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Separation of enantiomers of α-hydroxy acids by reversed-phase liquid chromatography after derivatization with 1-(9-fluorenyl)ethyl chloroformate

Bengt Fransson, Ulf Ragnarsson*

Department of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden

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Abstract

The application of the chiral reagent (+)-1-(9-fluorenyl)ethyl chloroformate, also known as (+)-FLEC and originally introduced for the separation of enantiomers of α-amino acids and the determination of their optical purity by HPLC, has been extended to enantioseparation within a set of α-hydroxy acids. Retention of the acidic diastereomeric carbonate derivatives thereby formed is conveniently regulated by the pH of the mobile phase and separation can generally be accomplished within about 30 min under isocratic conditions with tetrahydrofuran as organic modifier. Of six α-hydroxy acids so far examined five had separation factors of 1.24 or higher, whereas for malic acid it was in the range 1.10–1.15.

Keywords: Derivatization, LC; Enantiomer separation; Organic acids; Hydroxy acids; Fluorenylethyl chloroformate

1. Introduction

Chiral α-hydroxy acids occur abundantly in nature and have been isolated from animal tissues, plants and microorganisms [1]. Several of them are of considerable technological and commercial interest and are therefore prepared on a large scale by various methods including fermentation [2]. Many simple α-hydroxy acids can easily be made directly by the classical method from the corresponding amino acids [3], whereas for more complex ones asymmetric procedures are gaining in importance [4]. Some efficient general methods have recently been introduced in this context [5,6]. Chiral α-hydroxy acids often serve as convenient starting materials for the synthesis of homochiral compounds [2]. Reliable methods for the determination of the optical purity of α-hydroxy acids are consequently urgently required and a simple and convenient such, based on the application of the well-known reagent (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) and requiring only standard high-performance liquid chromatography (HPLC) equipment, is presented in this paper. FLEC was originally introduced for analysis of α-amino acids [7] and has been widely used in that context. The reagent as well as its enantiomer is commercially available.

With respect to the analysis of simple α-hydroxy acids, gas chromatography (GC) was first applied for the separation of diastereomeric derivatives of such on achiral stationary phases after introduction of

*Corresponding author. Fax: +46 18 552139, E-mail: urbki@bmc.uu.se

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various sets of suitable groups on the hydroxyl–carboxyl functions. Such sets included (−)-menthyloxycarbonyl–methyl ester [8], acetyl/different chiral esters [9,10], acetyl/(−)-menthyl ester [11] and trifluoroacetyl or trimethylsilyl/(+)-3-methyl-2-butyl ester [12]. These procedures are applicable, when a proper chiral column is not available but instead they obviously require a chiral reagent for derivatization. The first separation of enantiomers within the group of α-hydroxy acids was achieved by Bayer et al. [13,14] using a chiral GC column. The stationary phase used was a copolymer of dimethylsiloxane and (2-carboxypropyl)methylsiloxane, to the carboxylic groups of which a L-valine derivative had been coupled via its amino group. Separation of the enantiomers of lactic acid was originally accomplished after conversion to *O*-pentafluoropropionyl cyclohexylamides. More recently a large number of hydroxy acids were separated as simple esters with free hydroxyls on similar columns (Chirasil-Val) [15]. Since these columns are commercially available and applicable to all sorts of chiral substances, they have been widely used, although lately in competition with such containing cyclodextrin and similar materials as stationary phase [16]. Further relevant work based on the application of GC has been reviewed previously [17]. Among novel stationary phases applied to hydroxy acids, one with a tripodal selector bound to silica should be mentioned [18].

Several α-hydroxy acid enantiomers have been separated by capillary zone electrophoresis after addition of cyclodextrins [19,20] or the macrocyclic antibiotic ristocetin A [21] to the background electrolyte. Much work has recently been devoted to separation of this group of substances by HPLC using various columns and techniques. Pre-column derivatization with 2-quinoxaloyl chloride followed by separation on a cyclodextrin (Cyclobond) column has been reported [22]. Separation of the mandelic acid enantiomers on a similar column has been reviewed [23]. Three approaches involved the use of a chiral normal-phase [24] or reversed-phase column, in the latter case with the selector covalently [25] or ionically [26] bound to silica, whereas in another one the chiral selector was present in the mobile phase [27]. A chiral column was also used to separate lactic, malic and mandelic acid enantiomers in a recent paper [28]. In addition to silica columns such based on graphitic carbon, either with (2*R*,3*R*)-dicyclohexyl tartrate as chiral selector [29] or surface modified with a chiral amino acid derivative [30] have been examined and successfully applied for the separation of a few α-hydroxy acids.

2. Experimental

2.1. Materials, equipment and methods

Our L-lactic acid originated from Chemical Dynamics (South Plainfield, NJ, USA), D-lactic acid was a gift from Rhône-Poulenc (Melle, France), D- and L-malic acid and α-hydroxyisovaleric acid were purchased from Fluka (Buchs, Switzerland) and mandelic acid from Sigma (St. Louis, MO, USA), whereas D- and L-α-hydroxyisocaproic acid and 3-phenyllactic acid were made from the corresponding α-amino acids in connection with our earlier work [31]. (+)-1-(9-Fluorenyl)ethyl chloroformate, (+)-FLEC, was obtained from Akzo Nobel (Bohus, Sweden). According to the specification of the manufacturer, its optical purity was >99.9% and chemical purity >98%.

The liquid chromatography system consisted of a Model LC 10 AD solvent delivery system, a Model SPD 10A variable-wavelength detector (both Shimadzu) and a Rheodyne 7725 injector (Cotati, CA, USA). The column was a C_8, 3 μm, 15 cm cartridge system including a 1 cm reversed-phase integral guard column (Phase Separations, Clwyd, UK), The wavelength was set at 265 nm and the sensitivity at 0.01 or 0.02 absorbance unit at full scale. All separations were run at a nominal flow-rate of 1.0 or 2.0 ml/min at room temperature.

Buffers were prepared from acetic acid and 1 *M* sodium hydroxide to an ionic strength of 0.05 *M*. Isocratic binary mobile phases consisting of such buffers with tetrahydrofuran (THF) as organic modifier were used.

For the validation of the result in Fig. 2, a gas–liquid chromatography (GLC) experiment with a chiral capillary column (Chirasil-D-Val, 25 m×0.25 mm I.D., film thickness 0.11 μm) at 70°C after derivatization with isopropanol was performed [15].

2.2. Derivatization

(+)-FLEC reagent (50 μl; 18 m*M* in acetone) in a 2-ml test tube was flushed with nitrogen until the acetone had been removed, whereupon a solution of the α-hydroxy acid(s) (50 μl; 2 m*M* in pyridine) was added and the tube was kept in an ultrasonic bath (35 kHz, 120–240 W, type RK 106, Bandelin, Berlin, Germany) for 5 min. After the pyridine had been carefully removed by flushing with nitrogen, the remaining material was dissolved in borate buffer (1.00 ml; 0.5 *M*, pH=6.85) and 10–25 μl thereof injected without prior removal of the excess derivatization reagent.

In one case D,L-α-hydroxyisovaleric acid was reacted with (+)-FLEC and the areas for the diastereomeric products determined by an automatic integrator: The relative value found in this case was 1.00, indicating similar reactivity for the enantiomers.

3. Results and discussion

Six simple enantiomeric pairs of α-hydroxy acids (**1**) have been studied in this context, i.e., lactic acid, α-hydroxyisovaleric acid, α-hydroxyisocaproic acid, mandelic acid, 3-phenyllactic acid and malic acid. On reaction with (+)-FLEC, the absolute configuration of which is unknown, products of the general structure (**2**) are formed (Fig. 1), suitable for chromatographic separation.

By contrast with amino acids, which can and as a consequence of their zwitterionic nature must be derivatized with FLEC in aqueous buffers [7], the much lower nucleophilicity of hydroxyl in comparison with that of amino groups requires an organic solvent in this step. Using pyridine and a chloroformate, we recently succeeded to convert serine and threonine hydroxyls into analogous carbonate functions [32].

Individual derivatives, **2**, were injected onto a reversed-phase column and chromatographed in buffers under isocratic conditions with tetrahydrofuran as organic modifier. Peaks were monitored by a UV detector, although in the pioneering work dealing with amino acids detection was by fluorescence [7]. The first five α-amino acids gave rise to similar chromatograms. A typical such for the mixture of

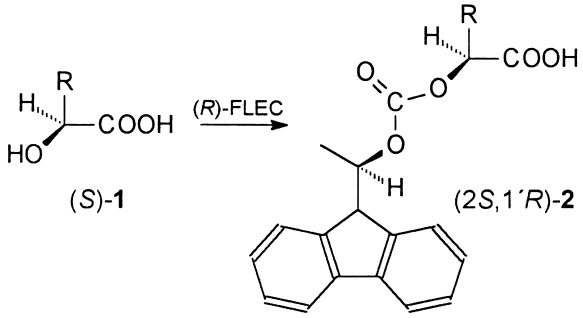

Fig. 1. Derivatization of an (*S*)-α-hydroxy acid with FLEC [drawn for (*R*)-FLEC].

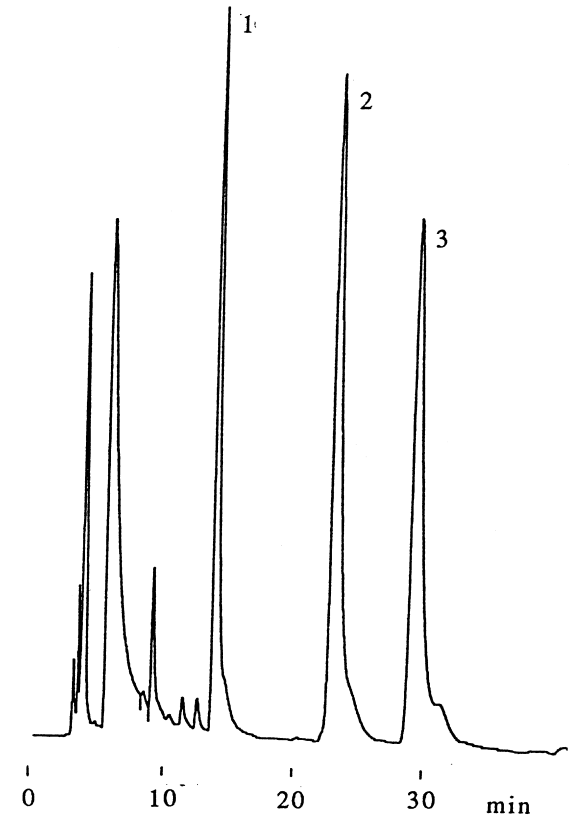

Fig. 2. Separation of α-hydroxyisovaleric acid enantiomers by reversed-phase liquid chromatography after derivatization with (+)-FLEC. Column: Spherisorb C_8, 3 μm (150×4.6 mm); mobile phase 0.05 *M* acetate, pH 3.80–THF (60:40), flow-rate 1.00 ml/min; detection wavelength 265 nm. Peak 1: (±)-1-(9-fluorenyl)ethanol; peaks 2 and 3: D- and L-hydroxyisovaleric acid.

Table 1
Capacity factors (k') and separation factors (α) for some α-hydroxy acids after derivatization with (+)-FLEC[a]

α-Hydroxy acid	k' (D)	k' (±)[b]	k' (L)	α
Lactic acid[c]	15.59		19.67	1.26
Lactic acid[d]	9.41		11.71	1.24
Lactic acid[e]	8.58		10.62	1.24
(±)-1-(9-Fluorenyl)ethanol[c]		27.64		
(±)-1-(9-Fluorenyl)ethanol[d]		22.50		
(±)-1-(9-Fluorenyl)ethanol[e]		7.15		
α-Hydroxyisovaleric acid[f]	5.19		6.86	1.32
α-Hydroxyisocaproic acid[f]	7.47		9.97	1.33
Mandelic acid[f]	4.30		5.64	1.31
3-Phenyllactic acid[f]	4.87		6.59	1.35
(±)-1-(9-Fluorenyl)ethanol[f]		2.70		
Malic acid[c]	12.12		13.70	1.15
Malic acid[g]	4.88		5.38	1.10
Malic acid[h]	4.88		5.36	1.10
(±)-1-(9-Fluorenyl)ethanol[g]		28.64		
(±)-1-(9-Fluorenyl)ethanol[h]		28.54		

[a] Column: Spherisorb C_8, 3 μm, 150×4.6 mm.
[b] (±)-1-(9-Fluorenyl)ethanol co-chromatographs with (+)-FLEC.
[c] Mobile phase: 0.05 *M* acetate, pH 4.35–THF (76:24); flow-rate: 2.00 ml/min; t_0=1.68 min.
[d] Mobile phase: 0.05 *M* acetate, pH 4.50–THF (76:24); flow-rate: 1.00 ml/min; t_0=4.03 min.
[e] Mobile phase: 0.05 *M* acetate, pH 3.80–THF (68:32); flow-rate: 1.00 ml/min; t_0=3.93 min.
[f] Mobile phase: 0.05 *M* acetate, pH 3.80–THF (60:40); flow-rate: 1.00 ml/min; t_0=3.73 min.
[g] Mobile phase: 0.05 *M* acetate, pH 4.50–THF (76:24); flow-rate: 2.00 ml/min; t_0=1.68 min.
[h] Mobile phase: 0.05 *M* acetate, pH 5.00–THF (76:24); flow-rate: 2.00 ml/min; t_0=1.68 min.

D- and L-hydroxyisovaleric acid is shown in Fig. 2. The results from all chromatography experiments are collected in Table 1.

As shown in Table 1, the first five α-hydroxy acid enantiomeric pairs are well separated within about 30 min giving α values in the range 1.24–1.35. Excess FLEC shows up as a broad peak, the position of which can be shifted to appear before or after the hydroxy acids. In all systems investigated, the chiral alcohol derived from FLEC co-chromatographs with FLEC. Consequently, assignment of configuration is simple and straightforward using this procedure. In all cases so far using (+)-FLEC, the D-isomer of the α-hydroxy acid appears before the L-isomer.

The new method can obviously be used to determine small amounts of enantiomeric impurities present in α-hydroxy acids and this was a major aim of our work, since we are using them as precursors for the synthesis of ^{15}N-labelled amino acid derivatives [31]. Commercial FLEC is very well resolved and previously allowed us to monitor optical purity up to 99.9% enantiomeric excess (e.e.) using an automatic integrator [33]. A typical example obtained for a sample of D-lactic acid is given in Fig. 3.

In comparison with the other α-hydroxy acids investigated in this context, malic acid gave a more complex chromatogram (Fig. 4). This is presumably due to the competing formation of cyclic anhydrides, and their subsequent reactions with alcohol derived from FLEC. Such reactions are well documented in the literature [34]. Peak 4 in this chromatogram is tentatively assigned to anhydrides and peak 3 to monoesters which are both broadened but not further resolved. These assignments are supported by the facts that k' is essentially constant (19.93/20.39/20.32) for peak 4 in the three chromatography systems with pH 4.35/4.50/5.00, whereas that for peak 3 is sensitive to changes in pH (19.07/16.37/15.80). Besides, the ratio between the integrated peak areas initially varies considerably.

As a consequence the yield of simple malic acid diastereomers in the standard derivatization procedure is lower than for the monocarboxylic acids. The resolution is also noticeably lower for malic acid,

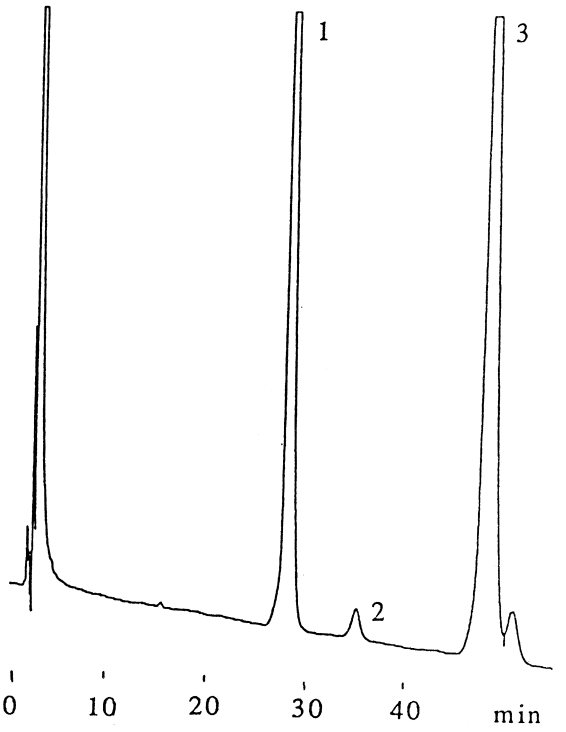

Fig. 3. Determination of the optical purity of D-lactic acid by reversed-phase liquid chromatography after derivatization with (+)-FLEC. Column: Spherisorb C_8, 3 μm (150×4.6 mm); mobile phase 0.05 *M* acetate, pH 4.35–THF (76:24), flow-rate 2.00 ml/min; detection wavelength 265 nm. Peaks 1 and 2: D- and L-lactic acid; peak 3: (±)-1-(9-fluorenyl)ethanol. The purity of D-lactic acid corresponds to 95.7% e.e.. The result was validated by GLC on a chiral capillary column [15]. The value found by this method was 96.0% e.e. [k'=11.51 (L) and 12.58 (D); α=1.09].

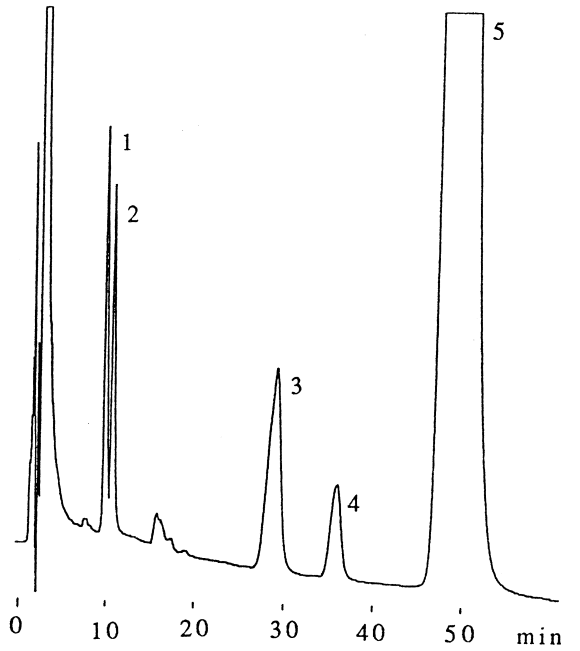

Fig. 4. Separation of malic acid enantiomers and by-products by reversed-phase liquid chromatography after derivatization with (+)-FLEC. Column: Spherisorb C_8, 3 μm (150×4.6 mm); mobile phase 0.05 *M* acetate, pH 4.50–THF (76:24), flow-rate 2.00 ml/min; detection wavelength 265 nm. Peaks 1 and 2: D- and L-malic acid; peak 3: presumably monoester; peak 4: presumably anhydride and peak 5: (±)-1-(9-fluorenyl)ethanol.

indicating a need for further optimization in this case, particularly for determination of the optical purity.

4. Conclusions

As demonstrated above, α-hydroxy acids like α-amino acids can be derivatized with FLEC to give diastereomers suitable for separation by reversed-phase HPLC but in this case the sample preparation must be conducted in the absence of water. Based on experiences from six investigated examples, the corresponding acidic, rather hydrophobic derivatives are suitable for chromatographic resolution which can be monitored by their inherent UV absorbance. The separations can be regulated by variation of the pH of the elution buffer and require a powerful organic modifier for the desorption of the components as well as the reagent. Practically useful results were obtained for all α-hydroxy acids studied. For five of them the separation factors were at least as high as those reported for the corresponding amino acids. For mandelic acid it was 1.31, whereas no data for phenylglycine is available.

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