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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF OPTICALLY ACTIVE METABOLITES AND DRUGS ON A NOVEL CHIRAL STATIONARY PHASE

HARTMUT FRANK, GRAEME J. NICHOLSON and ERNST BAYER

Institut für Toxikologie, Wilhelmstrasse 56 and Institut für Organische Chemie, Auf der Morgenstelle 18, 74 Tübingen (G.F.R.)

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SUMMARY

Chirasil-Val, a novel chiral polysiloxane-type stationary phase is capable of separating the enantiomers of optically active drugs and metabolites of several compound classes; α-amino acids, α-amino alcohols, glycols, aromatic and aliphatic α-hydroxy carboxylic acids and amines. Due to their high thermal stability, columns coated with Chirasil-Val may be coupled to a mass spectrometer. Potential applications of the new stationary phase include analysis of the optical purity of enantiomeric drugs, determination of the configuration of metabolites, and quantitation of optically active drugs and metabolites using the unnatural enantiometer as internal standard. Direct separation of enantiomers on Chirasil-Val is especially useful if only minute amounts of the optically active compounds are available for analysis.

INTRODUCTION

Stereoselectivity is an inherent phenomenon of biological systems. In most cases optical enantiomers exhibit different biological activities due to differences in strength of interaction with the corresponding receptor, different transport mechanisms and metabolism along different pathways [1]. Well known examples are the sympathomimetic α-phenyl-β-aminoalcohols, e.g. ephedrine, epinephrine, phenylephrine and others [2]. L-DOPA is rapidly absorbed and extensively metabolized in intestinal tissues, whereas D-DOPA is absorbed more slowly and not metabolized at all [3]. The D-enantiomer of penicillamine is applied in cases of cystinuria and mercury poisoning, the L-enantiomer is toxic [4]. L-Lactic acid is a normal constituent of human urinary samples, whereas presence of D-lactic acid signals metabolic disorder [5]. Only the L-enantiomer of N-phthaloyl glutamic acid, a metabolite of thalidomide, is teratogenic [6].

Basically, gas chromatographic (GC) analysis of optically active compounds

from biological samples can be performed in two ways: by derivatization with a pure enantiomer of an optically active reagent and separation of the resulting diastereomers on a nonchiral stationary phase [7, 8], or by direct separation of the enantiomers on a chiral stationary phase [9, 10].

Analysis via the detour of diastereomers has four principal shortcomings: (a) choice of derivatization agents is limited by the requirement of optical activity, (b) the derivatization reagent must be optically pure, (c) differences in the reaction kinetics of derivatization between two enantiomers leads to systematic errors in quantitative analysis and (d) enhanced probability of racemization at one of the asymmetric carbons during derivatization increases the error of quantitative determinations.

Direct separation of enantiomers on a chiral stationary phase offers the advantage that reagents commonly used for derivatization in GC may be applied. The two enantiomers to be separated interact with the chiral stationary phase via hydrogen bonds to form diastereomeric association complexes with differences in their solvation enthalpy of several hundred calories [11].

The low temperature range over which the hitherto known chiral compounds are suitable as GC stationary phases prevented the general use of the direct separation of enantiomers. Most of the phases have melting points of 80—100° and only a few are of sufficiently low volatility at temperatures above 130°. At elevated temperatures such columns show increased bleeding and deteriorate rapidly also combination with a mass spectrometer is unsatisfactory due to a high background in the mass spectra.

We recently synthesized a chiral stationary phase, referred to below as Chirasil-Val [12, 13], possessing low volatility and high thermal stability by coupling L-valine *tert.*-butyl-amide to a copolymer of dimethylsiloxane and carboxy-alkyl-methyl-siloxane units of appropriate viscosity and molecular weight. Chirasil-Val opens up a new temperature range for the analysis of optical isomers and consequently additional compounds of low volatility are now amenable to this method. Also, Chirasil-Val offers, for the first time, the possibility of coupling a mass spectrometer to a GC column capable of separating optical enantiomers.

EXPERIMENTAL

Preparation of capillaries

Capillaries of 0.3 mm I.D. made from borosilicate glass are dynamically coated with 0.5% colloidal silicic acid in acetone [14] and dried thoroughly at 200—250° with carrier gas flowing. They are coated with Chirasil-Val in methylene chloride by the static method [15], conditioned by programming to 210° with a slow hydrogen stream and kept there overnight.

In order to ensure that deactivation is always complete and to elute high boiling compounds, the capillaries are routinely kept at 200° overnight. Under these conditions the average life span of a column exceeds 6 months.

Esterification of carboxyl groups

A 500-μg amount of carboxylic acid is placed in a screw-cap vial, dissolved in 200 μl 2 *N* hydrogen chloride in anhydrous isopropanol and heated to

90° for 30 min. If the carboxyl group is sterically hindered (e.g. valine) 1 h is preferable. Excess reagent is removed under a gentle stream of nitrogen with moderate heating.

N, O, S-Acylation with pentafluoropropionic anhydride

A 250-μl volume of ethyl acetate and 50 μl pentafluoropropionic anhydride are added to the sample in a heavy-walled screw-cap vial. Air is displaced with nitrogen and the mixture is heated to 110° for 10 min. Excess reagent and solvent are removed with a gentle stream of nitrogen. The dry residue is dissolved in an appropriate volume of methylene chloride.

O-Pentafluoropropionyl lactic acid cyclohexylamide

A 1-mg amount of lactic acid (D or L) is dissolved in 100 μl methanol and 100 μl methylene chloride. An ethereal solution of diazomethane (or diazomethane gas) is added until the solution stays yellow for 2 min. Most of the solvent is evaporated, the concentrated solution is diluted with 100 μl methanol and 5 μl of cyclohexyl amine are added. After 30 min at 50° the solvent is removed under a gentle stream of nitrogen and the residue is O-acylated as described above.

Analysis of penicillamine

Penicillamine is desulfurized with Raney nickel according to ref. 16 and derivatized as described above, resulting in N-pentafluoropropionyl valine isopropylester.

GC is performed on a Carlo Erba Model 2101 gas chromatograph with glass capillaries 20 m × 0.3 mm coated with Chirasil-Val, film thickness ca. 0.1 μm. Hydrogen is used as carrier gas, inlet pressure 0.35 bar. Other conditions as given in the corresponding legend.

Peak areas are measured with the electronic integrator Spectra Physics System I.

GC—mass spectrometry is performed on a Varian Model 2740-1 gas chromatograph interfaced to a mass spectrometer Varian MAT 711 by an open split. The mass spectrometer is set to a resolution of approximately 1000; ionizing voltage 70 eV; ionizing current 0.8 mA; multiplier voltage as given in the mass spectra; interface temperature 220°; ion source temperature 220°.

RESULTS AND DISCUSSION

Chirasil-Val was synthesized primarily for the purpose of analyzing the optical purity of amino acids from synthetic peptides, but it is well suited to the separation of enantiomers of a large number of optically active compounds occurring in biological systems.

The general structure of the phase is given in Fig. 1. L-Valine *tert.*-butylamide is linked to the polysiloxane via the highly stable carboxamide group. Each chiral moiety is separated from the next by approximately 7 dimethylsiloxane units, thus avoiding interaction between neighbouring valine residues by hydrogen bonding. This seems to be an important factor for resolution and thermal stability. Phases with a smaller number of "diluting" dimethyl-

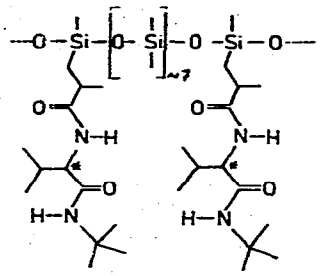

Fig. 1. Structure of the chiral stationary phase Chirasil-Val.

siloxane groupings were thermally less stable and, due to high melting temperatures, unsuitable as stationary phases. The phases synthesized so far can be used over a temperature range from 70° to 230°, for a short period even to 250°. Racemization of the chiral moiety is tolerable at 230°; after 24 h at this temperature approximately 3% of L-valine is racemized.

For derivatization we prefer perfluoroacyl derivates [17] whenever feasible, i.e. for amines, alcoholic and phenolic hydroxyl groups and thiols. These are among the most volatile derivatives used in GC. This is of importance, since the free enthalpy-differences decrease with increasing temperature.

In Fig. 2, the separation of the N, O-pentafluoro-propionyl derivatives of a number of sympathomimetic drugs and epinephrine metabolites is shown. Baseline resolution for most of the compounds is achieved but resolution factors vary considerably. They are mainly dependent upon the functional

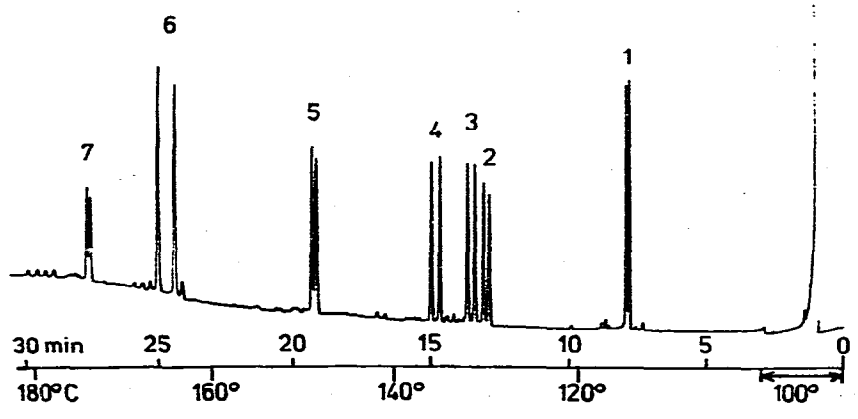

Fig. 2. GC separation of the enantiomers of several sympathomimetic drugs and epinephrine metabolites as N, O-pentafluoropropionyl-derivatives. Chromatographic conditions: 20 m × 0.3 mm Chirasil-Val, injector and detector 220°, carrier gas hydrogen 0.38 bar. Peaks: 1 = phenyl-2-methylamino-propanol (ephedrine), 2 = 1-(4'-hydroxyphenyl)-2-methylamino-propanol (suprifen), 3 = 3'-methoxy-4'-hydroxyphenylglycol, 4 = 1-phenyl-2-aminoethanol, 5 = 1-(3'-methoxy-4'-hydroxyphenyl0-2-methylaminoethanol (metanephrine), 6 = 3,4-dihydroxyphenylalanine isopropyl ester (DOPA), 7 = 1-(3',4'-dihydroxyphenyl)-2-amino-ethanol (norepinephrine). In all cases investigated the D-enantiomer is eluted prior to the L-enantiomer.

groups and their relative location within the molecule. Amino acids, as N-pentafluoro-propionyl amino acid isopropylesters, exhibit the highest resolution factors. Hydrogen-donating amino groups and hydrogen-accepting carbonyl groups of the N-acyl and ester moiety are in optimal sequence and similar spatial relationship for "fitting" onto the "hydrogen-bonding-matrix" of the stationary phase. The amino acid DOPA therefore exhibits one of the largest resolution factors. Enantiomers of the 1-phenyl-2-amino alcohols are not as well separated due to the dissimilar spatial relationship of the hydrogen-donating and accepting moieties of solute and solvent. If the amino group carries an additional substituent, no hydrogen bond can be formed at this site after acylation, and one point of an optimal "three-point-fit" is lost, with consequent reduction in the resolution factors. The same applies to the phenylglycols.

As already indicated, the enantiomers of optically active drugs and metabolites often differ greatly in their efficacy and activity. D-Ephedrine for instance is the isomer with the highest pressor-activity. If D-ephedrine racemizes at the asymmetric carbon adjacent to the amino group the depressor-active D-pseudoephedrine is produced. Clearly, an ephedrine preparation which contains significant amounts of D-pseudoephedrine may falsify the results of a study of its adrenergic activity. In such cases Chirasil-Val offers the possibility of analyzing the optical purity in a simple, time-saving manner with a high degree of sensitivity and accuracy.

A further example investigated was D-penicillamine; since this drug is administered often in doses of up to 1 g per day, an impurity even of a fraction of a percent of the toxic L-enantiomer may be hazardous. We therefore developed a method for analysis of the optical purity of this compound.

In order to test the linearity and accuracy of the method, samples of D-penicillamine are spiked with amounts of 0—10% of L-penicillamine and converted to valine with Raney-nickel [16]. The enantiomers are separated isothermally at 110° with a resolution factor $\alpha_{L/D}$ of 1.134. In Fig. 3 the percentage of L-penicillamine found is plotted against the amount of L-penicillamine added. Each value represents the mean of five determinations within the indicated standard deviations. The line obtained by the least square method has a slope of 0.9945 indicating a nearly perfect correlation of values-found with amount-spiked. The intercept of 0.55% is in very good agreement with the value obtained for the sample not spiked: 0.56 ± 0.04%. Percentages of L-penicillamine present in different preparations of D-penicillamine are listed in Table I.

In principle, the determination of the enantiomer-ratio by GC alone may be falsified by a compound with the same retention time as one of the enantiomers. This may occur especially in the analysis of a component in a drug formulation containing other additives. In such a case the reliability of the analysis is greatly improved by mass fragmentography, monitoring an ion typical for the compound to be quantitated. Due to its low volatility, Chirasil-Val is well suited for combination with a mass spectrometer. Fig. 4a shows the electron impact mass spectrum of 2.4 ng of L-DOPA, esterified, N, O-acylated and chromatographed isothermally at 180°. Fig. 4b shows the background mass spectrum of Chirasil-Val shortly after emergence of the DOPA

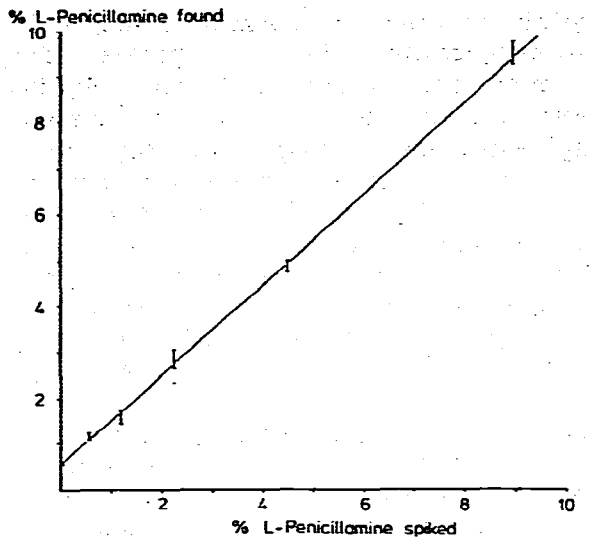

Fig. 3. Quantitative determination of L-penicillamine impurities in D-penicillamine spiked with differing amounts of L-penicillamine ($n = 5$).

TABLE I

DETERMINATION OF L-PENICILLAMINE IN D-PENICILLAMINE

Sample	L-Enantiomer (%)	Standard deviation ($n = 6$)
1	0.57	0.05%
2	0.56	0.04%
3	0.54	0.06%
4	0.35	0.08%
5	0.52	0.05%

peak. The background ions are mainly those expected for a poly-dimethylsiloxane.

For analysis of L-DOPA, the base peak at *m/e* 472 is chosen. In Fig. 5 an actual quantitation of the enantiomer ratio of a commercial L-DOPA is presented. For quantitative analysis by mass fragmentography especially in the picogram range, absence of disturbing background ions is a prerequisite. Chirasil-Val possesses only a few background ions of a very low intensity above *m/e* 360. Quantitation of minute amounts of L-DOPA by mass fragmentography therefore is feasible, as demonstrated in Fig. 6, depicting the mass fragmentogram of 30 pg of L-DOPA with a signal-to-noise ratio of 10.

Other compounds amenable to separation on Chirasil-Val are asymmetric amines, glycols and α-hydroxy carboxylic acids. For the latter, conversion to the corresponding ester often leads to insufficient separation of the enantiomers. This is due to the absence of a nitrogen-attached hydrogen which is

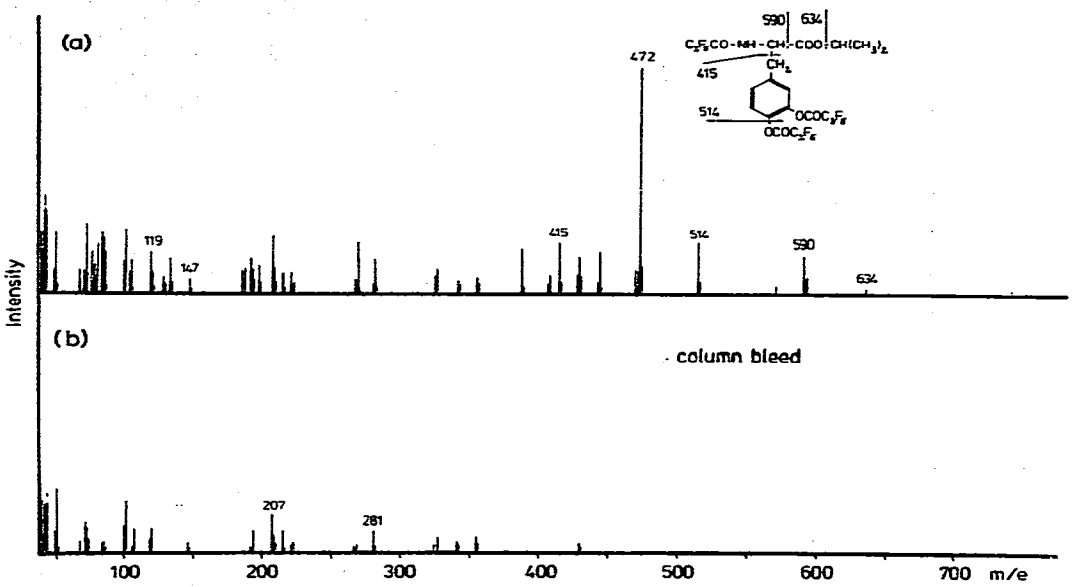

Fig. 4. (a) Electron impact mass spectrum of 2.4 ng commercial L-DOPA, converted to the isopropylester and N, O-pentafluoropropionyl derivative injected on a capillary 20 m × 0.3 mm Chirasil-Val, 180° isothermal, carrier gas Helium, 0.32 bar, mass spectrometer Varian-Mat 711, interface 220°, ion source 220°, 70 eV, multipl. volt 2.4 kV. (b) Background mass spectrum of Chirasil-Val, 2 min after emergence of L-DOPA. Conditions as in (a).

shown to be important for generation of a strong diastereomeric association complex between solvent and solute. If, however, the carboxylic acids are converted to the corresponding O-pentafluoropropionyl carboxylic amides, astonishingly high resolution factors are achieved. In Fig. 7 the separation of the enantiomers of the O-pentafluoropropionyl lactic acid cyclohexylamide is shown. The resolution factor for this simplest α-hydroxy carboxylic acid is in the same range as those achieved for α-amino acids on Chirasil-Val. As is the case for amino acids, the D-form of lactic acid is eluted prior to the L-enantiomer.

Another powerful application of such optically active stationary phases is the quantitation of optically active metabolites in biological samples by GC. Often the choice of a proper internal standard presents difficulties, and incomplete derivatization or decomposition of the derivative lower the accuracy of determination. Metabolites are usually present as only one enantiomer. By adding the optical enantiomer as internal standard [18], all the problems arising from incomplete recovery, incomplete derivatization, hydrolysis, thermal decomposition and shifting response factors are eliminated. The internal standard has the same solubility, reactivity and chemical stability as the compound to be quantitated. The mass spectra of both internal standard and compound to be analyzed are identical, which is of importance for selected ion recording.

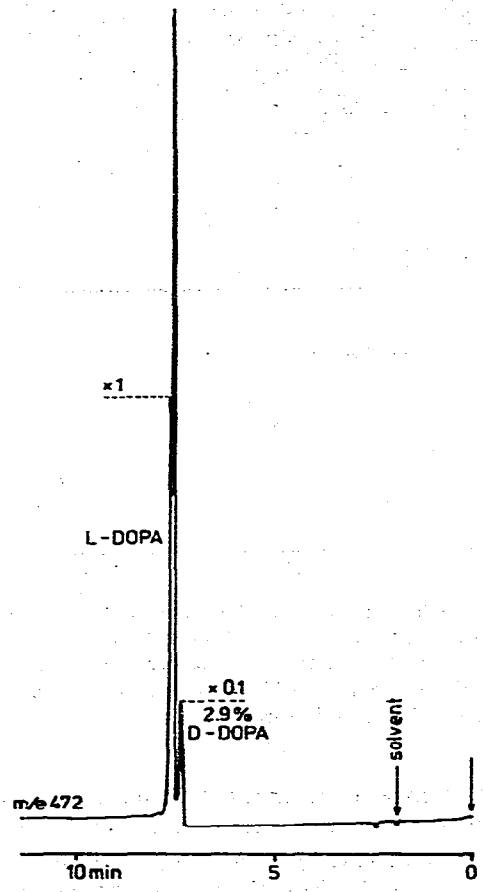

Fig. 5. Selected ion recording of 10 ng L-DOPA, chromatographed on a capillary 16.5 m × 0.3 mm Chirasil-Val at 190°. Other conditions as in Fig. 4.

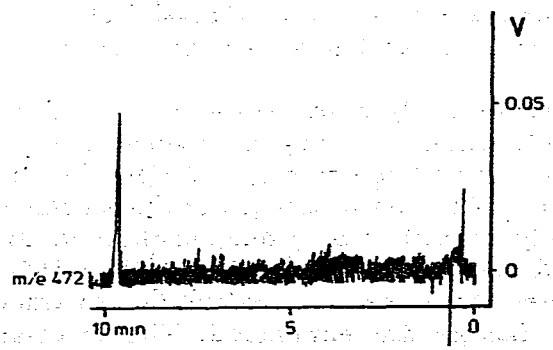

Fig. 6. Selected ion recording of 30 pg L-DOPA, chromatographed on a capillary 16.5 m × 0.3 mm Chirasil-Val. Mass spectrometer Varian MAT 711, sensitivity 0.01 V/cm, filter 5, multipl. volt. 2.6 kV. Other conditions as in Fig. 4.

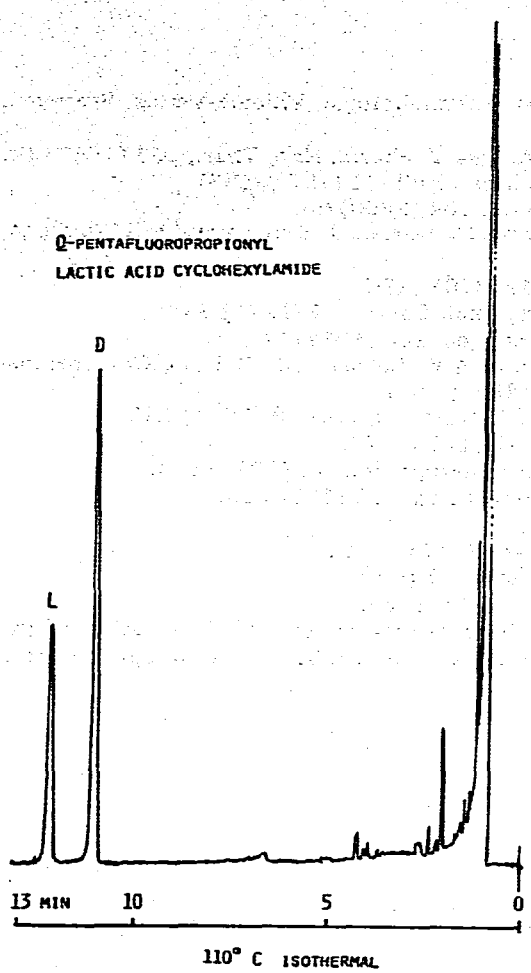

Fig. 7. Gas chromatographic separation of the enantiomers of O-pentafluoropropionyl lactic acid cyclohexyl amide. Conditions: 20 m × 0.3 mm Chirasil-Val, injector and detector 220°, carrier gas hydrogen 0.3 bar, 110° isothermal.

CONCLUSIONS

Chirasil-Val proves to be a valuable stationary phase for the separation of optical isomers and quantitative determination of the optical purity of various classes of enantiomeric drugs and metabolites by GC. The stationary phase is well suited for combination with mass spectrometry. Further, Chirasil-Val may be applied to the assignment of configurations to optically active metabolites and their quantitation using the unnatural enantiomer or the racemic mixture as an internal standard.

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