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Sensitive enantiomeric separation of aliphatic and aromatic amines using aromatic anhydrides as non-chiral derivatizing agents

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ABSTRACT

New pre-column derivatizing reagents: phthalic anhydride, 3-nitrophthalic anhydride, diphenic anhydride, 1,8-naphthalic anhydride and diphenylmaleic anhydride have been developed for resolving chiral compounds having amine groups. Although all of these agents produce derivatives with high molar absorptivities, the later two also fluoresce. Upon derivatization, aromatic analytes containing free carboxylic groups are produced. Both of these moieties enhance chiral recognition on cyclodextrin-based columns. The derivatization reaction is carried out at room temperature by shaking a buffered aqueous solution of a sample with an acetonitrile solution of the reagent. The reaction is fast and proceeds without any detectable racemization. The labeled compounds have favorable chromatographic properties which are demonstrated by resolution of a number of chiral compounds on cyclodextrin-bonded phases operated with non-aqueous polar organic eluents. The selectivity and good efficiency of this system contributes to its high sensitivity and in its applicability for detecting low levels of enantiomeric impurities. The detection limit is in the picomole range and less than 0.1% enantiomeric impurities can be determined in some cases

INTRODUCTION

Sample derivatization in HPLC has became a popular means of increasing the selectivity and the sensitivity of detection in numerous analytical determinations. Generally the derivatizing agent is a highly absorbent or fluorescent moiety, which is essential in cases where highly sensitive measurements are necessary. Previous work has reported the applicability of a number of derivatizing agents such as N-(9-fluorenylmethoxycarbonyl)glycine chloride [1–3], N-carbethoxyphthalimide, N-phthaloylglycine chloride [4], dansyl chloride [5] and 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate [6–9] for the efficient optical resolution of a number of amino acids and small peptides on Cyclobond columns. When using π-acidic chiral stationary phases, other non-chiral derivatizing agents such as β-naphthyl chloroformate [10] and α-naphthylisocyanate [11] have been shown to be useful.

In this paper we present the resolution of a number of chiral compounds having amine functional groups after derivatizing them with different anhydrides which are effective new pre-column derivatizing reagents. 3-Nitrophthalic anhydride has been used as a suitable blocking agent in polypeptide syntheses. It is an energetic acylating agent attaching to even relatively unreactive amino groups [12]. In initial chromatographic experiments we found that the resulting 2-carboxy-3-nitrobenzoyl derivatives of a number of chiral amines can be readily separated into enantiomers using polar, non-aqueous solvents and β-cyclodextrin-based stationary phases. Fur-

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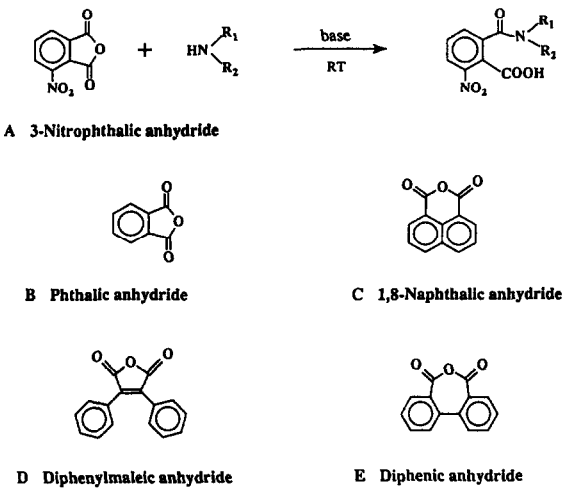


Fig. 1. Derivatization chemistry and molecular structure of anhydrides used in this study. RT = Room temperature.

ther we have extended the procedure and developed a general protocol using different anhydrides with highly absorbent or fluorescent moieties for enantiomeric separation of compounds having amine groups. The derivatization reaction and the structures of anhydrides used are outlined in Fig. 1.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a pump (LC-6A; Shimadzu, Kyoto, Japan), a system controller (SCL-6B, Shimadzu), Chromatopac (CR 601, Shimadzu), UV detector (SPD-6A, Shimadzu) and injector valve (Rheodyne, Cotati, CA, USA). The columns were 250 × 4.6 mm and were packed with *R*(−)-1-(1-naphthyl)ethyl carbamoylated-β-cyclodextrin (RN-β-CD) and β-cyclodextrin bonded to 5-μm spherical silica gel (Astec, Whippany, NJ, USA).

Chemicals

Samples of racemates and/or enantiomers investigated were purchased from different sources (Fluka, Buchs, Switzerland; Sigma, St. Louis, MO, USA; and Aldrich, Milwaukee, WI, USA). Acetonitrile, methanol and water were of OmniSolv grade and supplied from EM Science (Gibbstown, NJ, USA). Acetic acid and triethylamine were HPLC grade from Fisher (St. Louis, MO, USA). Anhydrides used as derivatizing agents were: diphenic and 3-nitrophthalic (Sigma), 2,3-diphenylmaleic, phthalic and 1,8-naphthalic (Aldrich).

Procedure

Derivatization was performed in 1% aqueous Na_2CO_3–acetonitrile solution (1:1, v/v) at room temperature. The derivatizing agent (anhydride) can be dissolved in acetonitrile (*ca.* 0.1%) before addition. Approximately 0.5 to 1.0 ml of this solution can be added per ml of analyte sample. All analytes in this study were dilute aqueous amines (from 10^{-3} to 10^{-5} *M*). The samples were shaken and allowed to react for *ca.* 3 min. Longer reaction times gave no quantitative change for the analyte, therefore it was assumed that the reaction was complete. After derivatization samples were acidified with a 50% acetic acid solution. Following acidification, the samples were diluted 10–50 times with acetonitrile and 5 μl were injected.

RESULTS AND DISCUSSION

Derivatization of chiral primary and secondary amines with suitable achiral anhydrides (shown in Fig. 1) results in highly absorbent or fluorescent compounds suitable for HPLC enantiomeric analysis on Cyclobond columns. As can be seen from Table I and Fig. 2 the resulting conjugates are readily enantioresolved. One set of HPLC conditions, *e.g.*, native β-cyclodextrin bonded phase operated with an acetonitrile–triethylamine–glacial acetic acid mobile phase, mixture was suitable for most of the solutes studied.

Recently it has been found that applicability of non-aqueous polar organic eluents in conjunction with cyclodextrin columns provides high enantioselectivity towards many classes of chiral compounds [1–3,8,9,13–16]. The eluents consist mainly of neat acetonitrile with small amounts of triethylamine, glacial acetic acid and methanol as modifiers. It has been postulated that the enantiomeric separation can be achieved due to the external complex formation between the solute and the cyclodextrin molecule. The chiral recognition arises from stereoselective hydrogen bond-

TABLE I

ENANTIOMERIC SEPARATION OF AMINE DERIVATIVES ON CYCLODEXTRIN (CD) COLUMNS USING A NON-POLAR ORGANIC MOBILE PHASE CONSISTING OF ACETONITRILE–TRIETHYLAMINE–ACETIC ACID (1000:4:2, v/v/v)

No.	Solute	k'_1	α	Anhydride	Column[a]
1	1-Methylbutyl-amine	6.41	1.04	1,8-Naphthalic	β-CD
2	*sec*-Butylamine	2.97	1.07	Phthalic	β-CD
3	2-Amino-3,3-dimethylbutane	3.12	1.18	3-Nitrophthalic	β-CD
		5.07	1.22	Phthalic	β-CD
		8.86	1.12	1,8-Naphthalic	β-CD
		2.42	1.14	Diphenic	β-CD
		5.35	1.22	Diphenylmaleic	β-CD
4	1,3-Dimethyl-butylamine	6.64	1.12	1,8-Naphthalic	β-CD
		1.49	1.05	Diphenic	β-CD
		2.06	1.05	Diphenylmaleic	β-CD
5	2-Ethylhexyl-amine	2.15	1.07	3-Nitrophthalic	β-CD
		2.77	1.11	Phthalic	β-CD
		2.14	1.11	Diphenylmaleic	β-CD
6	2-Aminoheptane	6.26	1.15	1,8-Naphthalic	β-CD
		0.56	1.20	Diphenylmaleic	β-CD
		2.27	1.11	3-Nitrophthalic	RN-β-CD
		3.06	1.20	Phthalic	RN-β-CD
7	3-Aminoheptane	5.06	1.16	1,8-Naphthalic	β-CD
		2.30	1.19	Phthalic	RN-β-CD
		1.82	1.14	3-Nitrophthalic	RN-β-CD
8	1-Cyclohexyl-ethylamine	4.03 (*R*)	1.25	3-Nitrophthalic	β-CD
		6.45 (*R*)	1.10	Phthalic	β-CD
		9.92 (*R*)	1.14	1,8-Naphthalic	β-CD
		3.13 (*S*)	1.09	Diphenic	β-CD
		6.52 (*S*)	1.09	Diphenylmaleic	β-CD
9	Naphthylethyl-amine	2.18	1.21	Phthalic	β-CD
10	2-Methyl-piperidine	8.85	1.24	Phthalic	β-CD
		2.87	1.06	Diphenic	β-CD
		5.21	1.25	Diphenylmaleic	β-CD
11	3-Methyl-piperidine	7.37	1.09	Phthalic	β-CD
		6.17	1.09	Phthalic	RN-β-CD
12	Norephedrine	2.46 (+)	1.14	Phthalic	β-CD
		2.68 (+)	1.17	1,8-Naphthalic	β-CD
		1.60 (−)	1.11	Diphenic	β-CD
13	Phenylalanine methyl ester	1.57 (D)	1.10	3-Nitrophthalic	β-CD
		1.61 (L)	1.07	Phthalic	β-CD
14	α-Phenylethyl-amine	1.98 (L)	1.07	3-Nitrophthalic	β-CD
		1.80 (L)	1.04	Phthalic	β-CD

[a] β-CD refers to the β-cyclodextrin bonded phase column (*i.e.*, Cyclobond I). RN-β-CD refers to the *R*(−)-1-(1-naphthyl)ethyl carbamoylated-β-cyclodextrin bonded phase column (*i.e.*, Cyclobond I-RN).

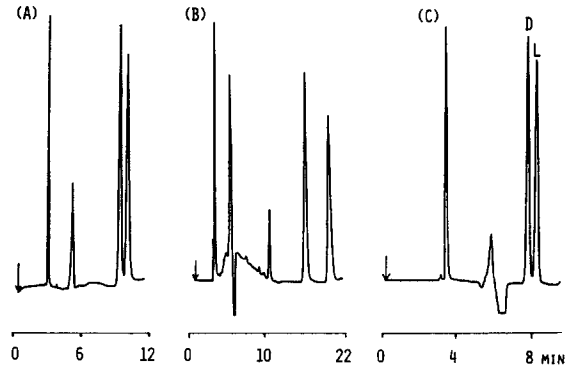


Fig. 2. Enantioseparation of amino compounds after derivatization with appropriate anhydride reagents. A β-cyclodextrin column (Cyclobond I) operated with a polar organic mobile phase consisting of acetonitrile–triethylamine–acetic acid (1000:4:2, v/v/v) was used. (A) Phthalic anhydride derivative of 2-ethylhexylamine; (B) diphenylmaleic anhydride derivative of 2-methylpiperidine; (C) 3 nitrophthalic anhydride derivative of phenylalanine methyl ester.

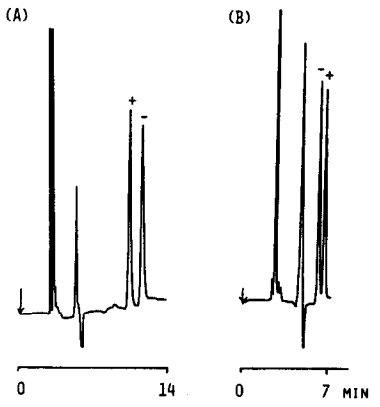


Fig. 3. Enantioseparation of (±)-norephedrine on β-cyclodextrin column. The eluent was: acetonitrile–triethylamine–acetic acid (1000:4:2, v/v/v). The derivatizing agents were (A) 1,8-naphthalic anhydride and (B) diphenic anhydride.

ings between donor and acceptor sites of the analyte with the secondary hydroxyl groups at the mouth of the cyclodextrin cavities (in the case of native cyclodextrin bonded phase) as well as other polar moieties at the mouth of the cyclodextrin cavity (in the case of derivatized cyclodextrin bonded phases). The strength of the interaction between the cyclodextrin stationary phase and the solute and therefore the retention and stereoselectivity is determined mainly by the structure of the analyte and the competitive interaction of mobile phase components [1,8,9,14]. This is confirmed by the chromatographic retention behavior found in this study.

As can be seen from Table I and Fig. 3 the retention parameters obtained on native β-cyclodextrin bonded phase depend very strongly on the derivatizing agent used. The "tagging" agent strongly effects the retention and selectivity. In some cases a reversal in elution order occurs when different anhydride derivatizing agents are used, *i.e.*, norephedrine in Fig. 3 for example. It indicates that both the chiral amine and achiral anhydride moiety are closely associated with the cyclodextrin. This is not surprising since as shown in Fig. 1 the derivatization reaction provides not only an aromatic moiety for easy photometric detection but also a free carboxylic group capable of hydrogen bond formation with the β-cyclodextrin stationary phase. In fact the presence of the carboxylic acid functional group is generally necessary for chiral recognition of these compounds in this separation mode. This is one reason anhydrides were chosen as derivatizing agents.

A comparison of retention data for 2-amino-3,3-dimethylbutane derivatized with diphenic, 1,8-naphthalic and diphenylmaleic anhydrides is shown in Fig. 4. Clearly the enantioseparation is affected by the size of the aromatic substituent. It seems that the diphenic moiety contributes to

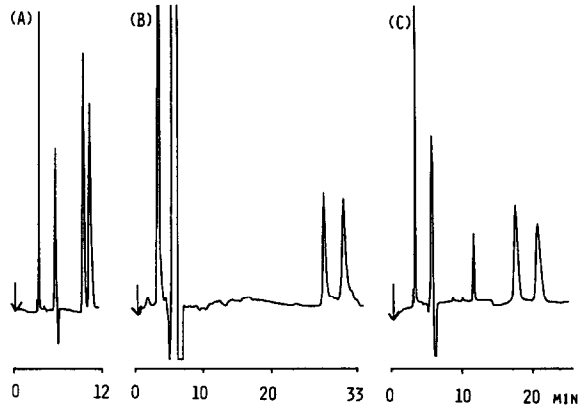


Fig. 4. Enantioseparation of 2-amino-3,3-dimethylbutane on β-cyclodextrin column in conjunction with non-aqeous mobile phase. Eluent: acetonitrile–triethylamine–acetic acid (1000:4:2, v/v/v). The derivatizing agents were (A) diphenic anhydride, (B) 1,8-naphthalic anhydride and (C) diphenylmaleic anhydride.

steric hindrance which weakens the strength of hydrogen bonding resulting in decreased retention. Similar tendencies were found in all instances for amines derivatized with diphenic anhydride.

The influence of small structural changes on the enantioselectivity is presented in Fig. 5. As can be seen from Table I and Fig. 5, 1,3-dimethylbutylamine can be separated into enantiomers on a β-cyclodextrin column after reacting with a number of different anhydride derivatizing agents. In contrast to the 1,3-dimethylbutylamine, the 1-methylbutylamine can be only partially resolved after its derivatization with phthalic anhydride. Similar behavior is observed for 2-methyl- and 3-methylpiperidine.

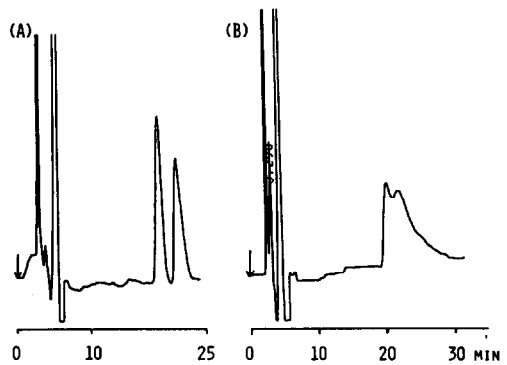


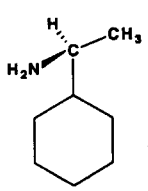
Fig. 5. Enantioseparation of: (A) 1,3-dimethylbutylamine and (B) 1-methylbutylamine after their derivatization with 1,8-naphthalic anhydride. The eluent was: acetonitrile–triethylamine–acetic acid (1000:4:2, v/v/v).

In some cases the interactions with native β-cyclodextrin bonded stationary phase were insufficient to obtain the enantioseparation of the functionalized amines. This problem was overcome by using derivatized cyclodextrin stationary phases to provide additional adsorption (binding) sites for stereoselective interactions with the solutes. The applicability of the carbamoylated β-cyclodextrin column (RN-β-CD) with the aromatic substituted amines resulted in selectivity enhancements in those cases where chiral recognition on the native β-cyclodextrin stationary bonded phase was unsatisfactory.

The labeling of non-chromophoric amines with aromatic anhydrides also results in highly absorptive or fluorescent adducts suitable for sensitive photometric detection. Table II lists the extinction coefficient of the derivatizing agents used and the detection limits found for different 1-cyclohexylethylamine conjugates in a typical

TABLE II

EXTINCTION COEFFICIENTS (ε_{240}), DETECTION LIMITS, EXCITATION AND EMISSION WAVELENGTHS FOR DIFFERENT ANHYDRIDE DERIVATIVES OF 1-CYCLOHEXYLETHYL AMINE



S(+)-1-cyclohexylethylamine

The column was a β-CD bonded phase (Cyclobond I) and the polar organic mobile phase consisted of acetonitrile–triethylamine–glacial acetic acid (1000:4:2, v/v/v).

Anhydride	ε_{240}	Sensitivity (pmol)[a]	k'	λ_{ex} (nm)	λ_{em} (nm)
Diphenic	13 818	50	3.13	–	–
2,3-Diphenylmaleic	7738	30	6.52	270	356
3-Nitrophthalic	8040	300	5.03	–	–
Phthalic	5883	300	7.11	–	–
1,8-Naphthalic	9333	150	11.93	293	384

[a] These sensitivites were determined at 240 nm using UV detection. When using fluorescence detection with 2,3-diphenylmaleic anhydride or 1,8-naphthalic anhydride, these limits were almost three orders of magnitude lower.

non-aqueous mobile phase system. Using UV detection at 240 nm the detection limits were found to be in the picomole range; it differs from 300 pmol estimated for nitro- and phthalic conjugates to low picomole range found for diphenylmaleic, 1,8-naphthalic and diphenic anhydrides. Using fluorescence detection, the limits for diphenylmaleic and 1,8-naphthalic anhydride derivatives were orders of magnitude lower (Table II).

The high selectivity and efficiency of the system coupled with the aromatic chromophores enabled enantiomeric purity determinations of functionalized amines at trace levels. Fig. 6 shows a chromatogram used for the evaluation of the enantiomeric purity of a (−)-norephedrine commercial standard after its derivatization with 1,8-naphthalic anhydride. As little as 0.156% (S.D. 0.044) of the (+)-enantiomer was determined with good precision in the presence of large amounts of corresponding (−)-enantiomer.

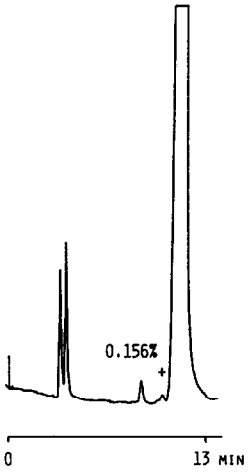


Fig. 6. Chromatogram used for evaluation of enantiomeric purity of (−)-norephedrine commercial standard (Aldrich) after its derivatization with 1,8-naphthalic anhydride. A β-cyclodextrin column was used. The eluent was: acetonitrile–triethylamine–acetic acid (1000:4:2, v/v/v).

Table III gives comparison data for enantiomeric trace analysis of (*R*)- and (*S*)-cyclohexylethylamine standards obtained after their precolumn labeling with different derivatizing agents. Also shown in Table II and Fig. 3 is that the elution order of enantiomers can be controlled by selection of an appropriate derivatizing agent. This is of great practical importance in the design of appropriate methodologies. As reported previously the sensitivity, accuracy and precision of determination can be improved significantly when trace enantiomers are eluted before major components [14]. Two pairs of derivatizing agents were chosen for the evaluation of optical purity of cyclohexylethylamine enantiomers; nitro- and phthalic anhydrides for *S* enantiomer and diphenic and diphenylmaleic anhydrides for the *R* enantiomer (see Fig. 7). Excellent agreement was found between the methods employed. This indicates not only the high accuracy of this method but also that the derivatizing steps induced essentially no racemization.

The derivatizing reaction is fast and is carried

TABLE III

THE COMPARISON OF ENANTIOMERIC TRACE ANALYSIS OF COMMERCIAL (*R*)- AND (*S*)-1-CYCLOHEXYLETHYLAMINE STANDARDS (FLUKA) OBTAINED AFTER THEIR PRECOLUMN LABELING WITH DIFFERENT DERIVATIZING AGENTS

Column: β-cyclodextrin. Eluent: acetonitrile–triethylamine–acetic acid (1000:4:2, v/v/v).

Solute	Derivatizing agent	% of opposite enantiomer	Standard deviation ($n = 5$)
(*R*)-1-Cyclohexylethylamine	2,3-Diphenyl maleic	1.316	0.049
	Diphenic	1.355	0.061
(*S*)-1-Cyclohexylethylamine	Phthalic	2.377	0.055
	3-Nitrophthalic	2.255	0.046

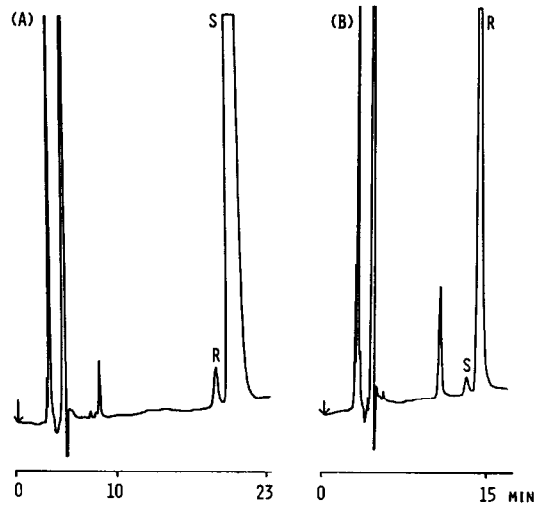


Fig. 7. Chromatogram used for evaluation of enantiomeric purity of cyclohexylethylamine commercial standard (Fluka). A β-cyclodextrin column was used. The eluent was: 1000 ml acetonitrile + 4 ml triethylamine + 2 ml acetic acid. (A) 3-nitrophthalic anhydride derivative of (S)-1-cyclohexylethylamine: (B) diphenylmaleic anhydride derivative of (*R*)-1-cyclohexylethylamine.

out at room temperature by shaking a buffered aqueous solution of the sample with an acetonitrile solution of reagent. Derivatizaiton is possible in non-aqueous solution as well. The excess of derivatizing reagents and their hydrolysis products are eluted close to the dead volume in the chromatographic systems used in this study. Consequently they do not interfere with the enantiomeric separations (see Fig. 3). The derivatives were found to be stable for several days at room temperature, after which an unidentified peak appeared in the chromatogram. In conclusion, the high reactivity of the reagents, the ease of the derivatizing reactions, the ability to change elution order and the ease of detection of the adducts combined with the resolving power of cyclodextrin bonded phases operated with non-aqueous eluents make the described method sensitive and effective.

ACKNOWLEDGEMENT

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