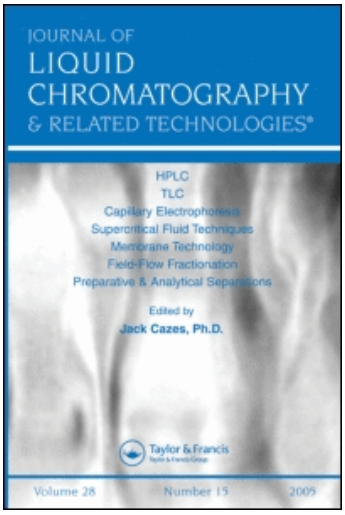

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HPLC Resolution of Hydroxyl Carboxylic Acid Enantiomers Using 2-Quinoxaloyl Chloride as a New Precolumn Derivatizing Agent

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HPLC RESOLUTION OF HYDROXYL CARBOXYLIC ACID ENANTIOMERS USING 2-QUINOXALOYL CHLORIDE AS A NEW PRECOLUMN DERIVATIZING AGENT

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ABSTRACT

Chiral α-hydroxy carboxylic acids were reacted using 2-quinoxaloyl chloride to form UV and fluorescent derivatives. Under mild conditions either in aqueous or non-aqueous conditions reaction proceeds quickly and without racemisation. The labelled compounds have been resolved into enantiomers on β-cyclodextrin bonded stationary phase operated with polar organic eluents. The high sensitivity and selectivity of the method has been used for the determination of low levels of enantiomeric impurities in commercial "pure" samples.

INTRODUCTION

Several hydroxy carboxylic acids have been known to show significant biological activities. Their determination in physiological fluids has been used in metabolism studies and in the identification of several disorders [1-3]. The majority of diagnostically useful organic acids are optically active. Thus the enantiomeric ratio determination of these compounds may provide more detailed information on biological processes and disorders in living organism. Previously several studies concerning the enantioseparation of organic acids were reported. However, these separations are not yet routine and an accurate, precise and sensitive method is still needed [4-7].

This paper presents the applicability of 2-quinoxaloyl chloride having a highly absorptive and fluorescence aromatic moiety as a new pre-column derivatisation reagent for α-hydroxy carboxylic acids. The resulting conjugates were optically resolved on β-cyclodextrin bonded stationary phase using non-aqueous polar eluents.

EXPERIMENTAL

A HPLC system consisting of a pump (Waters 600), UV detector (Programmable Absorbance Detector 785A, ABI),

integrator (HP3390A, HP) and a Rheodyne injection valve with a 250x4.6 mm Cyclobond I 2000, 5 μm, column (Technicol, Stockport, UK) was used.

Mobile phase was prepared from HPLC grade acetonitrile, methanol, triethylamine and glacial acetic acid (Fisons, UK). Flow rate was 1 ml/min with the column kept at room temperature throughout the studies. UV detection was carried out at 315 nm.

Derivatising reagent, 2-quinoxaloyl chloride, was purchased from Aldrich and α-hydroxy carboxylic acids (free or as a salt) were supplied by Aldrich and Sigma.

Approximately 0.5 mg of acid and 2-quinoxaloyl chloride was reacted in 2 ml vial containing 1 ml of acetonitrile and one drop of triethylamine for 30 min at room temperature. In the case of poor solubility of acid a few drops of water were added or the reaction was performed in 50% aqueous acetonitrile. Reaction mixture was diluted five folds with acetonitrile and 5 μl was injected into a column.

RESULTS

Derivatisation Chemistry

The structure of 2-quinoxaloyl chloride and the derivatisation reaction are shown in FIGURE 1. The reaction is

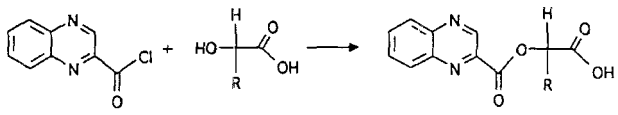

FIGURE 1. Derivatisation chemistry

fast and proceeds under mild conditions (see Experimental) at room temperature either in aqueous or non-aqueous solutions.

As can be seen the labelling with the tagged agent introduces not only an aromatic moiety for easy UV or fluorescent detections but also provides two nitrogen atoms which can interact with the secondary hydroxy groups at the mouth of the cyclodextrin cavity and may contribute to the overall retention and the steric discrimination of the resultant conjugates.

Retention Behaviour

The labelled compounds have favourable chromatographic proprieties and can be easily resolved into enantiomers on β-cyclodextrin bonded silica columns operated with non-aqueous polar organic mobile phases. TABLE 1 lists the retention parameters of derivatised α-hydroxy carboxylic acids obtained under optimal conditions. The neat acetonitrile with small amounts of triethylamine and acetic acid as modifiers has been used as a mobile phase for the enantioseparation of

TABLE 1

Separation Data for Racemic Mixtures of α -Hydroxy Carboxylic Acids (R-CH(OH)-COOH) on β -Cyclodextrin Column operated with Polar Organic Eluents

Acid name	R	k'_1	α	R_S	Eluent
Lactic	$-CH_3$	3.88	1.16	2.41	A
α-Hydroxybutyric	$-CH_2-CH_3$	3.72	1.17	2.49	A
α-Hydroxyvaleric	$-(CH_2)_2-CH_3$	3.61	1.17	2.40	A
α-Hydroxyisovaleric	$-CH-(CH_3)_2$	3.77	1.15	2.16	A
α-Hydroxycaproic	$-(CH_2)_3-CH_3$	3.77	1.15	2.16	A
α-Hydroxyisocaproic	$-CH_2-CH-(CH_3)_2$	4.04	1.13	2.10	A
α-Hydroxycaprylic	$-(CH_2)_5-CH_3$	3.61	1.14	2.01	A
α-Hydroxycapric	$-(CH_2)_7-CH_3$	3.50	1.14	2.02	A
α-Hydroxylauric	$-(CH_2)_9-CH_3$	3.44	1.14	1.98	A
α-Hydroxymyristic	$-(CH_2)_{11}-CH_3$	3.33	1.13	1.95	A
α-Hydroxypalmitic	$-(CH_2)_{13}-CH_3$	3.20	1.14	1.95	A
α-Hydroxystrearic	$-(CH_2)_{15}-CH_3$	3.01	1.13	1.86	A
α-Hydroxyarachidic	$-(CH_2)_{17}-CH_3$	2.82	1.12	1.72	A
α-Hydroxybehenic	$-(CH_2)_{19}-CH_3$	2.53	1.11	1.46	A
α-Hydroxyhexacosanoic	$-(CH_2)_{23}-CH_3$	2.23	1.12	1.34	A
Hexahydromandelic	$-C_6H_{11}$	1.94	1.46	4.03	B
Malic	$-CH_2-COOH$	3.40	1.11	1.58	B

Eluent:

A - acetonitrile + triethylamine + acetic acid, 1000 + 5 + 2.5, v/v/v

B - acetonitrile + methanol + triethylamine + acetic acid, 750 + 250 + 10 + 5, v/v/v/v

homologous alkyl α-hydroxy carboxylic acids. The elution of hexahydromandelic and malic acids in a reasonable time required the addition of as much as 25% (v/v) of methanol to the mobile phase. The L enantiomers were eluted prior to the D enantiomers in all cases.

As can be seen from these data the k' values of the solutes examined decrease in the order of increasing hydrophobicity of the side chain, R, on the chiral carbon atom. FIGURES 2 and 3 show the change of chromatographic parameters with increasing carbon number in the straight side chain. The results clearly indicate that the increasing length of the alkyl side chain influences the chromatographic separation in two ways: it decreases the retardation as well as the ratio of mass transfer distribution between mobile and stationary phases, which results in increasing plate height. Both factors decrease the separation of α-hydroxy carboxylic acid homologues in spite of nearly unchanged chiral discrimination, α, exhibited by the stationary phase.

The data presented above are in a good agreement with the model of chiral discrimination in water - free systems. Briefly, it has been postulated that in non-aqueous systems the inclusion complex formation is suppressed as the cyclodextrin cavity is largely occupied by the acetonitrile molecules. The chiral recognition on native cyclodextrin bonded phases arises from stereoselective hydrogen bondings

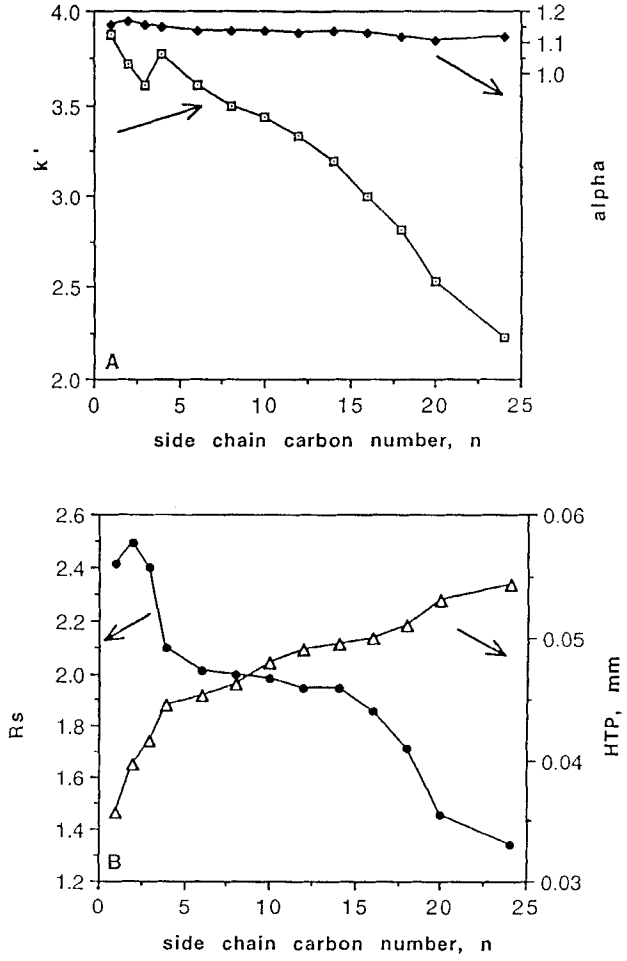

FIGURE 2. Change in chromatographic enantioseparation parameters of homologues α-hydroxy carboxylic acids with increasing carbon number (n) in the straight alkyl chain on the chiral centre. Eluent: acetonitrile + triethylamine + acetic acid, 1000 + 5 + 2.5, v/v/v.

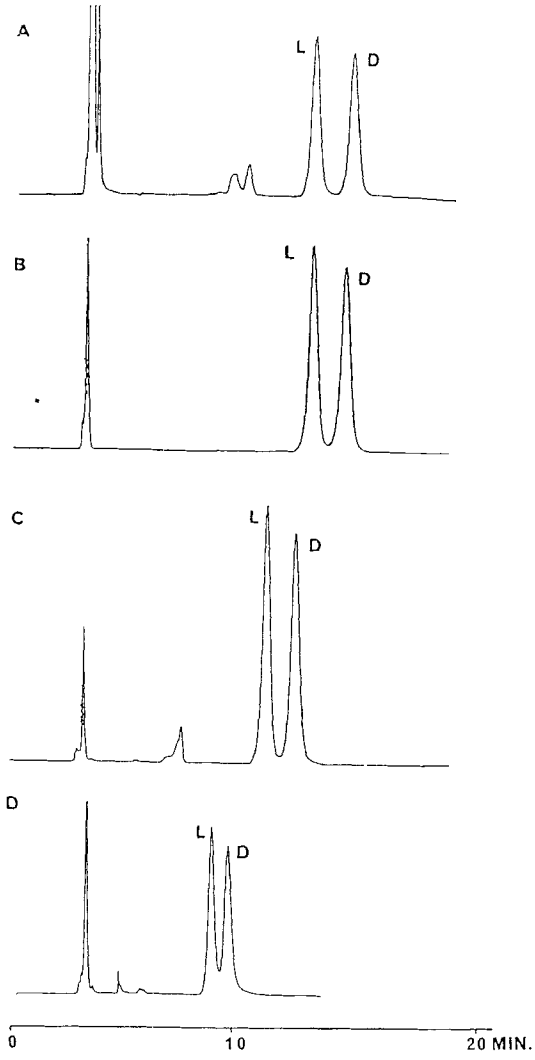

FIGURE 3. Chromatograms representing the change in enantioseparations of selected α-hydroxy carboxylic acids with the increasing hydrophobicity of the straight alkyl side chain on the chiral centre. Eluent as in FIG. 2.
Test compounds: (A) - lactic acid, (B) - caprilic acid, (C) - palmitic acid, (D) - hydroxyhexacosanoic acid.

between analyte and the secondary hydroxyl groups at the mouth of the cyclodextrin cavity [8-10].

This is further supported by the retention behaviour observed in the current study. Nearly unchanged enantioselectivity exhibited by the stationary phase towards the homologues carboxylic acids as well as the high symmetry of the peaks and the same elution order for all analytes investigated lead to the assumption that the chiral discrimination is caused by the same type of stereointeraction e.g. by hydrogen bondings between the hydroxyl groups of the β-cyclodextrin and the polar groups in the functionalised hydroxy carboxylic acids. The increasing length of the alkyl substituent, R, tends to decrease the strength of the hydrogen bonding complexation between the stationary phase and the solute owing to the increasing hydrophobicity of the later, but only slightly influences the stereoselectivity, (see TABLE 1 and FIGURE. 2A), which indicates that the straight aliphatic chain on the chiral carbon atom does not cause any significant hindrance for the stereoselective bonding formation. The influence of the side chain structure on the enantioseparation of racemic α-hydroxy carboxylic acids is presented in FIGURES 4 and 5. As can be seen in FIGURE 4 the enantiomers of α-hydroxycaproic acid with a straight alkyl group are eluted faster from the column than their isomers with a branched side chain due to their higher hydrophobicity. However, the bulkiness of the side chain influences the stereorecognition. In

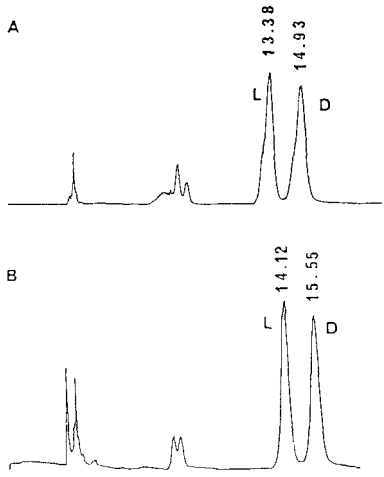

FIGURE 4. Chromatograms representing the enantioseparations of α-hydroxycaproic (A) and α-hydroxyisocaproic (B) acids. Chromatographic conditions as in FIG. 2.

spite of the longer retardation the chiral discrimination of the cyclodextrin phase towards the iso-caproic acid enantiomers is lower, probably due to the steric hindrance of the branched side chain. The same trend has been found in the separation of α-hydroxyvaleric and α-hydroxyisovaleric acids racemates (see TABLE 1). This effect is more pronounced (see FIGURE 5) under different mobile phase conditions, when the polar organic eluent contains significant amounts of methanol (10% v/v) as the third modifier. In this case the stereorecognition is affected not only by the analyte structure but also by competitive adsorption of methanol molecules. The influence of the solute structure on the chiral discrimination on the

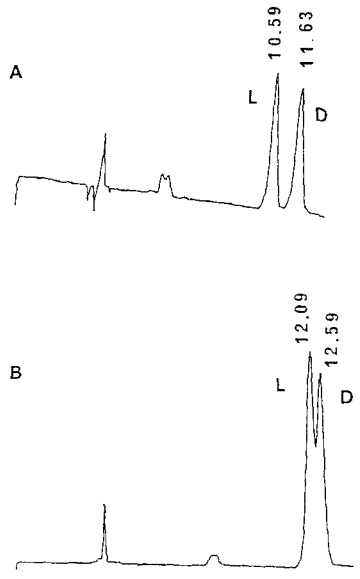

FIGURE 5. Enantioseparation of α–hydroxyvaleric (A) and α-hydroxyisovaleric (B) acids obtained on β-cyclodextrin stationary phase operated with eluent consisting of acetonitrile + methanol + triethylamine + acetic acid, 900 + 100 + 6 + 0.4, v/v/v/v.

native cyclodextrin in the water free system is also exemplified in FIGURE 6. As can be seen the change of the position of the hydroxy group in butyric acid significantly influences both the retardation and the enantioselectivity; in contrast to the excellent base line separation of α-hydroxybutyric acid, β-hydroxybutyric acid enantiomers can be only slightly recognized under the same chromatographic conditions.

The data presented above indicate that in the case of native cyclodextrin, with its homogenous (in energies)

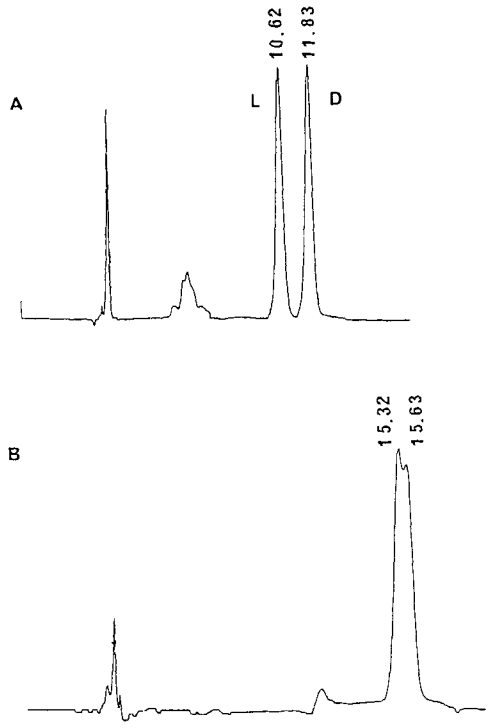

FIGURE 6. Influence of the solute structure on the chiral discrimination of native β -cyclodextrin stationary phase. Test compounds: α-hydroxybutyric acid (A), β–hydroxybutyric acid (B). Eluent: acetonitrile + methanol + triethylamine + acetic acid, 900 + 100 + 6 + 0.4, v/v/v/v.

adsorption sites, retardation and selectivity are mainly determined by the number and configuration of the groups in the analyte suitable for hydrogen bond formation with the secondary hydroxy groups of the cyclodextrin stationary phase. Note, that in the case of malic acid (see TABLE 1) containing two carboxylic groups significant amounts of methanol as well

TABLE 2

Optical Purity of Commercial Samples of L- and D-α-Hydroxy Carboxylic Acids

Acid Name	Source	Content of opposite enantiomer, %	Eluent	Standard deviation (n=4)
D-Lactic	Sigma	1.4	A	0.02
L-Lactic	Sigma	0.1	A	0.03
L-α-Hydroxyisovaleric	Fluka	-	A	-
L-α-Hydroxyisocaproic	Sigma	-	A	-
D-Malic	Aldrich	3.5	B	0.05
L-Malic	Aldrich	14.7	B	0.10
(+)-Hexahydromandelic	Aldrich	1.1	B	0.04
(-)-Hexahydromandelic	Aldrich	0.6	B	0.09

Eluent:

A - acetonitrile + triethylamine + acetic acid, 1000 + 5 + 2.5, v/v/v

B - acetonitrile + methanol + triethylamine + acetic acid, 750 + 250 + 10 + 5, v/v/v/v

as triethylamine and acetic acid were used to weaken the strength of complexation between the cyclodextrin and the analyte by the competitive adsorption of modifier molecules at the available adsorption (binding) sides. The competitive adsorption of the mobile phase components can be used for the optimisation of separation factors in chromatographic systems consisting of cyclodextrin stationary phase and water free eluents and has been previously used in the separation of many different classes of compounds [11-14].

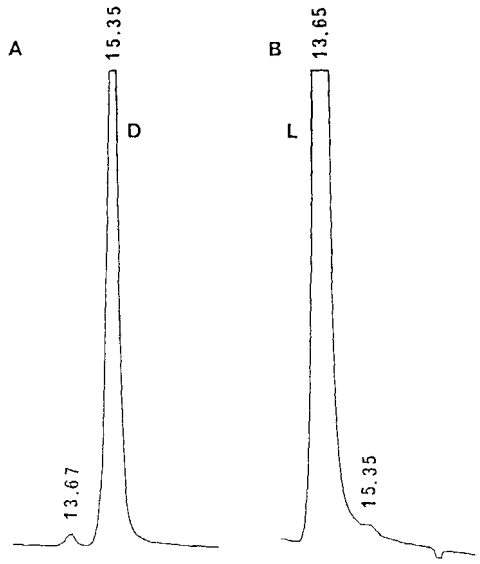

FIGURE 7. Chromatograms used for enantiomeric purity evaluations: D-lactic acid (A), L-lactic acid (B). Conditions as in TABLE 2.

Practical Application of the Method

The labelling with 2-quinoxaloyl chloride converts aliphatic α-hydroxy carboxylic acids into derivatives suitable for sensitive UV detection. The method has been used for determination of enantiomeric purity in a number of commercial samples, the results are presented in TABLE 2. Some level of impurities has been found in most samples investigated. The chromatograms used for the evaluation of optical purity of some commercial samples are shown in FIGURES 7 and 8. As can be seen from the data presented the

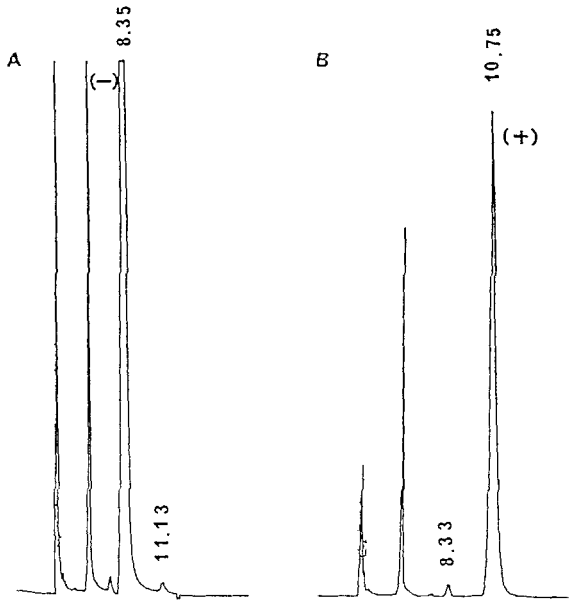

FIGURE 8. Chromatograms used for enantiomeric purity evaluations: (-)-hexahydromandelic acid (A), (+)-hexahydromandelic acid (B). Conditions as in TABLE 2.

procedure developed in this study enabled the determination of enantiomeric composition functionalised α-hydroxy carboxylic acids at the trace level. High selectivity and efficiency of the system as well as high symmetry of the peaks further contribute to the high accuracy and sensitivity of the method. In the case of L-lactic acid as low as 0.1% of the D enantiomer could be determined. Surprisingly high contamination with opposite enantiomer has been found in malic acid "optically pure" commercial samples. On the other hand no contamination has been detected in L-α-hydroxyisovaleric and L-α-hydrohyisocapric acids, which suggest that the derivatisation reaction induced essentially no racemisation.

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