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Journal of Chromatography A, 828 (1998) 191–198

JOURNAL OF CHROMATOGRAPHY A

Determination of the absolute configuration of α-hydroxyglycine derivatives by enzymatic conversion and chiral high-performance liquid chromatography

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Abstract

We describe an approach for facile determination of the absolute configuration of enantiomerically chromatographed racemates by combining enzymatic conversion and chiral chromatography. The method involves initial rapid development of chiral HPLC methods using polar organic eluents with polysaccharide chiral phases. We present here evidence for using the stereospecific peptidylamidoglycolate lyase (PGL, E.C. 4.3.2.5) to determine the absolute configuration of α-hydroxyglycine derivatives. The racemic solute was incubated with PGL, lyophilized and then enantiomerically chromatographed using the CHIRALPAK®AD™ column. Based on the specificity of the enzyme reaction, the unreacted enantiomer was assigned the absolute configuration *R*.

Keywords: Enantiomer separation; Absolute configuration; α-Hydroxyglycine derivatives; Peptidylamidoglycolate lyase (PGL)

1. Introduction

An increased understanding of the stereoselectivity of catalytic proteins and an enhanced awareness of the detrimental effects of the presence of unreactive enantiomers have led to an increased demand for the use of enantiomerically pure drugs. This is evident from the recent dramatic increase in the number of chiral drugs marketed as single enantiomers. Before 1990 there were few examples of drugs developed as pure enantiomers, though the majority of the synthetic drugs developed were chiral [1]. In 1997 the number had increased to about 51% of synthetic drugs being developed as enantiomerically pure while about 17% were still marketed as racemates [2]. The issue of chirality has spread from drug development to other areas such as food additives and agrochemicals where synthetic compounds are developed to interact with the biological environment [3].

The development of economical and efficient methods for obtaining enantiomerically pure compounds has arisen from the increased concern for the potentially adverse effects of racemic mixtures. High-performance liquid chromatography (HPLC) has proven useful for resolving racemates and for determining the optical purity of enantiomers and their absolute configuration. HPLC has, therefore, become the method of choice. There are three main strategies for chiral resolution by HPLC that have evolved [4,5]: (i) derivatization of the analytes with chiral reagents to form resolvable diastereomeric derivatives [6]; (ii) use of chiral additives in the mobile phase [7,8]; and (iii) development of chiral

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00651-7

stationary phases [9–12]. Chiral stationary phases (CSPs) permit direct resolution of pure enantiomers on both an analytical and a preparative scale. Recovery of the resolved analytes can be difficult when using derivatization of the analytes or chiral additives in the mobile phase, whereas CSPs elute pure enantiomers directly from the column and thus eliminate the recovery process. A large number of CSPs have become commercially available for specific and for general purposes. A class of CSPs that have attracted a lot of attention consists of chiral polysaccharide derivatives adsorbed to a porous silica gel. The polysaccharide derivative based CSPs have proven to be very effective in resolving a wide range of racemates, including aromatic hydrocarbons, amines, carboxylic acids, alcohols, amino acid derivatives, and many commercially available drugs [10–12].

The determination of the absolute configuration of chiral molecules has historically required laborious methods such as determination of the X-ray crystal structures of diastereomeric derivatives made with a chiral derivatizing agent of known configuration [13]. Other widely used methods involve determining the NMR of diastereomeric derivatives or diastereomeric complexes. When diastereomeric derivatives are formed a difference in the chemical-shift is induced which is structure dependent [14,15]. A similar effect is achieved when forming diastereomeric complexes with a chiral solvent [16] or with a chiral lanthanide complex [17]. Methods such as optical rotatory dispersion and circular dichroism are limited because in order to provide information about the absolute configuration they require comparison against enantiomeric standards of closely related analogs [18]. The traditional methods for determination of absolute configuration are very sensitive to the purity of the enantiomer — small impurities can prevent crystal formation, preclude definitive proton assignments, or prohibit optical signals. More recently, various HPLC-based methods have been developed which are analogous to the HPLC-based methods developed for separating the enantiomers. Many of the HPLC-based methods are limited either by requiring derivatization [19,20] or comparison against enantiomeric standards [21,22]. A more direct approach involves utilizing enzymes of known stereospecificity. We present here a method utilizing

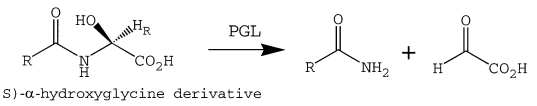

Fig. 1. PGL catalyzed conversion of a (*S*)-α-hydroxyglycine derivative to form the corresponding amide product and glyoxylate.

stereoselective enzyme catalysis by peptidylamidoglycolate lyase (PGL, E.C. 4.3.2.5) to determine the absolute configuration of enantiomers of α-hydroxyglycine derivatives, which have been resolved by chiral chromatography. PGL is a mammalian enzyme, which mediates the final step in the activation of glycine-extended peptides by catalyzing the cleavage of a C–N bond to produce the bioactive amidated peptide and glyoxylate (Fig. 1). PGL is stereospecific towards the *S*-configuration at the α-carbon of the α-hydroxyglycine [23]. Our method involves incubating PGL with a racemic mixture followed by enantiomeric resolution on a CHIRALPAK®AD™ column. A key aspect of our method is that it can be used to determine absolute configuration without using enantiomeric standards of the α-hydroxyglycine analytes. We demonstrate the applicability of this method to a whole series of acyl α-hydroxyglycine derivatives, and we anticipate that the method is easily applicable to a wide variety of enzyme systems.

2. Materials and methods

2.1. Materials

Cinnamoyl α-hydroxyglycine, 1-naphthylacetyl α-hydroxyglycine, phenylacetyl α-hydroxyglycine, 3-phenylpropionyl α-hydroxyglycine, 4′-methoxyphenylacetyl α-hydroxyglycine, and 4′-nitrophenylacetyl α-hydroxyglycine were synthesized in our laboratory by Dr. Dongsheng Ping [24,25]. Trifluoroacetic acid (TFA), perchloric acid ($HClO_4$), HPLC grade isopropanol (IPA), ethanol (EtOH) and acetonitrile (MeCN) were purchased from Fisher Scientific (Pittsburgh, PA, USA) whereas HPLC grade hexane was purchased from J.T. Baker (Phillipsburg, NJ, USA). Cinnamamide and benzamide were purchased from Aldrich (Milwaukee, WI,

USA). *N*-benzoyl α-hydroxyglycine and sodium 2-(*N*-morpholino)ethanesulfonate (MES–Na buffer) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Enzyme purification

PGL was purified from an Sf9/BEV expression system as described by Moore [26].

2.3. Enzyme assays

Assays were performed in 150 m*M* MES–Na buffer, pH 7.0, containing racemic, purified *R*- or *S*-enantiomer of cinnamoyl α-hydroxyglycine and enzyme in a total volume of 170 μl at 37°C. After 40 min. the reaction was quenched by the addition of 20 μl 3 *M* $HClO_4$. The quenched reaction mixture was analyzed quantitatively for cinnamamide by injection without prior filtration onto reversed-phase HPLC. HPLC analyses were performed at room temperature using a LDC Constametric III system equipped with a LDC Spectromonitor 3100 variable wavelength detector and a Rheodyne 7125 injection valve (20 μl loop). Separations were achieved on a Spherisorb C8 5 micron reversed-phase column (250×4.6 mm, Alltech, Deerfield, IL, USA) fitted with an ALL-guard C8 5 micron guard column (7.5×4.6 mm, Alltech, Deerfield, IL, USA). The mobile phase was 30% (v/v) acetonitrile, 70% (v/v) deionized water, and 0.1% (v/v) TFA. The column was operated at a flow-rate of 1.5 ml min^{-1}. Cinnamoyl α-hydroxyglycine and cinnamamide were detected at 278 nm. The cinnamoyl α-hydroxyglycine eluted at 2.5 min and the cinnamamide eluted at 3.8 min.

2.4. Absolute configuration by chiral chromatography

Assays were performed in 150 m*M* MES–Na buffer, pH 7.0, containing 7 m*M* cinnamoyl α-hydroxyglycine or 5 m*M* *N*-benzoyl α-hydroxyglycine and enzyme in a total volume of 3 ml at 37°C. Aliquots (20 μl) were withdrawn from the incubation mixtures and analyzed qualitatively for amide by injection directly onto reversed-phase HPLC. HPLC analyses for cinnamamide were performed as described above. HPLC analyses for benzamide were performed under similar conditions as described for cinnamamide. The mobile phase was 20% (v/v) acetonitrile, 80% (v/v) deionized water, and 0.1% (v/v) TFA and the wavelength was 225 nm. When complete conversion was reached (as determined by a 1 to 1 ratio between the amide and the α-hydroxyglycine peaks) the reaction mixtures were lyophilized. Lyophilized reaction mixture and racemic mixture were analyzed by chiral chromatography on a CHIRALPAK®AD™ column (4.6×250 mm, Chiral Technologies, PA, USA) equipped with a CHIRALPAK®AD™ guard column (4.6×50 mm, Chiral Technologies, PA, USA). The chromatography was performed on a Waters™ LC Module I Plus system equipped with a 486 tunable UV/Vis detector, a 600E multisolvent delivery system, and a 715 autosampler with a 225 μl syringe. The system is controlled from the Millennium Chromatography Manager software package (Waters, Milford, MA, USA). HPLC analyses were performed at room temperature. The column was operated at a flow-rate of 1.0 ml min^{-1} using the solvent systems described in Table 1. The α-hydroxyglycine derivatives were detected at the wavelengths described in Table 1.

2.5. Optical rotation

Optical rotation was determined after collecting the purified enantiomers from the CHIRALPAK®AD™ column, by using a JASCO DIP-360 digital polarimeter (JASCO, Easton, MD, USA).

3. Results

The ability of the CHIRALPAK®AD™ column to resolve racemic mixtures of *N*-benzoyl α-hydroxyglycine was investigated under different elution conditions. Two classes of eluting conditions were attempted, a conventional ‘normal-phase’ eluent consisting of hexane with alcohol and TFA as modifiers, and a ‘polar organic’ eluent consisting of acetonitrile with alcohol and TFA as modifiers. Fig. 2 depicts the optimal resolution obtained with each of the eluting conditions. The conditions selected were 92.5% hexane with 7.5% ethanol and 0.15%

Table 1
Chromatographic conditions for chiral HPLC resolution of α-hydroxyglycine derivatives

α-Hydroxyglycine derivative	Mobile phase	% (v/v/v)	Detection UV, nm
N-Benzoyl α-hydroxyglycine	Hexane/EtOH/TFA	92.5/7.5/0.15	225
N-Benzoyl α-hydroxyglycine	MeCN/IPA/TFA	95/5/0.1	225
Cinnamoyl α-hydroxyglycine	MeCN/IPA/TFA	90/10/0.1	278
3-Phenylpropionyl α-hydroxyglycine	MeCN/IPA/TFA	90/10/0.1	210
1-Naphthylacetyl α-hydroxyglycine	MeCN/IPA/TFA	98/2/0.1	210
Phenylacetyl α-hydroxyglycine	MeCN/IPA/TFA	98/2/0.1	210
4′-Methoxyphenylacetyl α-hydroxyglycine	MeCN/IPA/TFA	95/5/0.1	210
4′-Nitrophenylacetyl α-hydroxyglycine	MeCN/IPA/TFA	95/5/0.1	210

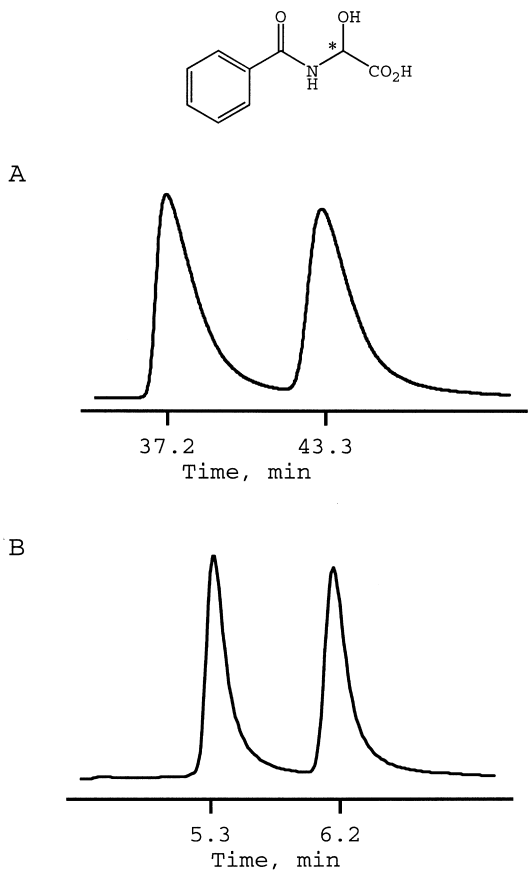

Fig. 2. Chiral resolution on the CHIRALPAK®AD™ column. *N*-benzoyl α-hydroxyglycine was resolved using 92.5% (v/v) hexane with 7.5% (v/v) EtOH and 0.15% (v/v) TFA (A) or 95% (v/v) MeCN with 5% (v/v) isopropyl alcohol and 0.1% (v/v) TFA (B). UV absorbance (*Y*-axes) was monitored at 225 nm. All further chromatographic conditions are in Table 1 and Section 2.

TFA (Fig. 2A) and 95% acetonitrile with 5% isopropanol and 0.1% TFA (Fig. 2B). The two types of eluents are not miscible. Therefore, the column was conditioned by flushing with 100% isopropanol between eluents. The polar organic eluent gave retention times of 5.3 and 6.2 min for the two enantiomers with a resolution of 1.53 whereas the normal-phase eluent gave retention times of 37.2 and 43.3 min with a resolution of 1.18. The amount of TFA modifier greatly affected the retention times for both elution conditions. Without TFA, *N*-benzoyl α-hydroxyglycine did not elute from the column. For the normal-phase eluent, use of ethanol instead of isopropanol as the alcohol component of the mobile phase gave shorter retention times and better resolution for *N*-benzoyl α-hydroxyglycine. In both cases, an increase in the amount of alcohol caused a decrease in the retention times (results not shown).

The absolute configuration of the resolved enantiomers was determined by exploiting PGL's ability to stereospecifically convert the *S*-enantiomer of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine. PGL was incubated with racemic mixtures of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine respectively until complete conversion was obtained as observed by reversed-phase HPLC. Subsequently, the reaction mixtures were lyophilized. Fig. 3 shows chromatograms of the racemic mixtures of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine and their lyophilized enzymatic conversions. As can be seen from Fig. 3B and D, only the non-reactive enantiomer remained after the enzymatic conversion. Based on the previously demonstrated stereospecificity of PGL [23], we can assign the absolute configuration *R*

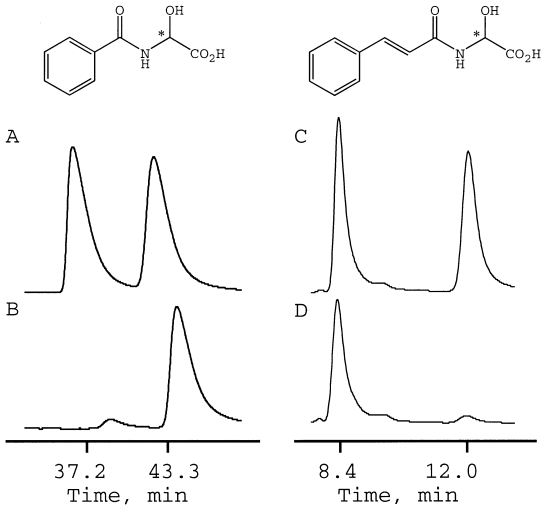

Fig. 3. Absolute configuration of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine as determined by PGL. PGL was incubated with racemic mixtures of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine until conversion of the *S*-enantiomer was complete. Racemic mixtures of *N*-benzoyl α-hydroxyglycine without (A) and with (B) PGL catalyzed conversion and cinnamoyl α-hydroxyglycine without (C) and with (D) PGL catalyzed conversion were resolved on the CHIRALPAK®AD™ column. UV absorbance (*Y*-axes) of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine was monitored at 225 nm and 278 nm, respectively. All further chromatographic conditions are in Table 1 and Section 2.

to the unreacted enantiomer (peak 2 for *N*-benzoyl α-hydroxyglycine and peak 1 for cinnamoyl α-hydroxyglycine) and the absolute configuration *S* to the reactive enantiomer (peak 1 for *N*-benzoyl α-hydroxyglycine and peak 2 for cinnamoyl α-hydroxyglycine). We further determined for both compounds that the *S*-enantiomer exhibited a negative optical rotation and the *R*-enantiomer exhibited a positive optical rotation.

The absolute configuration was confirmed by PGL's stereoselective catalysis of the conversion of each of the purified enantiomers. PGL's stereospecificity was demonstrated by incubating PGL with a racemic mixture of cinnamoyl α-hydroxyglycine until no further conversion was observed. After quantitative analysis by HPLC it can be seen that only 51% of the racemic mixture of cinnamoyl α-hydroxyglycine was converted to cinnamamide in the presence of PGL (Fig. 4A). Fig. 4B and C show that only 10% of the purified *R*-enantiomer was

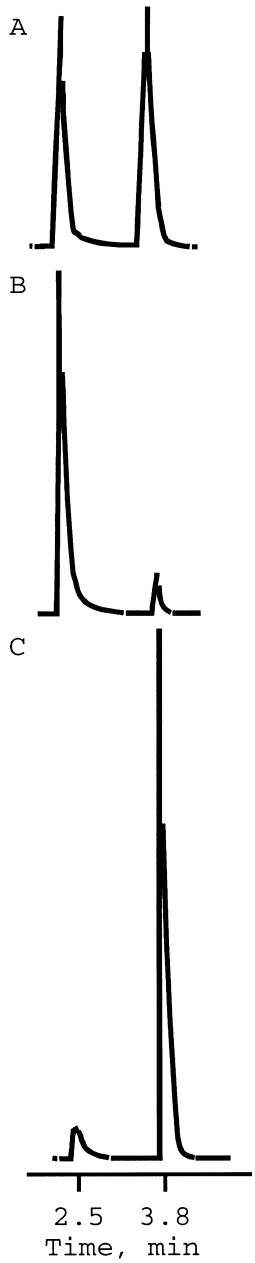

Fig. 4. Absolute configuration was confirmed by stereospecific conversion by PGL. PGL was incubated with racemic (A), purified *R*- (B) and *S*-enantiomers (C) of cinnamoyl α-hydroxyglycine until no further change was observed. Aliquots of each of the reaction mixtures were analyzed by reversed-phase HPLC. UV absorbance (*Y*-axes) was monitored at 278 nm. All further chromatographic conditions are in Table 1 and Section 2.

converted while 94% of the purified *S*-enantiomer was converted to cinnamamide in the presence of PGL. In the absence of PGL, there was a non-enzymatic conversion of the purified enantiomers of 7% and 5% respectively.

The CHIRALPAK®AD™ column with a polar organic mobile phase can be used to resolve the enantiomers of not only *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine but also a whole series of acyl α-hydroxyglycine derivatives. Fig. 5 shows the resolution of racemic mixtures of 1-naphthylacetyl α-hydroxyglycine, 4′-nitrophenylacetyl α-hydroxyglycine, 4′-methoxyphenylacetyl α-hydroxyglycine, 3-phenylpropionyl α-hydroxyglycine, and phenylacetyl α-hydroxyglycine. The optimal elution conditions for each of the acyl α-hydroxyglycine derivatives was determined by varying the ratio of acetonitrile and isopropanol from 90/10 to 98/2. All mobile phase systems contain 0.1% TFA as a modifier (Table 1).

4. Discussion

The use of CSPs allows for rapid development of enantiomeric separation, which in turn allows for direct recovery of the purified enantiomers. The CHIRALPAK®AD™ column consists of silica gel coated with polyamylose tris(3,5-dimethylphenylcarbamate). The separation of the enantiomers of *N*-benzoyl α-hydroxyglycine shown here using both the normal-phase and the polar organic elution systems demonstrate the advantages of the polar organic eluent very clearly. When using the polar organic eluent we observed much shorter retention times combined with a higher resolution, sharper peaks, and less tailing. The chromatographic advantages of operating the CHIRALPAK®AD™ column under the polar organic elution system has been demonstrated previously with other analytes [27]. The polysaccharide matrix of the CHIRALPAK®AD™ column allows for enantiomeric resolution through a number of mechanisms, none of which are currently fully understood.

Enzymes show a high degree of selectivity with respect to the identity of their substrates. Many enzymes are highly stereoselective. Techniques based on enzyme catalysis are playing an increasing role in producing chiral compounds and separating racemic mixtures [28]. However, enzyme catalysis is particularly useful for determining enantiomeric purity and absolute configuration because many enzymes discriminate completely between enantiomers. Herein, we have described a direct enzyme-based method for facile determination of absolute configuration of α-hydroxyglycine derivatives. Our method is based on the stereochemistry of PGL as well as PGL's ability to convert *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine to their corresponding amides and glyoxylate [23,24]. Our results illustrate the advantages of combining enantiomeric separation with stereoselective enzymatic conversion for determining absolute configuration. In three steps (enzymatic conversion of the reactive enantiomer of a racemic mixture, lyophilization and enantiomeric separation by chromatography) the absolute configuration is identified. Determination of the absolute configuration is obtained by comparing the elution profiles of racemic mixtures of the analyte before and after enzymatic conversion. It is important to emphasize that our method does not require the availability of enantiomeric standards of the analyte. Resolution of the individual components of complex mixtures is routinely achieved by HPLC [29,30]. This along with the high degree of enzymatic selectivity allows our method to determine the absolute configuration of pure analytes as well as analytes in mixtures where the impurities would otherwise preclude definitive assignments to be made with more traditional methods. Due to the high sensitivity of chromatographic separation and enzyme catalysis our method allows for accurate determination of absolute configuration to be made when only microgram or nanogram quantities of the analyte are available for analysis.

The development of chiral drugs requires pharmacological activity and toxicological assessment for both enantiomers. Our method simultaneously gives conditions for purifying both enantiomers along with establishing their absolute configuration. By using the CHIRALPAK®AD™ column with the polar organic eluent at room temperature, we ensure the maximum stability of the α-hydroxyglycine derivatives [31]. The purified enantiomers are easily recovered by evaporating the mobile phase under vacuum at room temperature.

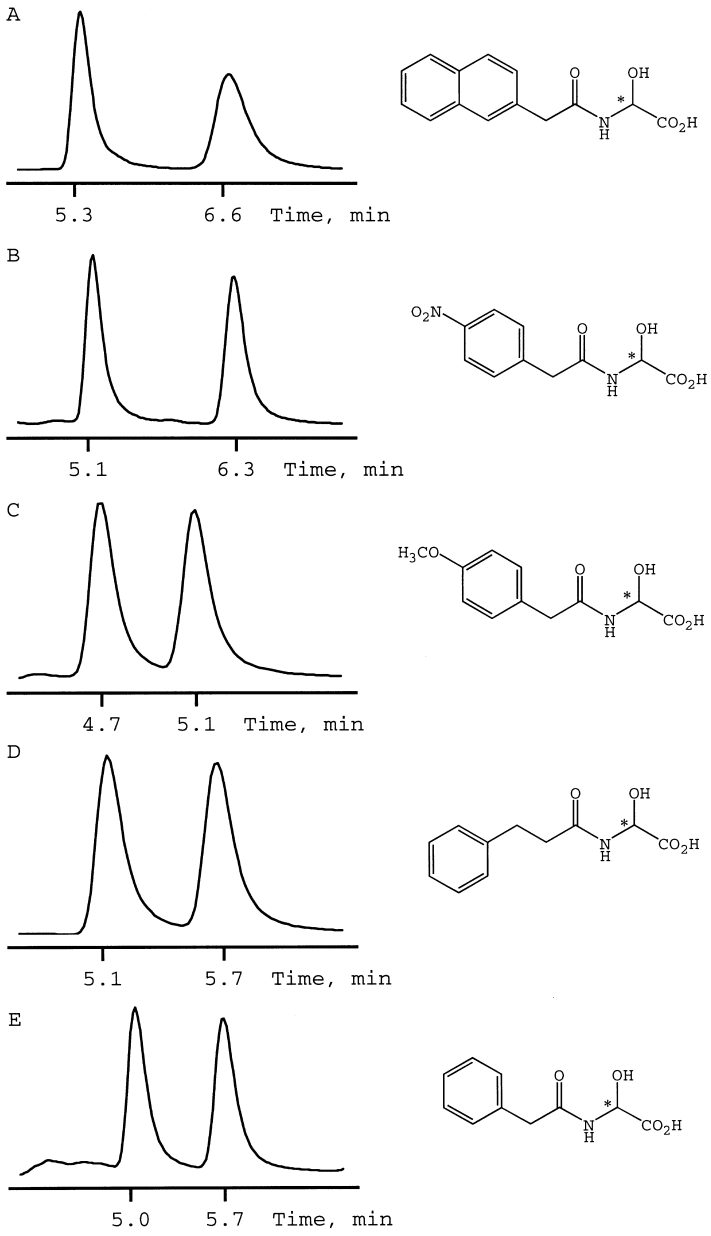

Fig. 5. Chiral resolution of a group of α-hydroxyglycine derivatives on the CHIRAIJPAK®AD™ column. Resolution of (*R*/*S*)-1-naphthylacetyl α-hydroxyglycine (A), (*R*/*S*)-4′-nitrophenylacetyl α-hydroxyglycine (B), (*R*/*S*)-4′-methoxyphenylacetyl α-hydroxyglycine (C), (*R*/*S*)-3-phenylpropionyl α-hydroxyglycine (D), and (*R*/*S*)-phenylacetyl α-hydroxyglycine (E). UV absorbance (*Y*-axes) was monitored as described in Table 1. All further chromatographic conditions are in Table 1 and Section 2.

It has previously been shown that PGL can stereospecifically catalyze the conversion of a whole series of α-hydroxyglycine derivatives [24]. We have demonstrated here that the CHIRALPAK®AD™ column with the polar organic elution system can be used to separate the enantiomers of a series of acyl α-hydroxyglycine derivatives. Therefore, our method for determination of absolute configuration of the

purified enantiomers of *N*-benzoyl α-hydroxyglycine and cinnamoyl α-hydroxyglycine can easily be expanded to also determine the absolute configuration of the resolved enantiomers of the whole series of acyl α-hydroxyglycine derivatives.

This simple method of combined enzyme catalysis and chiral chromatography provides a rapid, practical and sensitive way to determine the absolute configuration of purified enantiomers along with the conditions for enantiomeric separation, and the method is easily applied to a wide variety of enzyme systems.

Acknowledgements

We thank Dr. Dongsheng Ping for synthesizing most of the acyl α-hydroxyglycine derivatives used, and Dr. Charlie D. Oldham and Dr. Allison B. Moore for many technical discussions and recommendations. We gratefully acknowledge the financial support of the National Institutes of Health (GM40540) and the Molecular Design Institute.

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