0.95		4.25	

crease in chiral sites on packings induces different orientations in chiral polymers and lowering of selectivity in the case of packings from chiral poly(triphenylmethyl methacrylate).

Table III shows the enantioselectivity of non-bonded stationary phases. The stationary phase was prepared by the suspension and evaporation method[7]. Similarly to the polymer-bonded chiral stationary phase, poly(Leu-BG) containing L-leucine gave higher selectivity than poly(MG) from poly(γ-methyl-L-glutamate) homopolymer.

In order to estimate the recognition site on the stationary phase, terminal amino groups of a polyamino acid chain were benzyloxycarbonylated:



and the enantioselectivity was examined. As shown in Table IV, the enantioselectivity shows a slight decrease on the P-Leu12 stationary phase, but was almost unchanged on P-Leu67. This indicates that the recognition site is on the polyamino acid chain of the stationary phase.

The secondary structure of the polyamino acid chain in the stationary phase was estimated by Fourier transform (FT)-IR spectroscopy using diffuse reflectance. The spectra show that both the poly(Leu-BG) and the poly(MG) spheres are formed from the α-helix component in spite of their different selectivities (amide I, 1650 cm^{-1}; amide V, 610 cm^{-1}). This indicates that the stereospecificity of leucine residues on the α-helical polymer chain is a significant factor for higher selectivity.

TABLE IV

INFLUENCE ON THE SEPARATION COEFFICIENT OF THE PROTECTION OF N-TERMINAL AMINO GROUPS BY BENZYLOXYCARBONYLATION

Dipeptide		*P-Leu67*				*P-Leu12*			
R	*Chirality*	*Non-treated*		*Protected*		*Non-treated*		*Protected*	
		k'	α	*k'*	α	*k'*	α	*k'*	α
Cbz	L-L	13.51	1.30	10.84	1.33	0.93	1.45	0.61	1.38
	D-D	17.56		14.43		1.35		0.84	
Eoc	L-L	8.95	1.30	7.05	1.34	0.64	1.58	0.38	1.34
	D-D	11.68		9.47		1.01		0.51	

ACKNOWLEDGEMENT

We thank Dr. K. Tamaki and Mr. T. Kobayashi of Mitsui Toatsu Chemicals for the preparation of NCA monomers.

REFERENCES

1 Y. Okamoto, I. Okamoto and H. Yuki, *Chem. Lett.*, (1981) 835.
2 Y. Okamoto, S. Honda, I. Okamoto and H. Yuki, *J. Am. Chem. Soc.*, 103 (1981) 6971.
3 Y. Okamoto, M. Kawashima and K. Hatada, *J. Am. Chem. Soc.*, 106 (1984) 5357.
4 H. Kiniwa and Y. Doi, *Polym. Preprints Jpn.*, 35 (1986) 650, 651.
5 K. Yamada, H. Shosenji, H. Ihara and Y. Otsubo, *Tetrahedron Lett.*, 27 (1979) 2529.
6 Y. Okahata, *Membranes*, 12 (1987) 339.
7 H. Ihara, T. Yoshinaga and C. Hirayama, *J. Chromatogr.*, 362 (1986) 197.
8 Y. Okamoto and K. Hatada, *J. Liq. Chromatogr.*, 9 (1986) 369.

Journal of Chromatography, 450 (1988) 277–280
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 738

Note

Preparative chiral separation in an aqueous two-phase system by a few counter-current extractions

BÖRJE SELLERGREN* and BJÖRN EKBERG
Department of Pure and Applied Biochemistry, Lund Institute of Technology, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
PER-ÅKE ALBERTSSON
Department of Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
and
KLAUS MOSBACH
Department of Pure and Applied Biochemistry, Lund Institute of Technology, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
(Received June 16th, 1988)

The increasing need for optically pure substances in the pharmaceutical industry has led to the development of several techniques for analytical and preparative chiral separations[1–3]. In liquid chromatography, preparative separations have been demonstrated using cyclodextrins, triacetylcellulose, polyamides, ligand exchange and Pirkle type phases and recently also imprinted polymers[3]. Using proteins as chiral stationary phases, a variety of racemic compounds have been resolved[4]. However, due to a low sample load capacity, only a few examples can be found where proteins have been used for preparative chiral separations[5].

By employing bovine serum albumin (BSA) in an aqueous two-phase system[6] we have demonstrated the possibility of semipreparative chiral separations, using a counter-current distribution apparatus[7,8]. In this report we demonstrate that only a few counter-current extractions (eight) are required for a preparative scale separation of D- and L-Kynurenine. A complete procedure is presented where the enantiomers are isolated by ultrafiltration, leaving the phase polymers and the BSA for possible reuse. The separation is simply carried out in separation funnels and no other equipment is required.

MATERIALS AND METHODS

Chemicals and phase system

Dextran 40 was obtained from Pharmacia (Uppsala, Sweden), poly(ethylene glycol), PEG 8000 from Union Carbide (New York, NY, U.S.A.) and BSA (No. A-7906) and D,L-Kynurenine from Sigma (Milwaukee, WI, U.S.A.). The phase system was 8% (w/w) Dextran 40, 7% (w/w) PEG 8000 and 6% (w/w) BSA in 0.05 *M* sodium phosphate buffer, pH 8.5, containing 0.1 *M* sodium chloride. A 1.5-l volume of this system was shaken and then allowed to settle. The resulting phase volume ratio was 1.15 (volume upper phase/volume lower phase).

0021-9673/88/$03.50

Counter-current extractions

In eight separatory funnels (500 ml) were placed 80 ml of the upper phase (PEG-containing). The first funnel contained in addition 70 ml of the lower phase. To this were added 100 mg of D,L-Kynurenine. After shaking and settling, the lower phase was transferred to the second funnel and an additional 70 ml of the lower phase was added to the first funnel. This completed the first transfer. Next, the first and second funnels were shaken and, after settling, the lower phase of the second funnel was transferred to the third and the lower phase of the first funnel was transferred to the second. Thereafter fresh lower phase was added to the first funnel. This completed the second transfer. In this way eight transfers were completed.

Optical purity analysis

An high-performance liquid chromatographic (HPLC) method, based on the one described by Allenmark *et al.*[4], was developed using an analytical BSA column. The column (200 mm × 5 mm I.D.) containing Si 500 (Mèrck) (20–45 μm) was prepared as described elsewhere[5]. The eluent was 0.05 *M* sodium phosphate, pH 8, containing 2% *n*-propanol, the flow-rate was 1.5 ml/min and the elution was monitored at 257 nm. In Fig. 1 are seen typical elution profiles of the pooled fractions (after ultrafiltration) and of the racemate itself.

Removal of the phase polymers and salt

After checking the optical purity and the amount of substance in each fraction, fractions 2–4 and 7 and 8 were pooled. One part of water was added and the pH was lowered to about 4 with acetic acid. Part of the solution was then recycled through an ultrafiltration unit, as described elsewhere[9] (Pelicon laboratory cassette system; Millipore, Göteborg, Sweden) with a 10^4 molecular weight cut off. After freeze drying of the filtrate, a small amount of methanol was added in order to extract the substance from the salt-containing solid. The optical purity of the substance was then estimated from Fig. 1 and the amount was estimated by comparing the peak area with that of a known amount of substance. This showed that about 15 mg of racemate had been separated from the phase polymers.

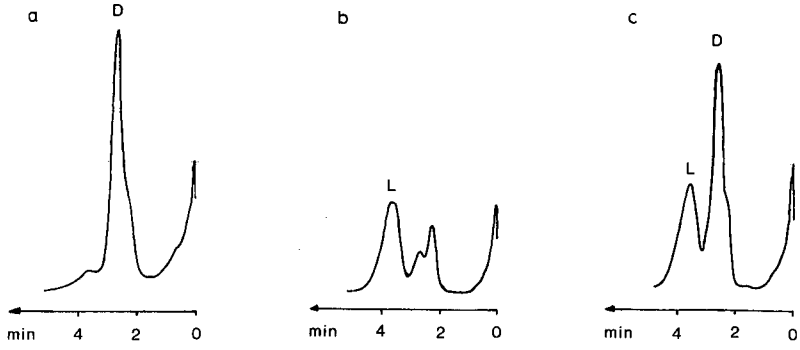


Fig. 1. HPLC elution profiles of fractions 2—4 (a), 7, 8 (b), and pure D,L-Kynurenine (c) on a BSA–silica column. Eluent: 0.05 *M* sodium phosphate buffer, pH 8.0, containing 2% *n*-propanol. Flow-rate: 1.5 ml/min.

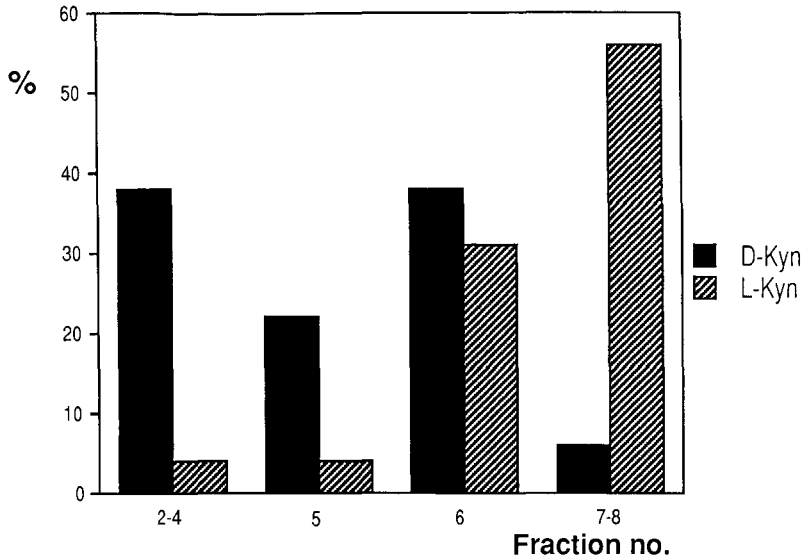


Fig. 2. Enantiomeric distribution in the pooled fractions. Percentage based on the total amount of each enantiomer applied.

RESULTS AND DISCUSSION

The enantiomeric distribution (Fig. 2) and the substrate recovery (Table I) of the pooled fractions shows that about half (50 mg) of the D,L-Kynurenine applied can be obtained in high enantiomeric purity (≈90%). To increase further the yield, either additional extraction steps or recycling is necessary. The latter is possible since the polymers of the phase system (including BSA) are recovered after the ultrafiltration step. This is promising for a large scale continuous process.

The procedure described has several obvious advantages. No immobilization of the protein to a stationary phase is needed, which allows easy testing for chiral selectivity of proteins in general. Some racemic compounds which are not resolved by columns with BSA adsorbed onto silica[5] can be resolved in our system, *i.e.*, D,L-2-methylsulphinylbenzoic acid (17 mg, separation coefficient 1.8 and resolved peak maxima)[8]. Furthermore, the number of extraction steps can be chosen according to

TABLE I

TOTAL AMOUNT AND OPTICAL PURITY OF D,L-KYNURENINE IN THE POOLED FRACTIONS

Only a part of the total amount was separated from the phase polymers.

Fraction	*D,L-Kynurenine (mg)*	*Optical purity (%)**
2–4	22	90 (D)
5	13	83 (D)
6	35	55 (D)
7, 8	30	90 (L)

* Refers to the enantiomer in parentheses.

the separation coefficient of the substance and in favourable cases only a few extractions are required to obtain high optical purity. Finally, these extractions can be carried out with simple equipment.

ACKNOWLEDGEMENT

We thank Dr. Folke Tjerneld and Mr. Per Erlandsson for their valuable assistance. We are grateful to Professor Stig Allenmark, University of Göteborg, for a sample of D,L-2-methylsulphinylbenzoic acid. This work was supported by the Swedish Board for Technical Development and the Swedish National Science Foundation.

REFERENCES

1 W. H. Pirkle and J. Finn, in J. D. Morrison (Editor), *Asymmetric Synthesis*, Academic Press, New York, 1983, p. 87.
2 W. Lindner, *Chromatographia*, 24 (1987) 97–107.
3 B. Sellergren, M. Lepistö and K. Mosbach, *J. Am. Chem. Soc.*, 110 (1988) 5853–5860.
4 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 316 (1984) 617—624.
5 P. Erlandsson, L. Hansson and R. Isaksson, *J. Chromatogr.*, 370 (1986) 475–483.
6 P.-Å. Albertsson, in *Partition of Cell Particles and Macromolecules*, Wiley, New York, 1986.
7 B. Ekberg, B. Sellergren and P.-Å. Albertsson, *J. Chromatogr.*, 333 (1985) 211–214.
8 B. Sellergren, B. Ekberg, P.-Å. Albertsson and K. Mosbach, *Pat. Appl.*, PCT/SE86/00315 (1986).
9 F. Tjerneld, I. Persson, P.-Å. Albertsson and B. Hahn-Hägerdal, *Biotechnol. Bioeng. Symp.*, No. 15 (1985) 419–429.

Journal of Chromatography, 450 (1988) 281–283
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 765

Note

Separation and determination of some amino acid ester enantiomers by thin-layer chromatography after derivatization with (*S*)-(+)-naproxen

NADIR BÜYÜKTIMKIN*
Faculty of Pharmacy, University of Istanbul, 34452 Istanbul (Turkey)
and
ARMIN BUSCHAUER
Institut für Pharmazie, Freie Universität Berlin, D-1000 Berlin 33 (F.R.G.)
(Received June 30th, 1988)

For the determination of the enantiomeric purity of amino acids, several chromatographic methods have been described, such as direct resolution of enantiomers on an optically active stationary or mobile phase, or separations based on the derivatization with a chiral reagent followed by chromatography of the diastereomers on a conventional stationary phase[1–4].

The aim of the present work is the separation and the evaluation of the enantiomeric composition of some amino acid esters on precoated thin-layer chromatographic (TLC) plates using highly UV-absorbing optically active α-alkyl-α-aryl acetic acids[5,6], here (*S*)-(+)-naproxen[6,7], as the derivatizating reagent.

EXPERIMENTAL

Solvents (analytical grade), thionyl chloride and TLC plates (20 cm × 20 cm) (Art. 5554) were obtained from E. Merck (Darmstadt, F.R.G.). (*S*)-(+)-Naproxen was obtained from Sigma (München, F.R.G.). Racemic and enantiomeric amino acids and some methyl esters were from Aldrich (Steinheim, F.R.G.) or E. Merck. Amino acid methyl esters not commercially available were prepared analogously to a known method[8].

Apparatus

Melting points were obtained with a Büchi apparatus and are uncorrected. IR spectra were obtained in potassium bromide discs with a Perkin-Elmer 1420 spectrometer. The chromatograms were scanned with a Camag II TLC scanner.

Preparation of (S)-(+)-naproxen chloride[9]

(*S*)-(+)-Naproxen (0.01 *M*) was dissolved in 20 ml of anhydrous toluene. A 5-ml volume of freshly distilled thionyl chloride was added and the solution was refluxed for 30 min. After cooling, the mixture was evaporated *in vacuo*. The residue was dissolved in dichloromethane and crystallized twice: m.p. 91–92°C; IR, 1785 cm^{-1} (C=O).

0021-9673/88/$03.50

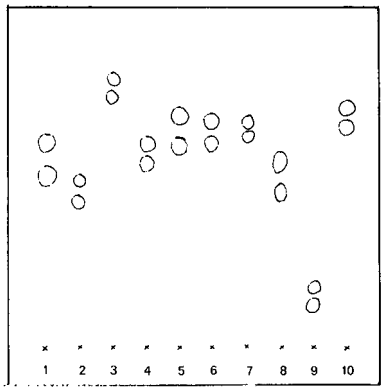


Fig. 1. Chromatogram of amino acid methyl esters after derivatization with (*S*)-(+)-naproxen chloride. Amino acids as in Table I.

Preparation of amino acid derivatives and chromatography

(*S*)-(+)-Naproxen chloride (0.02 m*M*) was dissolved in 5 ml dichloromethane, and 0.01 m*M* amino acid methyl ester and two drops of pyridine were added. The solution was refluxed for 30 min. After evaporation of the solution *in vacuo*, the residue was dissolved in 5 ml dichloromethane and was successively washed with 5 ml 0.1 *N* sodium bicarbonate, 5 ml 0.1 *M* hydrochloric acid and twice with 5 ml water, dried over anhydrous sodium sulphate and made up to 5 ml. The preparative synthesis and spectrometric data will be reported elsewhere.

A 5-μl volume of the dichloromethane solution was applied to the plates using a 25-μl Hamilton syringe. The plates were developed to a distance of 17 cm with the solvent systems toluene–dichloromethane–tetrahydrofuran [(5:1:1, v/v/v (I) or 5:1:2, v/v/v (II); ammonia atmosphere] in preequilibrated (15 min) glass chambers. After

TABLE I

hR_F OF RESOLVED DIASTEREOISOMERIC AMINO ACID METHYL ESTERS DERIVATIVES

Solvent: toluene–dichloromethane–tetrahydrofuran [5:1:2 (v/v), ammonia atmosphere]. Development time: 50 min. Solvent front: 17 cm. Temperature: 25°C.

(S)-(+)-Naproxen amide of amino acid	hR_F	
	R	*S*
1 Methionine	56	60
2 Asparagine	46	53
3 Leucine	79	85
4 Proline	57	64
5 Norvaline	63	73
6 Valine	63	71
7 Phenylalanine	66	69
8 α-Aminobutyric acid	49	57
9 Tyrosine	12	17
10 Norleucine	69	75

drying, the plates were evaluated by spectrodensitometry at 254 nm with a scanning speed of 1 mm/min. The enantiomer ratios were calculated by comparing the corresponding peak areas of integral values.

RESULTS AND DISCUSSION

In the present work ten enantiomeric amino acid esters were separated as (*S*)-(+)-naproxen amides by TLC. A representative chromatogram is shown in Fig. 1. The hR_F values of the diastereomeric pairs are listed in Table I.

The results show (*S*)-(+)-naproxen chloride to be a useful derivatizating reagent for the separation and determination of amino acid esters enantiomers. Diastereomers derived from *R*-amino acids were more strongly retained than derivatives of *S*-amino acids. The configurational stability of the derivatives is a marked advantage. In a comparative assay in dichloromethane, no measurable racemization was observed within 24 h under laboratory conditions. The high UV absorptivity of the reagent[3] and of the derivatives makes the assay very convenient since the amino acids, with the exception of aromatic ones, exhibit no appreciable UV absorption. No specific visualization agents are required. It is possible to detect an optical impurity of 1%. The accuracy and precision of the method was tested in the analysis of mixtures of some pure diastereomers, the ratio of the areas or the integral values of the diastereomeric peaks being used to give the experimental optical purities. With the exception of tyrosine (monoacyl derivative), multiple development did not improve the separation. The best results were obtained with the solvent system II.

The chromatographic resolution described provides a cheap, rapid, simple and sensitive method for the determination of amino acid esters. Optically active solvent systems or expensive chemically bound stationary phases are not required, as well as impregnating substances, which can influence negatively the separation and assay. The same derivatives were also used in gas chromatograhy (GC), the results of which will be reported elsewhere.

ACKNOWLEDGEMENTS

The present work was partly accomplished at the Institute for Pharmacy, with a research grant from Freie Universität Berlin to N.B., which is greatly acknowledged. The authors are grateful to Professor Dr. Walter Schunack for his support. Martin Langstädtler is thanked for his collaboration in TLC densitometric measurements.

REFERENCES

1 K. Günther, M. Schickedanz, K. Drauz and J. Martens, *Fresenius' Z. Anal. Chem.*, 325 (1986) 297.
2 R. Bhushan and I. Ali, *J. Chromatogr.*, 392 (1987) 460.
3 T. Fukuhara and S. Yuasa, *J. Chromatogr.*, 411 (1987) 502.
4 R. W. Souter, *Chromatographic Separation of Stereoisomers*, CRC Press, Boca Raton, FL, 1985; and references cited therein.
5 H. Weber, H. Spahn, E. Mutschler and W. Möhrke, *J. Chromatogr.*, 307 (1984) 145.
6 H. Spahn and W. Henke, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 329 (Suppl.) (1985) R11.
7 H. Spahn, D. Krauß and E. Mutschler, *Pharm. Res.*, 5 (1988) 107.
8 G. Gerhard and W. Schunack, *Arch. Pharm. (Weinheim Ger.)*, 313 (1980) 709.
9 F. U. Petracek (Riker Laboratories Inc.), *Ger. Pat.*, 2 322 125 (1973); *C.A.*, 80 (1974) 27024z.

Journal of Chromatography, 450 (1988) 284–290
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 735

Note

Rapid optical resolution of anionic metal complexes by gel permeation chromatography

MILAN STRAŠÁK*
Department of Analytical Chemistry, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava (Czechoslovakia)
and
SLAVOMÍR BYSTRICKÝ
Institute of Chemistry, Slovak Academy of Science, 842 38 Bratislava (Czechoslovakia)
(First received April 26th, 1988; revised manuscript received June 20th, 1988)

The most thoroughly investigated multidentate ligands are EDTA* and its analogues. Their cobalt(III) complexes are not only of interest for understanding their stereochemistry, but also for their applications in the bioinorganic field because they provide simple structural models of the active site of metalloproteins[1].

The flexible EDTA ligand is hexadentate in the complex $[Co(EDTA)]^-$, giving only one geometric isomer, the *cis*(N)-isomer (Fig. 1a). Structural parameters of this chelate system show notable departures from those for regular octahedral coordination and imply that the system is strained. The two glycinate (G) rings lying in the plane of the diamine ring are considerably more strained than the out-of-plane glycinate rings (R). The ethylenediamine "backbone" ring (E) of the $[Co(EDTA)]^-$ complex is locked in one conformation (δ for the structure having Λ configuration and *vice versa*)[2]. Other hexadentate ligands, structurally similar to EDTA, such as EDDS having two longer carboxylate chains, form isomers differing in the number of six-membered rings in the girdle plane. The optically active *SS*-EDDS ligand could theoretically form two isomers: *trans*(O_5) and *trans*(O_6) having opposite absolute configurations (Fig. 1b). Molecular mechanics calculations here demonstated that *SS*-EDDS is stereospecifically coordinated to form exclusively the *trans*(O_5) isomer[3].

In many cases where the number of donor atoms bound to the metal ion is less than six, the tetradentate ligands EBAA permit the possibility of further isomerism[4] (Fig. 1c). The separation and isolation of these geometrical isomers have been carried out either by column chromatography on an ion-exchange resin (Dowex, Amberlite)[5] and cellulose derivatives (Sephadex, Cellex)[6], or by fractional crystallization.

Although a number of metal complex cations have been resolved by chromatographic methods[7,8], few studies of the optical resolution of complex anions have

* Compound abbreviations: EDTA = ethylenediamine-N,N,N′,N′-tetraacetate; IPDTA = isopentanediamine-N,N,N′,N′-tetraacetate (3-methyl-1,2-diaminobutane-N,N,N′,N′-tetraacetate); PHEDTA = 1-phenylethylenediamine-N,N,N′,N′-tetraacetate; DBTA = 2,3-diaminobutane-N,N,N′,N′-tetraacetate; EDDS = ethylenediamine-N,N′-disuccinate; EBV = ethylenebisvaline; EBAA = ethylenebisaminoacidate; en = ethylenediamine; ox = oxalate; asp = aspartate.

0021-9673/88/$03.50

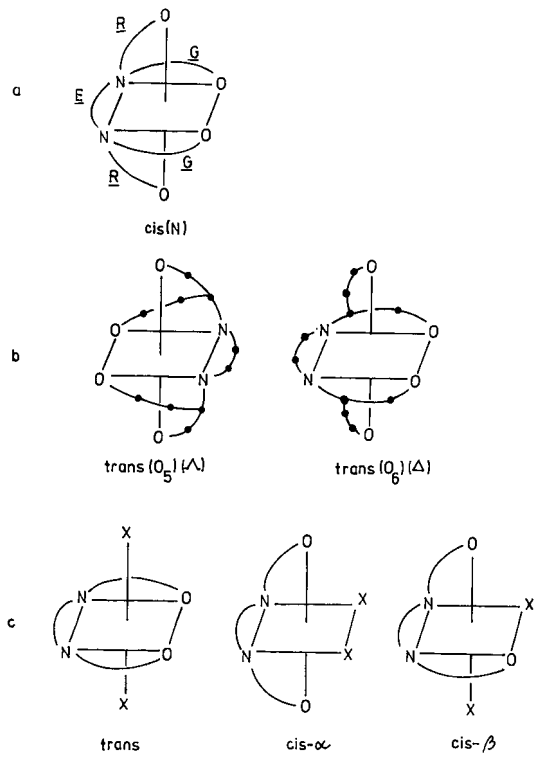


Fig. 1. Geometrical isomerism of six-coordinate complexes including (a) EDTA-type ligands, (b) *SS*-EDDS and (c) a flexible tetradentate EBAA-type ligand.

been reported, because of the lack of effective eluting agents. Tatehate *et al.*[9] have recently reported that when *Λ*-(+)$_{589}$-$[Co(en)_3]Cl_3 \cdot H_2O$ in 30% ethanolic solution is used as the eluting agent, some cobalt(III) aminopolycarboxylate anionic complexes can be resolved by column chromatography on DEAE-Sephadex A-25. Continuing our research in the field of metal ion complexes of amino acid deivatives, we recently reported the partial optical resolution of cobalt(III) chelate cationic complexes by gel permeation chromatography (GPC)[10]. The object of the present study is the rapid optical resolution of anionic metal complexes by GPC on a column of Sephadex G-10.

EXPERIMENTAL

All of the EDTA-type complexes and $[Co(EDDS)]^-$ were prepared by dissolving equimolar amounts of the corresponding racemic ligand, cobalt(II) hydroxide and potassium carbonate in water, followed by oxidation of the resulting solution by hydrogen peroxide in the presence of active charcoal. All the new compounds gave satisfactory elemental analyses. Their stereochemistry was determined by means of ^{13}C NMR, electronic absorption and infrared spectroscopy. The ligands were generally prepared by reactions of the diamines with bromoacetic acid.

$K[Co(rac\text{-}EBV)CO_3] \cdot 1/2\ H_2O$ was prepared by the following procedure

A mixture of 1.3 g (5 mmol) of *rac*-EBV in 5 ml of water and freshly prepared $K_3[Co(CO_3)_3]$ ("green solution"[11]) was stirred for *ca.* 20 min at 50°C. The colour of the solution changed from green to wine-red. After cooling, the reaction mixture was filtered, and, after addition of 5 ml of water, the filtrate was placed on an anion-exchange chromatographic column (25 cm × 3 cm) containing DEAE-Sephadex A-25-120 which had previously been equilibrated in distilled water. The column was eluted with water at a flow-rate of 1 ml/min. Two fractions were collected. The first (60 ml) was deep red, and evaporation of this fraction to dryness on a rotary evaporator gave 1.3 g of the *cis*-α isomer. The second, more strongly adsobed, band was collected in a 30-ml fraction, and evaporation of this purple fraction gave 0.2 g of the *cis*-β isomer. The compounds may be recrystallized without change from water or ethanol. Calc. for $K(CoC_{15}H_{26}N_4O_7) \cdot 1/2\,H_2O$ (M_r = 425.22): C, 36.68; H, 5.23; N, 6.58%. Found for the *cis*-α isomer: C, 37.10; H, 5.26; N, 6.4% found for the *cis*-β isomer: C, 36.72; H, 5.39; N, 6.46%.

Optical resolution

The racemic anionic complex (20–40 mg) dissolved in an appropriate aqueous solution (*ca.* 5 ml) was placed on the top of a column (60 cm × 2 cm I.D.) of Sephadex G-10 and was eluted with water. The elution rate was 0.3–0.4 ml/min. During elution the anionic complex was partially separated into two enantiomers.

Circular dichroism (CD) spectra were recorded on a Jobin-Yvon Dichrograph III. The concentrations of the solutions (10^{-4}–10^{-2} *M*) were determined from their absorption spectra recorded on a Specord UV–VIS spectrophotometer or by atomic absorption spectrometry (AAS).

RESULTS AND DISCUSSION

The resolution of $[Co(EDTA)]^-$ by several methods has been reported: selective adsorption on optically active quartz[12], spontaneous resolution of its ammonium or rubidium salts[2] and use of the resolving agent to form diastereoisomers[13]. The chromatographic technique is very effective to achieve the optical resolution of such anionic complexes[7,9]. The resolution by the column chromatography described of chiral complexes is based on the interactions between a chiral adsorbent and a chiral eluent. Gillard and Spencer[14] isolated anionic and neutral complexes of cobalt(III) with gylcyl-L-histidine by a combination of anion-exchange resin chromatography and GPC on Sephadex G-10. Later, Gillard *et al.*[15] used chromatography on a column of Sephadex G-10 to separate complexes of different charges and/or geometries. When *fac*-$[Co(ox)(L\text{-}asp)(en)]^-$ separated from the corresponding *mer* isomer on a column of Dowex 50W-X8 (H^+) was rechromatographed on a column of Sephadex G-15, its diastereoisomers were separately eluted with water[16].

Continuing our study on the stereochemistry and reactivity of metal complexes with amino acid derivatives, we have used GPC on a column of Sephadex G-10 to resolve some cobalt(III) anionic complexes. The degree of resolution achievable on one passage through the column is exemplified by the data in Table I. The $\Delta\varepsilon$ values obtained for optical isomers of $[Co(EDDS)]^-$ (Fig. 2) can be compared with $\Delta\varepsilon_{max}$ = −2.31 (548 nm)[4] and for those of *cis*-α-$[Co(EBV)CO_3]^-$ or *cis*-β-$[Co(EBV)CO_3]^-$ with $\Delta\varepsilon_{max} = \pm 2.39$ (587 nm) respectively for the pure enantiomers[17].

TABLE I

ABSORPTION (AB) AND CIRCULAR DICHROISM (CD) DATA FOR THE RESOLVED COMPLEXES

ε in l mol^{-1} cm^{-1}.

Complex	*AB*		*CD*	
	λ (nm)	*ε*	*λ (nm)*	*Δε*
Δ-[Co(PHEDTA)]$^-$	550	219	590	−0.26
			525	+0.68
	385	181	390	+0.12
Λ-Isomer	the same values		590	+0.17
			528	−0.23
			420	+0.07
			360	+0.06
Δ-trans-(O_5)-[Co(EDDS)]$^-$	523	180	610	−0.07
			540	+0.56
			472	−0.12
	384	69	420	−0.21
Λ-Isomer	the same values		609	+0.09
			540	−0.42
			490	+0.24
			413	+0.20
Δ-cis-α-[Co(EBV)CO_3]$^-$	549	65	595	−1.48
			493	+0.41
	394	92	418	−0.12
			370	−0.06
Λ-Isomer	the same values		595	+1.04
			490	−0.20
			420	+0.07
			373	+0.04
Δ-cis-α-[Co(EBV)CO_3]$^-$	546	79	587	−1.23
			485	+0.11
	390	110	420	−0.09
			363	−0.04
Λ-Isomer	the same values		587	+0.86
			483	−0.06
			420	+0.04
			362	+0.03
Δ-[Co(IPDTA)]$^-$	546	279	578	−0.13
			490	+0.31
	385	203	390	−0.15
			362	−0.08
Λ-Isomer	the same values		580	+0.08
			490	−0.21
			390	+0.23
			360	+0.24
Δ-[Co(DBTA)]$^-$	546	310	620	−0.21
			575	+0.14
			465	−0.11
	383	258	400	+0.09
Λ-Isomer	the same values		620	+0.08
			570	−0.10
			463	+0.07
			390	−0.07

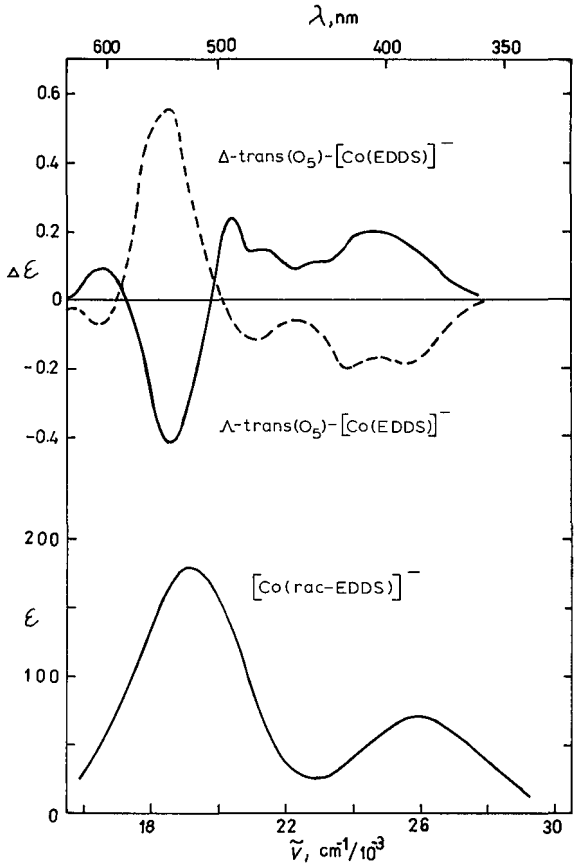


Fig. 2. CD and absorption spectra for $[Co(EDDS)]^-$.

Some observations can be made on the resolution of these complexes on the molecular sieve Sephadex G-10. It is interesting that, with all the complexes studied, the *Δ*-enantiomers have substantially greater optical purity (see Table I). The

TABLE II

CORRELATION BETWEEN THE SIGN OF THE CD PEAK OF THE FIRST ELUTED ENANTIOMERS AND THEIR ABSOLUTE CONFIGURATIONS

Isomer	*Sign of the longer wavelength CD peak*	*Absolute configuration*
$[Co(EDDS)]^-$	+	*Λ*
$[Co(PHEDTA)]^-$	−	*Δ*
$[Co(IPDTA)]^-$	−	*Δ*
$[Co(DBTA)]^-$	+	*Λ*
cis-α-$[Co(EBV)CO_3]^-$	+	*Λ*
cis-β-$[Co(EBV)CO_3]^-$	+	*Λ*

relationship between absolute configurations and elution order may be seen from Table II. In spite of the results from optical resolution of the cationic $[CoN_4O_2]^+$-type complexes[10], where the enantiomers with the *Λ*-configuration are eluted first, it is difficult to find a simple relationship between the elution order and the absolute configuration of the anionic complexes $[CoN_2O_4]^-$.

The electronic absorption and CD data for the complexes studied are summarized in Table I. Two adsorption bands were observed for each compound, suggesting an effectively cubic ligand field, but their CD curves show three components for each band in the case of the EDDS complex (Fig. 2) or two components for the cobalt(III) complexes with EDTA analogues (Fig. 3), respectively. Contributions to the CD spectra observed for these octahedral $[CoN_2O_4]^-$ complexes arise from (i) the configurational effect due to the arrangement of chelate rings around the cobalt(III) ion, (ii) the conformational effect (of the E chelate ring mainly), (iii) the vicinal effect of asymmetric carbon atoms which has the opposite sign to that for the asymmetric nitrogen.

From a comparison of the data in Table I and CD curves (see Figs. 2 and 3), the degree of optical resolution is better for EDDS and EBV complexes than for EDTA-type complexes. This is due to the fact that both EDDS and EBV ligands possess optically active centres at the coordinated nitrogen donor atoms as well as at the asymmetric carbon atoms, whereas alkyl and aryl substituted EDTA analogues

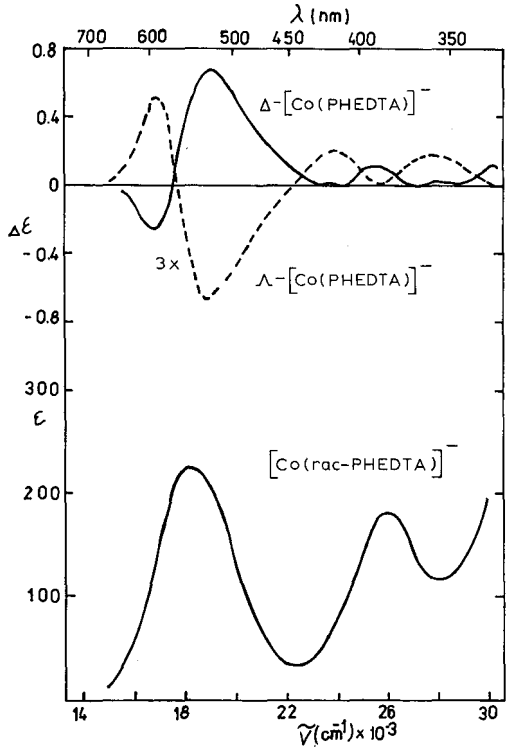


Fig. 3. CD and absorption spectra for $[Co(PHEDTA)]^-$.

possess optically active centres only at the asymmetric carbon atoms. Since the resolution by the mentioned column chromatography of chiral complexes is based on the interaction between a chiral adsorbent and a chiral complex, the chiral discriminations (chirodiastaltic interactions) between the chiral adsorbent and a chiral complex are greater for EEDS and EBV complexes. In contrast with the structural chirality of quartz surfaces, the chirality for the polydextrans seems to be intrinsic and local due to the presence of the C–O–H bond in the glucose ring[18]. Consequently, differential adsorption of the two enantiomers may be achieved.

This chromatographic method can be used to obtain partial resolution of racemic amino acid and related compounds in the form of the cobalt(III) complexes. The separation of optical isomers on the molecular sieve Sephadex G-10 is convenient and appears to be generally applicable for the partial resolution of non-labile chelate compounds. The method is rapid (several hours) and simple in operation (gravity flow with water as an eluent), and the molecular sieve in the column appears to be stable for several years.

The method should serve as a diagnostic tool for the identification of isomers and should permit the study of the optical activity of complexes that resist resolution.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. F. Pavelčík for supplying *rac*-EDDS and *rac*-DBTA and Dr. V. Novák for supplying *rac*-IPDTA.

REFERENCES

1 K. Igi, M. S. Urdea and J. I. Legg, *Inorg. Chem.*, 20 (1981) 3208 and references therein.
2 H. A. Weakliem and J. L. Hoard, *J. Am. Chem. Soc.*, 81 (1959) 549.
3 F. Pavelčík and J. Majer, *Collect. Czech. Chem. Commun.*, 43 (1978) 239.
4 D. J. Radanovič, *Coord. Chem. Rev.*, 54 (1984) 159.
5 F. Jursík, in Z. Deyl, K. Macek and J. Janák (Editors), *Liquid Column Chromatography*, Elsevier, Amsterdam, 1975, Ch. 51.
6 Y. Yoshikawa and K. Yamasaki, *Coord. Chem. Rev.*, 28 (1979) 205.
7 H. Yoneda, *J. Chromatogr.*, 313 (1984) 59.
8 H. Krebs, *Forschungsberichte des Wirtschafts und Verkehrsministerium Nordrhein Westfalen No. 270*, Westdeutscher Verlag, Köln, Opladen, 1956.
9 A. Tatehata, M. Fujita, K. Ando and Y. Asaba, *J. Chem. Soc., Dalton Trans.*, (1987) 1977.
10 M. Strašák and S. Bystrický, *J. Chromatogr.*, 403 (1987) 331.
11 M. Shibata, *Modern Synthesis of Cobalt(III) Complexes*, Akademie-Verlag, Berlin, 1983, p. 69.
12 D. H. Busch and J. C. Bailar, Jr., *J. Am. Chem. Soc.*, 75 (1953) 4574.
13 W. T. Jordan, B. J. Brennan, L. R. Froebe and B. E. Douglas, *Inorg. Chem.*, 12 (1973) 1827.
14 R. D. Gillard and A. Spencer, *J. Chem. Soc., Dalton Trans.*, (1972) 902.
15 R. D. Gillard, S. H. Laurie, D. C. Price, D. A. Pipps and C. F. Weick, *J. Chem. Soc., Dalton Trans.*, (1974) 1385.
16 M. Takeuchi and M. Shibata, *Bull. Chem. Soc. Jpn.*, 47 (1974) 2797.
17 F. Jursík and S. Abdel-Moez, *Collect. Czech. Chem. Commun.*, 52 (1987) 1488.
18 E. Alvira, L. Vega and C. Girardet, *Chem. Phys.*, 118 (1987) 233.

PUBLICATION SCHEDULE FOR 1988

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	435/1 435/2 435/3 436/1	436/2 436/3	437/1 437/2	438/1 438/2	439/1 439/2 440 441/1	441/2 442 443	444 445/1 445/2 446	447/1 447/2 448/1	448/2 448/3 449/1	449/2 450/1 450/2 450/3 452	The publication schedule for further issues will be published later.	
Bibliography Section		460/1		460/2		460/3		460/4		460/5		460/6
Cumulative Indexes, Vols. 401–450												451
Biomedical Applications	424/1	424/2	425/1 425/2	426/1 426/2	427/1	427/2 428/1	428/2 429	430/1	430/2 431/1	431/2	432	433 434

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

Dispatch. Before sending the manuscript to the Editor please check that the envelope contains three copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Notes and Letters to the Editor will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

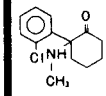
Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.

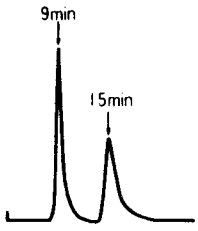
Chiral HPLC columns for optical resolution

CHIRALPAK
CHIRALCEL

●Examples of New chiral HPLC columns●

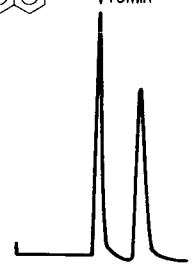
CHIRALCEL CA-1





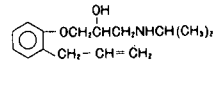
Column : 4.6mm ID × 250mm
Eluent : ethanol-H_2O (95 : 5)
Flow rate : 0.5ml/min
Detection : UV254nm

CHIRALCEL OD

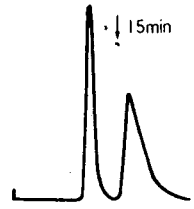


Column : 4.6mm ID × 250mm
Eluent : hexane-2-propanol-diethylamine (80 : 20 : 0.1)
Flow rate : 0.5ml/min
Detection : UV254nm

CHIRALCEL OD

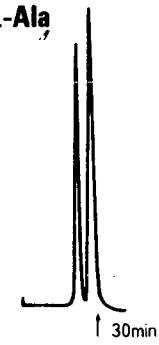


Sample : 150mg



Column : 20mm ID × 500mm
Eluent : hexane-2-propanol-diethylamine (80 : 20 : 0.1)
Flow rate : 12ml/min
Detection : UV254nm

CHIRALPAK WE



Column : 4.6mm ID × 250mm
Eluent : aq 0.25mM $CuSO_4$
Flow rate : 1.0ml/min
Detection : UV230nm Temp. 35°C

Name	Separation
CHIRALPAK OT(+) CHIRALPAK OP(+)	Compounds possesing aromatic group (see : Technical Brochure No.1)
CHIRALCEL OA CHIRALCEL OB CHIRALCEL OC CHIRALCEL OD CHIRALCEL OK CHIRALCEL CA-1	(see : Technical Brochure No.1, No. 3)
CHIRALPAK WH CHIRALPAK WM CHIRALPAK WE	DL-Amino acid or its derivative (see : Technical Brochure No. 2, No. 4)

■Size of column

Analytical Column	4.6mm (I.D.) × 250mm (L)
Precolumn	4.6mm (I.D.) × 50mm (L)
Semipreparative column	10mm (I.D.) × 250mm or (500mm)
	20mm (I.D.) × 250mm or (500mm)

■Preparation of pure enantiomer (~kg) with Chiral column are acceptable.

Please contact!

DAICEL CHEMICAL INDUSTRIES, LTD.

Tokyo
8-1, Kasumigaseki 3-chome,
Chiyoda-ku, Tokyo 100, Japan
Phone : 03(507)3151, 3189
Telex : 222-4632 DAICEL J
FAX : 03(507)3198

New York
Pan-Am Bldg., 200 Park Avenue,
New York, N.Y. 10166-0130, U.S.A.
Phone : (212)878-6765, 6766
Telex : (23)236154 DCC UR
FAX : (212)682-6632

DAICEL (U.S.A.), INC.
611 west 6th Street, Room 2152
Los Angeles California 90017, U.S.A.
Phone : (213)629-3656
Telex : 215515 DCIL UR
FAX : (213)629-2109

DAICEL (EUROPA) GmbH
Königsallee 92a.
4000 Düsseldorf 1, F.R. Germany
Phone : (0211)134158
Telex : (41)8588042 DCEL D
FAX : (0211)879-8329

11×83/