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Effect of sample derivatization in liquid chromatographic separations of amine and amino acid enantiomers using diamide-type stationary phases

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ABSTRACT

Thirteen chiral diamide-type stationary phases, including a Pirkle-type material, for the resolution of optical isomers in liquid chromatography were prepared and examined for their separability. These phases have both $\pi-\pi$ interactions and hydrogen bonding sites and are chemically connected with silica by way of aminopropylsilica. As a chiral centre for the phases, valine was examined in comparison with phenylglycine, which was used in Pirkle-type phases. As the samples, benzoyl-, dimethylbenzoyl- and dinitrobenzoyl-O-isopropyl derivatives were applied. The phase that showed the highest separability was the immobilized dimethylbenzoyl valine phase for N-dinitrobenzoyl-O-isopropyl derivatives. Good separability was also found with a 3,5-dichlorobenzoylvaline phase. Hydrogen bonding at the C-terminal amide was found to make little contribution to the separation.

INTRODUCTION

The importance of determining the stereoisomeric composition of compounds cannot be overemphasized in various fields related especially to biology and medicine. Chromatographic methods, such as GC, LC and supercritical fluid chromatography, offer distinct advantages over classical techniques in the separation and determination of stereoisomeric composition. A number of stationary phases for the liquid chromatographic separation of stereoisomers exist and many of them are now commercially available [1,2].

Separation using LC can be achieved through various chiral discriminators or selectors [3]. One type of selector that is very widely used is the diamide-type chiral stationary phases. Usually, the selectors are used in immobilized form on a support material such as silica gel.

In this work, thirteen diamide-type chiral stationary phases for LC were synthesized and examined for their properties in relation to sample derivatization.

There seems to be no intrinsic difference in the chiral recognition mechanisms between LC and GC. The basic philosophy for the molecular design of such selectors produced by various workers is based on hydrogen bonding and/or $\pi-\pi$ interactions [4–7]. It can be assumed that the difference between the manner of association of an optical isomer with a chiral diamide molecule and that of an antipode with the same chiral diamide molecule provides the chiral recognition.

The diamide molecule can be divided into three parts: the chiral centre (amino acid residue), N-terminal acyl moiety and C-terminal amide moiety. In amino acid enantiomer separations by GC, it is usually observed that the most effective diamide phases are obtained by using leucine and valine as the chiral centre.

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Poor stereoselectivity is generally observed with proline, phenylglycine and alanine [8–10].

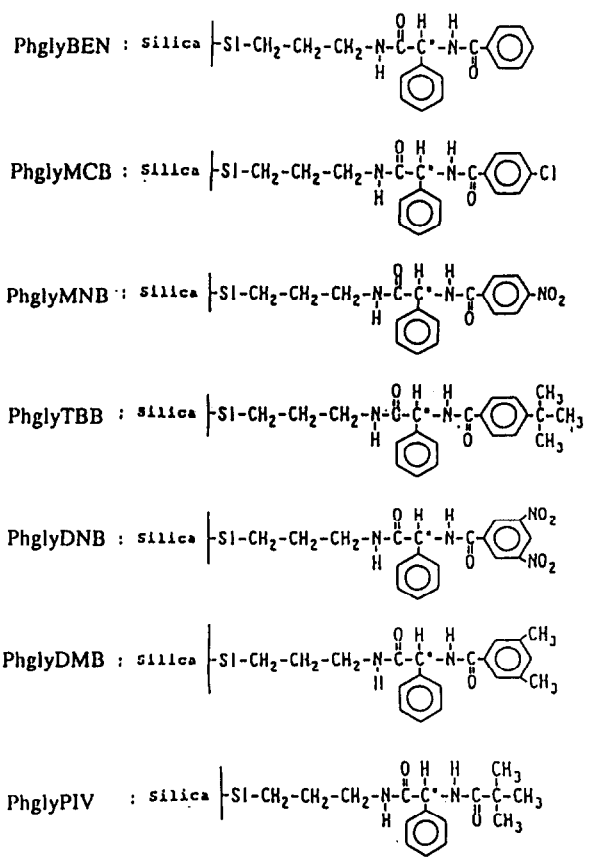
In typical Pirkle-type diamide phases, phenylglycine is the chiral centre and the samples are derivatized to dinitrobenzoyl amides.

In this work, from the $\pi-\pi$ interaction point of view, amine and amino acid samples were converted into three types of derivatives, benzoyl-, dinitrobenzoyl- and dimethylbenzoyl-amide, and compared for separability with a Pirkle dinitrobenzoyl-type stationary phase. In addition, phenylglycine in the Pirkle-type phases was changed to valine and the properties were examined.

EXPERIMENTAL

Chiral stationary phases

Diamide-type chiral stationary phases (CSPs) with thirteen different structures as shown in Fig. 1 were synthesized by a well known method [11].



A chiral amino acid, phenylglycine (optical purity >99%) or valine (optical purity >98%) in this study, was reacted overnight with an acid chloride in ice-cold 2 *M* NaOH solution. After washing with diethyl ether, 6 *M* HCl was added to adjust the pH to 2. The product was extracted with ethyl acetate and recrystallized. The N-acylamino acid obtained was further reacted with aminopropylsilica-immobilized silica (5 μm, Develosil-NH_2) (Nomura Chemical) in dry tetrahydrofuran in the presence of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinone for 8 h at room temperature. The CSP beads filtered with a Millipore 3-μm filter, then washed successively with tetrahydrofuran, acetone, heptane and diethyl ether.

Sample preparation

Amines and amino acids including 2-aminohexane (2-AH), α-phenylethylamine (α-PEA), phenylglycine (Ph-Gly), norvaline (Nr-val), nor-

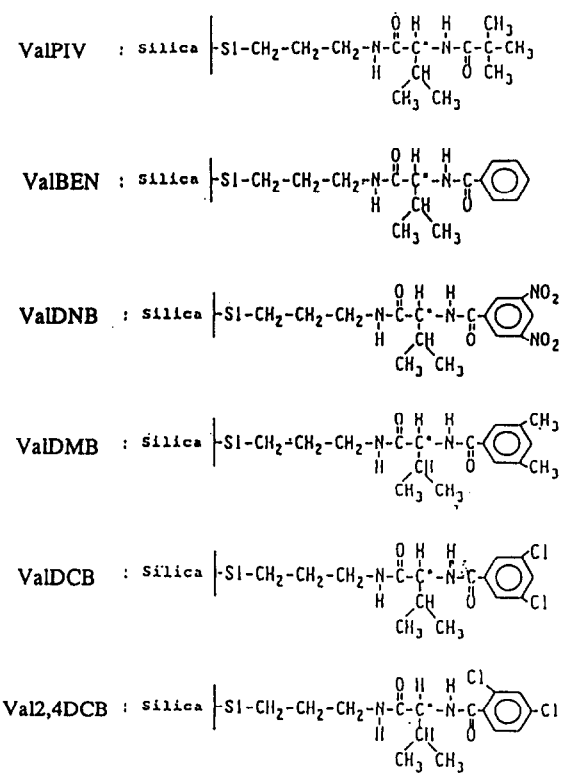


Fig. 1. Chiral stationary phases synthesized and their abbreviations.

leucine (Nr-Leu), α-aminobutyric acid (α-ABA) and β-aminobutyric acid (β-ABA) were used as samples. The carboxylic acid group was esterified with 2 *M* HCl–2-propanol solution at 120°C for 2 h under reflux. The amino group was treated with benzoyl (BEN), 3,5-dinitrobenzoyl (DNB) or 3,5-dimethylbenzoyl (DMB) chloride to obtain the corresponding benzylamides. After derivatization, the samples were purified column chromatographically using a silica gel column and hexane–ethyl acetate (10:1) as the mobile phase.

Chromatography

The chromatographic equipment consisted of a Shimadzu (Kyoto, Japan) Model LC-6A high-pressure pump, a Rheodyne (Cotati, CA, USA) Model 7125 sampling valve with a 25-μl loop, a Shimadzu Model SPD-6AV variable-wavelength UV–Vis spectrophotometric detector and a Shimadzu C-R5A or C-R6A integrator. In some instances, a Model CCPE high-pressure pump and a Model UV-8011 UV–Vis spectrophotometric detector from Tosoh (Tokyo, Japan) were also used. Stainless-steel HPLC columns (200 × 4.6 mm I.D. or 250 × 4.6 mm I.D.) were slurry packed with the prepared CSPs. The mobile phase was chosen as hexane–2-propanol (95:5) as the most appropriate and used at a flow-rate of 1.0 ml/min. The detector wavelength was set at 254 nm. All measurements were performed at room temperature.

Measurement of optical purity of the chiral phase

It is often required to compare the separation factors of the enantiomers with other phases or conditions. In that event, at least the optical purities of the chiral phases should be known. This was very difficult, however, in this study, because the amount of the immobilized chiral moiety was too small to be hydrolysed, derivatized and analysed by the conventional procedure. Therefore, we measured the optical purities before immobilization on the aminopropylsilica. As the immobilization reaction to silica was carried out at room temperature, it was considered safe to assume that the optical purities of the prepared phases were very close to those values.

For the determination of the optical purity of the chiral phases, a gas chromatographic method after hydrolysis and derivatization was employed. In brief, a small amount of stationary phase sample was placed in a glass tube containing 1 ml of 2 *M* HCl–2-propanol, then the tube was sealed and heated at 120°C for 6 h for hydrolysis and simultaneous isopropyl esterification. After completion of the reaction, the solvent was evaporated and the residue was dissolved in 0.1 *M* NaOH solution. Amino acid O-isopropyl esters were extracted with ethyl acetate and again the solvent was evaporated to the dryness. After dissolution in dichloromethane and trifluoroacetic anhydride, the ester was trifluoroacetylated at room temperature for 2 h. After evaporation, the derivatives were analysed for their enantiomer compositions by gas chromatography. A glass capillary column coated with N-stearoyl-L-valine-*tert.*-butyramide stationary phase and a flame ionization detector were used.

RESULTS AND DISCUSSION

As mentioned earlier, several interaction models have been proposed between solute molecules and chiral diamide molecules [4–7]. Two aspects should be pointed out: (1) The solute–solvent interaction consists of three points, namely π–π and two hydrogen bonds; and (2) the interaction of all three points occurs between one solute molecule and one diamide molecule.

This work was started with chiral phases having phenylglycine as the chiral centre for studying the effect of sample derivatization in HPLC. We then studied the necessity for a phenyl group as the acyl moiety and phenylglycine as the chiral moiety.

Although Pirkle and McCune [12–15] reported the use of leucine and valine as the chiral centre, we further examined the best combination of stationary phase and sample derivatives.

In Table I the optical purities of the CSPs prepared are presented. It has been stated that the optical purity could be 100% if the starting materials are optically pure [16]. Here, as shown in Table I, some of the Phgly-type phases have optical purities of less than 95%. In the case of LC phases, separation factors are affected not

TABLE I

OPTICAL PURITIES OF CSP MATERIALS SYNTHESIZED

CSP	Optical purity (%)	CSP	Optical purity (%)
PhglyBEN	78.2	ValBEN	98.8
PhglyMCB	83.2	ValDNB	96.2
PhglyMNB	80.4	ValDMB	95.8
PhglyTBB	93.2	ValDCB	99.8
PhglyDNB	100	Val2,4DCB	100
PhglyDMB	62.8	ValPIV	97.4
PhglyPIV	96.1		

only by the chiral group immobilized, but also by the retention on the silanol group of the silica support, residual free aminopropylsilyl groups, etc. Therefore, those separation factors obtained experimentally could not be corrected as in the case of GC [17]. In this paper, the results on the CSPs with optical purities higher than 95% are the main subjects of discussion.

CSPs with phenylglycine for the chiral centre

Using phenylglycine for the chiral centre of the CSPs, CSPs similar to the Pirkle type were synthesized first and compared in their separation properties for three kinds of sample derivatives.

Separation on PhglyDNB phase. It is very important in HPLC to derivatize the samples to achieve higher separability. We considered that although there is a resonance effect with the nitro group, the 3,5-dinitrobenzoyl-type phase PhglyDNB, which is exactly a Pirkle-type phase, is assumed to be a π-acceptor type and the 3,5-dimethylbenzoyl derivative is a π-donor type. Donor–acceptor interaction between the sample and stationary phase was considered to give favourable results.

The separation factors of three types of sample derivatives were measured and are presented in Table II. As expected, the DMB-type samples show higher separation factors than DNB-type samples. The results show, however, that BEN-type samples have the highest separation factors. It is favourable for the PhglyDNB phase to convert sample amines and amino acids to BEN type derivatives.

In addition, although the optical purity of the PhglyDMB synthesized was much lower than that of PhglyDNB, it showed the highest separability for DNB-type samples. With the PhglyDNB phase, π-donor–π-acceptor interaction enhanced the separability.

TABLE II

SEPARATION FACTORS OF THREE KINDS OF DERIVATIVES OF AMINES AND AMINO ACIDS ON PhglyDNB

Sample[a]	Derivative		
	BEN	DNB	DMB
2-AH	1.094	1.039	1.112
α-PEA	1.221	1.043	1.187
β-ABA	1.121	1.000	1.188
Asp	1.124	1.033	1.109
Ser	1.216	1.289	1.257
Ph-Gly	1.417	1.243	1.435
Val	1.442	1.137	1.329
Nr-Val	1.579	1.081	1.387
Thr	1.449	1.195	1.278
Phe	1.590	1.162	1.308
Ile	1.285	1.102	1.267
Nr-Leu	1.424	1.059	1.405
Tyr	1.238	1.400	1.497
Ala	1.526	1.162	1.417
α-ABA	1.345	1.109	1.344
Leu	1.752	1.073	1.597

[a] 2-AH = 2-aminohexane; α-PEA = α-phenylethylamine; Ph-Gly = phenylglycine; Nr-Val = norvaline; Nr-Leu = norleucine; α-ABA = α-aminobutyric acid; β-ABA = β-aminobutyric acid.

Effect of acylbenzene ring on the separation. In order to examine the effectiveness of the acylbenzoyl group in the chiral phase, pivalic acid was introduced into the acyl group. The results obtained are presented in Table III. As there is no benzoyl group in PhglyPIV and the $\pi-\pi$ interaction could not occur, the enantiomers are considered to be separated by the formation of two hydrogen bonds with CSP molecules. On the other hand, it was considered that a $\pi-\pi$ interaction of the sample benzoyl group with the benzene ring in the phenylglycine moiety of the stationary phase might possibly occur, which might be a disadvantage for effective separation. Further, the phenyl group in the chiral centre may disturb sterically the hydrogen bond formation.

CSPs with valine for the chiral centre

In the next step, based on the GC point of view [8–10], we used the valine as the amino acid residue in place of phenylglycine, *i.e.*, the phenylglycine in PhglyPIV was replaced with valine (ValPIV). The separation factors on ValPIV became much larger than those on PhglyPIV, especially when the samples were BEN or DMB derivatives, as shown in Tables IV–VI. It was considered that the benzene ring in the chiral centre is not so important for the separation of amino acid enantiomers, but the benzene ring in the acyl group is of use for attracting the sample molecules in the required interaction position.

TABLE III

SEPARATION FACTORS OF THREE KINDS OF DERIVATIVES OF AMINES AND AMINO ACIDS ON PhglyPIV

Sample[a]	Derivative		
	BEN	DNB	DMB
2-AH	1.000	1.104	1.000
α-PEA	1.029	1.000	1.000
β-ABA	1.000	1.000	1.000
Asp	1.000	1.122	1.000
Ser	1.000	1.000	1.000
Ph-Gly	1.077	1.133	1.000
Val	1.063	1.398	1.000
Nr-Val	1.099	1.375	1.000
Thr	1.094	1.146	1.000
Phe	1.105	1.272	1.000
Ile	1.000	1.395	1.000
Nr-Leu	1.121	1.397	1.000
Tyr	1.000	1.114	1.032
Ala	1.058	1.180	1.000
α-ABA	1.075	1.298	1.000
Leu	1.124	1.485	1.000

Search for favourable diamide-type CSP. It was assumed suitable for diamide-type CSPs to use α-amino acid with no phenyl group as the chiral centre, and to use a 3,5-disubstituted benzoic acid for the acyl group. As for the sample, similarly to PhglyDNB phase, if the acyl group of the CSP is a π-acceptor type, it might be favourable for it to be a π-donor type, and *vice versa*. The CSPs synthesized according to this concept are ValDNB, ValDMB and ValDCB. In Tables IV–VI, the separation factors for each sample form are presented. The separation factors for DNB-type samples on ValDMB are the highest of all, including PhglyDNB.

When the results on the 3,5-dichlorobenzoyl-type phase ValDCB were compared with those on the 2,4-dichlorobenzoyl-type Val2,4DCB, the former was much superior. The DMB- and BEN-type samples were better separated on ValDCB than on ValDMB.

Based on the GC work, we concluded that it is sufficient for amino acid enantiomer separation if there are two hydrogen bonds and the steric effect of the chiral centre available. This was proved by the results for DNB-type samples on ValDMB. Further, as shown with DNB-type samples on ValDCB and DNB-type samples on ValDMB, the solute–solvent $\pi-\pi$ interaction enhances the separability.

In Fig. 2, separation factors of various amine and amino acid samples on the CSPs prepared are compared. The horizontal axis is roughly in the order of increasing separation factor observed in GC [9]. The separation factors on the valine-type CSPs show a similar tendency to GC. They seem to have some mechanistic suitability for the separation of amino acid enantiomers as required in GC. Phenylglycine-type CSPs, however, have some points such as Nr-Val, Nr-Leu and Leu which are different from valine-type CSPs. We consider that phenylglycine-type

TABLE IV

SEPARATION FACTORS OF BEN DERIVATIVES OF AMINES AND AMINO ACIDS ON VAL-TYPE PHASES

Sample	Derivative					
	ValBEN	ValDMB	ValDCB	Val2,4DCB	ValDNB	ValPIV
2-AH	1.000	1.000	1.045	1.054	1.090	1.000
α-PEA	1.000	1.000	1.049	1.000	1.054	1.027
β-ABA	1.000	1.000	1.000	1.000	1.076	1.000
Asp	1.067	1.118	1.175	1.063	1.216	1.094
Ser	1.000	1.089	1.166	1.150	1.489	1.062
Ph-Gly	1.082	1.151	1.197	1.075	1.277	1.182
Val	1.174	1.331	1.378	1.262	1.491	1.310
Nr-Val	1.253	1.430	1.594	1.396	1.695	1.447
Thr	1.062	1.152	1.201	1.081	1.521	1.104
Phe	1.211	1.427	1.514	1.311	1.672	1.409
Ile	1.187	1.388	1.428	1.318	1.506	1.302
Nr-Leu	1.291	1.494	1.672	1.472	1.715	1.512
Tyr	1.138	1.250	1.308	1.048	1.919	1.354
Ala	1.163	1.306	1.437	1.154	1.754	1.263
α-ABA	1.193	1.350	1.462	1.222	1.654	1.273
Leu	1.353	1.592	1.839	1.608	1.938	1.617

stationary phases are not the most suitable diamide phase for amino acid enantiomer separation.

Supposedly, with Pirkle-type phases, a $\pi-\pi$ interaction and hydrogen bonding between C=O and N–H groups near the benzoyl group

TABLE V

SEPARATION FACTORS OF DNB DERIVATIVES OF AMINES AND AMINO ACIDS ON VAL-TYPE PHASES

Sample	Derivative					
	ValBEN	ValDMB	ValDCB	Val2,4DCB	ValDNB	ValPIV
2-AH	1.000	1.000	1.000	1.032	1.000	1.033
α-PEA	1.046	1.108	1.075	1.000	1.192	1.072
β-ABA	1.116	1.232	1.189	1.080	1.000	1.000
Asp	1.201	1.445	1.314	1.049	1.186	1.081
Ser	1.251	1.587	1.531	1.201	1.394	1.094
Ph-Gly	1.350	1.862	1.580	1.060	1.244	1.132
Val	1.518	2.221	1.985	1.000	1.372	1.246
Nr-Val	1.654	2.479	2.068	1.141	1.398	1.224
Thr	1.389	1.880	1.776	1.178	1.370	1.104
Phe	1.685	2.517	2.216	1.135	1.436	1.249
Ile	1.582	2.352	2.019	1.106	1.369	1.253
Nr-Leu	1.680	2.568	2.121	1.209	1.409	1.250
Tyr	1.647	2.376	2.202	1.063	1.756	1.278
Ala	1.517	2.143	1.915	1.000	1.383	1.174
α-ABA	1.550	2.269	1.960	1.045	1.411	1.194
Leu	1.822	2.829	2.413	1.234	1.424	1.324

TABLE VI

SEPARATION FACTORS OF DMB DERIVATIVES OF AMINES AND AMINO ACIDS ON VAL-TYPE PHASES

Sample	Derivative					
	ValBEN	ValDMB	ValDCB	Val2,4DCB	ValDNB	ValPIV
2-AH	1.000	1.000	1.000	1.000	1.135	1.000
α-PEA	1.000	1.027	1.076	1.000	1.042	1.045
β-ABA	1.000	1.000	1.077	1.000	1.103	1.000
Asp	1.082	1.131	1.211	1.000	1.161	1.000
Ser	1.050	1.127	1.287	1.137	1.476	1.091
Ph-Gly	1.084	1.164	1.245	1.000	1.222	1.154
Val	1.186	1.404	1.588	1.149	1.373	1.253
Nr-Val	1.298	1.549	1.721	1.292	1.550	1.341
Thr	1.105	1.215	1.407	1.080	1.556	1.138
Phe	1.247	1.498	1.634	1.219	1.479	1.288
Ile	1.224	1.480	1.548	1.216	1.390	1.263
Nr-Leu	1.363	1.649	1.812	1.356	1.576	1.393
Tyr	1.129	1.227	1.392	1.000	1.596	1.169
Ala	1.197	1.353	1.590	1.087	1.580	1.235
α-ABA	1.240	1.427	1.592	1.145	1.495	1.250
Leu	1.420	1.723	2.111	1.475	1.751	1.572

are the possible interactions, which is similar to the monoamide type interaction in GC [18,19].

Concerning the reason why monosubstituted benzoyl phases are inferior to the disubstituted type, we consider that in addition to the electronegativity of oxygen in the amide group, the angle of the phenyl group to the (>C=O)(>N–H) plane is different between the two, and the resulting interaction might not be same as that proposed by Pirkle and McCune [13]. We have not been able to confirm this assumption yet, but the results we have obtained with computer simulation work, which we have just started, are supportive.

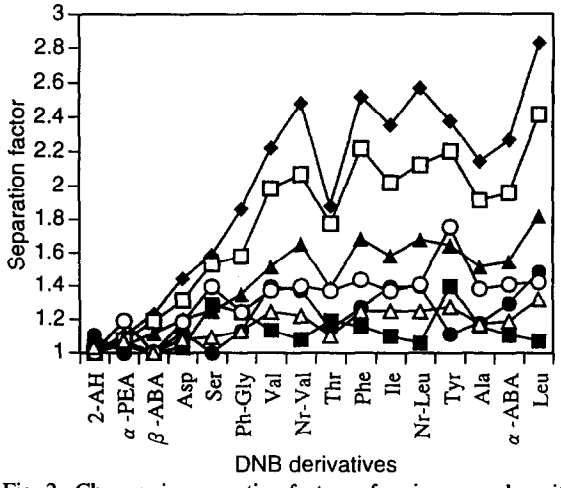


Fig. 2. Changes in separation factors of various samples with different CSPs. CSP: ■ = PhglyDNB; ● = PhglyPIV; ▲ = ValBEN; ◆ = ValDMB; □ = ValDCB; ○ = ValDNB; △ = ValPIV. Samples: DNB derivatives of amines and amino acids.

CONCLUSIONS

Thirteen CSPs for the HPLC separation of amine and amino acid enantiomers were synthesized and compared. The results showed that (1) on dinitrobenzoyl-type stationary phases, dimethylbenzoyl derivatives of samples were better separated than dinitrobenzoyl derivatives, and *vice versa*, (2) for amino acid samples, valine is superior to phenylglycine as the chiral centre of the stationary phase and (3) a π–π interaction between the benzene rings in the benzoyl groups of the sample and the acyl group in the stationary phase has an additional effect on the chiral separation.

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