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RESEARCH PAPER

Fast, sensitive LC–MS resolution of α-hydroxy acid biomarkers via SPP-teicoplanin and an alternative UV detection approach

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Abstract

Enantioseparation of α-hydroxy acids is essential since specific enantiomers of these compounds can be used as disease biomarkers for diagnosis and prognosis of cancer, brain diseases, kidney diseases, diabetes, etc., as well as in the food industry to ensure quality. HPLC methods were developed for the enantioselective separation of 11 α-hydroxy acids using a superficially porous particle–based teicoplanin (TeicoShell) chiral stationary phase. The retention behaviors observed for the hydroxy acids were HILIC, reversed phase, and ion-exclusion. While both mass spectrometry and UV spectroscopy detection methods could be used, specific mobile phases containing ammonium formate and potassium dihydrogen phosphate, respectively, were necessary with each approach. The LC–MS mode was approximately two orders of magnitude more sensitive than UV detection. Mobile phase acidity and ionic strength significantly affected enantioresolution and enantioselectivity. Interestingly, higher ionic strength resulted in increased retention and enantioresolution. It was noticed that for formate-containing mobile phases, using acetonitrile as the organic modifier usually resulted in greater enantioresolution compared to methanol. However, sometimes using acetonitrile with high ammonium formate concentrations led to lengthy retention times which could be avoided by using methanol as the organic modifier. Additionally, the enantiomeric purities of single enantiomer standards were determined and it was shown that almost all standards contained some levels of enantiomeric impurities.

Keywords Disease biomarkers · Hydroxy acids · Enantiomers · TeicoShell · Enantioselectivity · Mass spectrometry

Introduction

Alpha hydroxy acids (AHAs) have a wide range of applications. Their exfoliating and moisturizing properties make them useful in dermatology [1–3]. Mechanistically, the chelating ability of AHAs enables them to reduce calcium ion concentrations in the epidermis resulting in disruption of cellular adhesions and exfoliation [4]. Topical lactic acid solutions have shown to affect the epidermis and dermis and increase cell turnover based on the concentration of lactic acid in the solution [2]. In the food industry, phenyllactic acid isolated from bakery products was shown to have antifungal activity against molds [5]. Also, D-3-phenyl lactic acid exhibited antifungal activity against *Salmonella enterica* [6]. The change in concentration of D-lactic acid in fermented dairy products could be an indication of the bacterial activity [7].

More importantly, there are pathologic effects resulting from depletion or excess of single enantiomers of α-hydroxy acids in humans and/or other animals. L-Lactic acid is the natural form present in humans [8]. Some medical conditions are related to an imbalance of L-lactic acid levels in the human body. For example, hypoxia, sepsis, pancreatitis, thiamine deficiency, delirium tremens, and diabetic ketoacidosis could be indicative of hyperlactatemia (increased levels of L-lactic acid above the normal ranges) or more severely, lactic acidosis [9]. Low levels of L-lactic acid (hypolactatemia) are less common and could occur when there is an increase in pyruvate dehydrogenase activity by dichloroacetate [10]. D-Lactic acid is produced by various fungal and bacterial species [8]. Excessive consumption of highly fermentable concentrates by calves can increase levels of *Streptococcus bovis* in their rumen, which increases D-lactic acid production resulting in lower pHs (≤ 5) which destroys other useful rumen bacteria [11]. D-Lactic acidosis is a rare neurologic syndrome in humans. Its cause is unrelated to those that result in L-lactic acidosis as it occurs after jejunoileal

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bypass surgery or in individuals with short bowel syndrome [12]. Another study showed that elevated levels of D-lactate in the plasma and urine of patients with D-lactate dehydrogenase deficiency was accompanied by increased levels of D-2-hydroxyisovaleric acid and D-2-hydroxyisocaproic acid [13]. Moreover, it was found that D-lactic acid was elevated in the saliva, urine, and plasma of patients with diabetes [14]. Elevated levels of D-lactate anions in diabetic ketoacidosis have been related to an increased plasma anion gap (an imbalance between anions and cations in the plasma) [14].

L-2-Hydroxyglutaric aciduria, D-2-hydroxyglutaric aciduria, and combined D, L-2-hydroxyglutaric aciduria are disorders that are related to accumulated amounts of the corresponding 2-hydroxyglutaric acid enantiomers [15–18]. L-2-Hydroxyglutaric acid accumulation can occur due to gene mutations resulting in either a deficiency of L-2-hydroxyglutaric acid–metabolizing enzymes or increased activity of certain mitochondrial enzymes which produce L-2-hydroxyglutaric acid as a side product [15, 16]. The symptoms usually include developmental delays, neurological problems, hypoxia, and brain dysfunction [15–17, 19]. Additionally, L-2 hydroxy aciduria was thought to have a possible role in predisposing individuals to brain tumorigenesis and Wilms tumor [20]. The imbalanced D- and L-hydroxyglutaric acid levels resulting from IDH1/IDH2 genes (the gene encoding cytosolic isocitrate dehydrogenase-1/2) have been related to various diseases, including glioma, cardiomyopathy, kidney cancer, tumor suppressor gene mutation, and tumor growth [14, 21]. Increased D-hydroxyglutaric acid levels could also cause mitochondrial dysfunction by activating NMDA receptors and dysregulation of intercellular calcium ions [22].

Clearly, it is important to separate and measure the enantiomers of AHAs since the absolute quantities and ratios of such enantiomers are pertinent to different medical conditions. Previous studies have tried to separate hydroxy acid enantiomers using methods like capillary electrophoresis [23, 24], nuclear magnetic resonance (NMR) [25], and liquid chromatography [26–28]. In this study, baseline resolution was achieved for 10 α-hydroxy acids under 8 min. Different conditions were required for UV detection vs. mass spectrometric detection. In addition, the effect of mobile phase acidity, salt concentration, and organic modifier on enantiomeric separations of AHAs was investigated using a teicoplanin-based chiral stationary phase. Teicoplanin was first introduced in 1995 as a chiral selector for HPLC [29]. TeicoShell, which is teicoplanin bonded to superficially porous silica particles, is a chiral stationary phase that has been used in various modes of HPLC including normal phase mode, reversed phase mode, and the polar organic mode to separate amino acids, betablockers, and various acidic and neutral pharmaceutical compounds [30–34].

Materials and methods

Reagents and materials

All analytes in Table 1, potassium dihydrogen phosphate, and ammonium formate were purchased from Millipore-Sigma (formerly Sigma-Aldrich, St. Louis, MO). Lactic acid, 2-hydroxyglutaric acid, 2-hydroxyisovaleric acid, mandelic acid, 2-hydroxyisocaproic acid, and phenyllactic acid had a single enantiomer standard available to test the elution order. Optima™ LC–MS grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). The chiral stationary phase was obtained by covalently attaching the teicoplanin glycopeptide to silica gel via linkage chain [29]. TeicoShell HPLC column (15 cm × 3 mm i.d., on 2.7 µm superficially porous silica particles (SPPs)) was provided by AZYP, LLC, Arlington, TX.

Instrumentation

The UHPLC-UV instrument used was an Agilent 1290 Infinity series system (Agilent Technologies, Santa Clara, CA) equipped with a quaternary pump, an auto-sampler, and a diode array detector. The instrument was controlled by OpenLAB CDS ChemStation software (Rev. C.01.06, Agilent Technologies 2001–2014). The UHPLC-MS instrument was TSQ s II MS, triple quadrupole from Thermo Scientific. The instrument was controlled by Chromeleon 7 software.

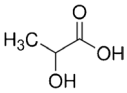
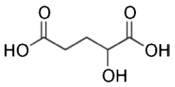
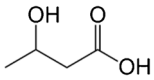
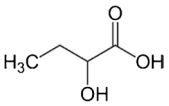
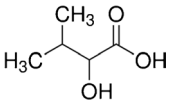
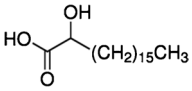
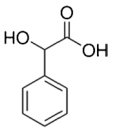
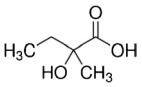
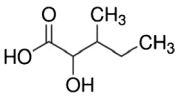
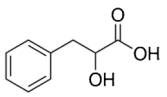
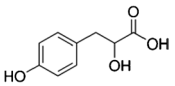
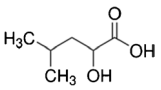
Methods

Mass spectrometry vs. UV spectroscopy detection

Since most of the investigated compounds in this study did not contain good chromophores, they must be analyzed with either specific selective detectors like mass spectrometers (MS) or with highly transparent salts (phosphate-based) in the mobile phase with which low-wavelength UV detection is feasible.

LC–MS analyses Analyte concentrations were ~ 0.2 to 0.02 mg/ml in 50/50 acetonitrile/water, except 2-hydroxystearic acid which was dissolved in methanol. Injection volumes were 0.1 to 0.5 µL. All experiments were done in selected ion monitoring (SIM) mode. The monitored m/z values are listed in Table 1. The negative ion voltage was set to 2500 V. Ion transfer tube temperature and vaporizer temperature were at 325 °C and 350 °C, respectively. Scan data rate was 250 Da/s with scan width of 10 m/z. For LC–MS analyses, ammonium formate plus formic acid was used to investigate the effect of acidity of the aqueous phase and salt concentration on the enantioseparation of AHAs. Acetonitrile and methanol were investigated as organic modifiers.

Table 1 Structure of the compounds analyzed in this study. *SIM*, selected ion monitoring mode

	Name	Structure	SIM m/z (Negative ion mode)	pKa*
1	Lactic acid		89	3.86
2	2-Hydroxyglutaric acid		147	3.28
3	3-Hydroxybutyric acid		215	4.41
4	2 -Hydroxybutyric acid		103	3.99
5	α-Hydroxyisovaleric acid (2-Hydroxy-3-methylbutyric acid)		117	4.14
6	2-Hydroxystearic acid		299	4.75
7	Mandelic acid		151	3.41
8	2-Hydroxy-2-methylbutyric acid		117	4.10
9	2-Hydroxy-3-methylvaleric acid		131	4.25
10	Phenyllactic acid		165	4.31
11	p-Hydroxyphenyllactic acid		181	3.58
12	2-Hydroxyisocaproic acid		131	4.26

*Source: ChemAxon

LC-UV analyses Analyte concentrations were ~ 2 mg/ml in 50/50 acetonitrile/water, except 2-hydroxystearic acid which was dissolved in methanol. Injection volumes were 0.3 µL. Sampling rate was at 40 Hz with response time of 0.13 s. Due to the low UV cutoff of most of the analyzed hydroxy acids, ammonium acetate or ammonium formate salts could not be added to the mobile phase when using a UV detector. Therefore, potassium dihydrogen phosphate was used as the additive. The mobile phase consisted of acetonitrile as the organic modifier and potassium dihydrogen phosphate with different concentrations (20 mM, 10 mM, 5 mM) and pH values (3, 4, 5, 6) as the aqueous solvent. The pH of the aqueous solution was adjusted with potassium hydroxide or phosphoric acid. It should be noted that the solubility of dihydrogen phosphate is limited in acetonitrile. Therefore, increasing the salt concentration in the aqueous phase or increasing the concentration of acetonitrile in the mobile phase could lead to precipitation.

Results and discussion

Optimized conditions using MS detection and formate-containing mobile phases

Table 2 lists the optimum conditions for separation of all of the AHAs in this study using formate-containing mobile phases and MS detection. 2-Hydroxy-2-methylbutyric acid was an analyte that did not separate despite its structural similarity to other analytes. The methyl group on the chiral center could have disrupted the interaction between the hydroxyl group and the stationary phase which led to loss of enantioselectivity. This also happened with quinine-based chiral stationary phases in other studies [28]. Regarding the elution order of the enantiomers, it was observed that the S enantiomers always eluted before the R enantiomers for analytes that had single enantiomer standards available. In order to obtain the optimized conditions, effects of salt concentration, acidity of the mobile phase, the organic modifiers, and the mobile phase flow rate were investigated as discussed in upcoming sections.

Retention behaviors with different organic modifiers

As observed in Fig. 1, the retention behavior of two different molecules that differed significantly in terms of hydrophobicity (i.e., lactic acid and 2-hydroxystearic acid) was investigated at different mobile phase compositions with

Table 2 Optimum conditions for chiral separation of α-hydroxy acids by LC-MS. HPLC column: TeicoShell. 15 cm × 3 mm i.d., 2.7 μm SPP. For information on all investigated mobile phases, see Table S2. k_1, retention; α, selectivity; *Rs*, resolution; *AF*, ammonium formate

Name	Optimum mobile phase condition	k_1	α	Rs
Lactic acid	85/15 ACN/AF 20 mM, pH 5, 0.3 ml/min	1.58	1.19	2.4
2-Hydroxyglutaric acid	90/10 MeOH/AF 100 mM, pH 5, 0.2 ml/min	0.44	2.56	1.7
2-Hydroxybutyric acid	85/15 ACN/AF 20 mM, pH 5, 0.3 ml/min	1.08	1.17	1.7
2-Hydroxyisovaleric acid	90/10 MeOH/AF 100 mM, pH 5, 0.15 ml/min	−0.13	NA*	1.5
2-Hydroxystearic acid	90/10 MeOH/AF 100 mM, pH 5, 0.3 ml/min	−0.10	NA*	2.3
Mandelic acid	85/15 ACN/AF 20 mM, pH 3, 0.3 ml/min	0.29	2.66	5.2
2-Hydroxy-3-methylvaleric acid (peaks 1, 2)	90/10 MeOH/AF 100 mM, pH 5, 0.1 ml/min	−0.22	NA*	2.0
2-Hydroxy-3-methylvaleric acid (peaks 2, 3)	90/10 MeOH/AF 100 mM, pH 5, 0.1 ml/min	0.36	1.52	1.5
Phenyllactic acid	85/15 ACN/AF 50 mM, pH 5, 0.3 ml/min	0.41	1.16	1.6
p-Hydroxyphenyllactic acid	85/15 ACN/AF 20 mM, pH 5, 0.3 ml/min	0.69	1.14	1.6
2-Hydroxyisocaproic acid	85/15 ACN/AF 20 mM, pH 5, 0.3 ml/min	0.52	1.29	1.6

*The first enantiomer of this analyte eluted before the dead time. Therefore, the k value was negative and selectivity was not calculated

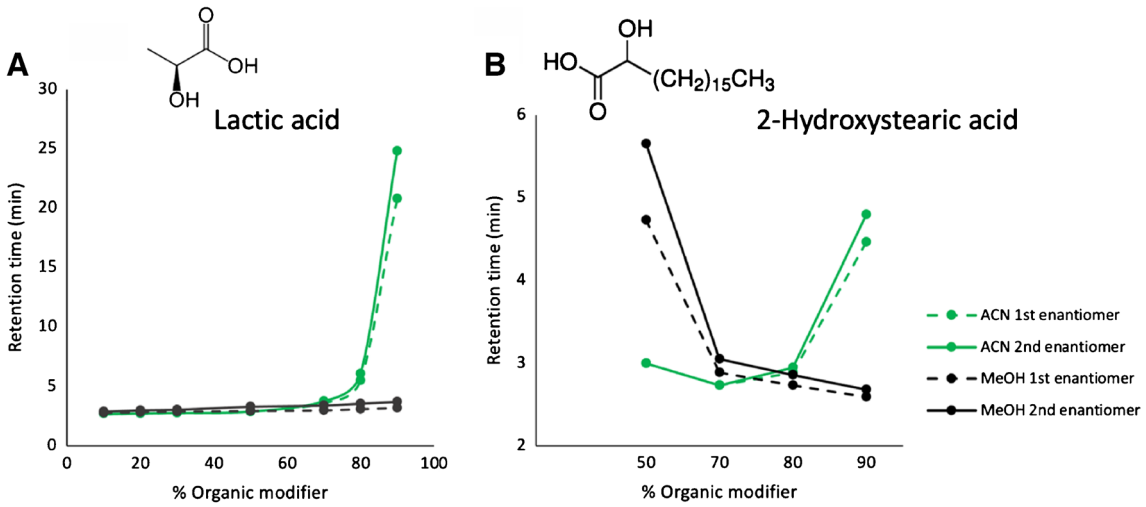


Fig. 1 **A**, **B** Retention behavior of lactic acid and 2-hydroxystearic acid in different percentages of organic modifiers, acetonitrile and methanol. The aqueous part of the mobile phase contained 20 mM ammonium formate, pH = 5. Flow rate 0.2 ml/min. Detection: mass spectrometry, negative ion mode, monitored m/z: 89 for lactic acid and 299 for 2-hydroxystearic acid

both methanol and acetonitrile as an organic modifier with 20 mM ammonium formate (pH = 5) as the aqueous solvent. As shown in Fig. 1A, for lactic acid, it was observed that increasing the organic modifier concentration increased the retention with both organic modifiers. With methanol-containing mobile phases, the lower retention of lactic acid resulted in peak overlap with impurities including those of similar mass (at 90% MeOH concentration). This can be avoided by using acetonitrile as the organic modifier. The retention behavior of lactic acid was similar to that of hydrophilic interaction liquid chromatography (HILIC) mode since the retention increases by increasing the percentage of the organic modifier in the mobile phase. The increase in retention by increasing acetonitrile content in the mobile phases can be related to increased hydrogen bonding interactions between the chiral stationary phase and the analyte. On the other hand, for methanol-containing mobile phases due to hydrogen bonding ability of methanol, retention was much lower. This has been observed with cyclodextrin- and cyclofructan-based stationary phases as well [35–40]. Table S3 of Supplementary Information (SI) shows that by increasing the organic modifier concentration, the enantioresolution increased for lactic acid.

As observed in Fig. 1B, the hydrophobic molecule, 2-hydroxystearic acid, did not elute at high aqueous phase compositions (> 55% water) given its poor solubility in water. With methanol-containing mobile phases, typical reversed phase retention behavior was observed. For acetonitrile-containing mobile phases, at low organic concentrations, reversed phased type behavior was observed. However, high acetonitrile concentrations increased retention (i.e., HILIC behavior). Table S3 of SI shows that for 2-hydroxystearic acid in methanol-containing mobile phases, the enantioresolution improved at higher aqueous concentrations. However, by increasing the aqueous content of the mobile phase, the peaks broadened due to the increased hydrophobic effect [41]. With acetonitrile-containing mobile phases, increasing the organic modifier increased enantioresolution for 2-hydroxystearic acid.

Ion-exclusion was another factor affecting the retention behavior which is observed when ionic analytes elute at/or earlier than the dead time [42]. It occurs due to electrostatic repulsion between analytes and the stationary phase [42].

Notably, the solubility of 2-hydroxystearic acid is greater in methanol than acetonitrile; therefore, in high concentration acetonitrile mobile phases, the loadability decreases. Thus, injecting too high an amount of analyte with such a mobile phase resulted in split peaks and poor peak shapes (Fig. 2B). By lowering the amount of analyte injected into the system, this issue can be resolved (see Fig. 2A).

Effect of the mobile phase acidity with formate-containing mobile phases

The effect of eluent acidity has been studied for a group of aromatic hydroxy acids and their derivatives using a teicoplanin-based stationary phase with 5-µm silica particles [27]. It was shown that by increasing the pH of the aqueous solvent, the enantioselectivity and retention increased up to a certain point and then decreased or remained unchanged [27]. The pKas of compounds in this study ranged between 3.3 and 4.8. The investigated pH values for the aqueous solvents were 3, 4, 5, and 6. As shown in Fig. 3A, with ammonium formate–containing mobile phases, the retention values showed an initial increase and then a slight decrease when increasing the pH of the aqueous component, except 2-hydroxyglutaric acid which always exhibited increased retention. Figure 3B indicates that increasing the pH of the aqueous solvent increased the resolution values up to pH of 5. The resolutions decreased from pH 5 to 6, except for 2-hydroxyglutaric acid which showed a continuous increase in resolution by decreasing the acidity of the mobile phase. As observed in Fig. 3C, the selectivity values slightly increased from an aqueous solvent pH of 3 to 4 and then showed little to no change subsequently. The chromatographic data for all other analytes is listed in SI Table S2.

There are a few ionizable moieties in the teicoplanin structure. This molecule consists of four fused macrocyclic rings, containing seven aromatic rings with ionizable phenolic

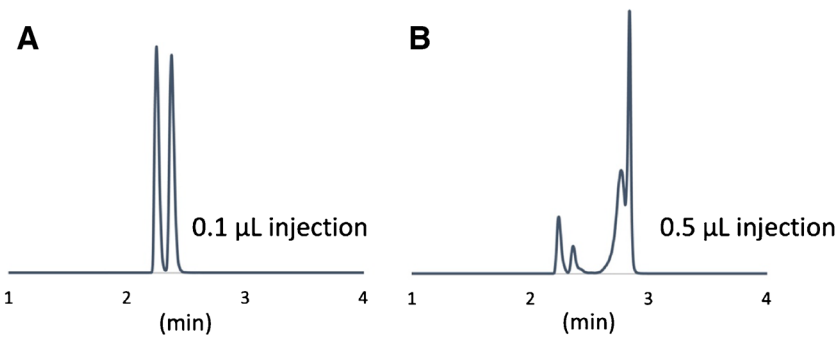


Fig. 2 Effect of amount of injection, i.e., solubility, on peak shape for 2-hydroxystearic acid. Mobile phase: 94/6 acetonitrile/20 mM ammonium formate pH = 6. Flow rate 0.3 ml/min. Detection: mass spectrometry, negative ion mode, monitored m/z: 299. **A** Injection volume: 0.1 µl; **B** injection volume 0.5 µl

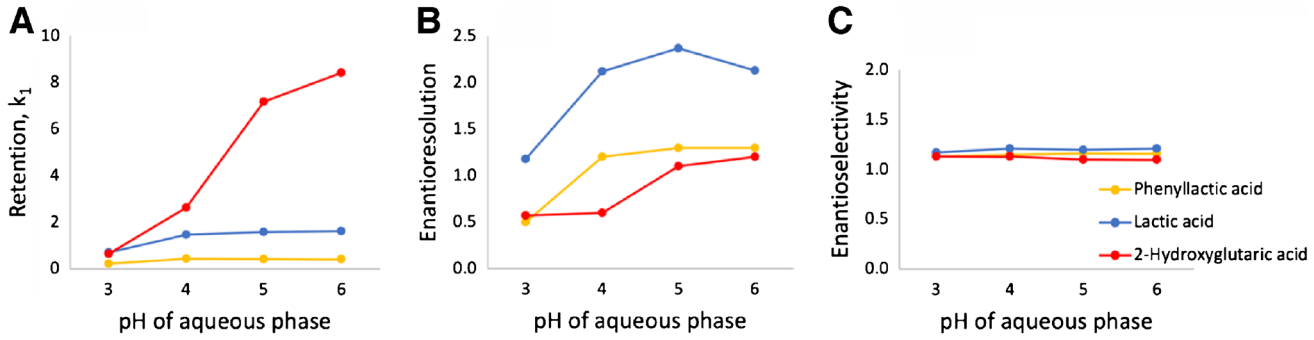


Fig. 3 Observed trends on effect of mobile phase acidity on **A** retention (k_1), **B** enantioresolution, and **C** enantioselectivity. Mobile phase: 85/15 acetonitrile/ammonium formate 20 mM, flow rate 0.3 ml/min. Detection: mass spectrometry. See "Materials and methods" for detection conditions

moieties [29]. Additionally, the teicoplanin molecule contains a primary amine and a carboxylate group, which are ionizable and can have different charges based on the acidity of the mobile phase [29]. Thus, the charge state of stationary phase changes with mobile phase acidity/basicity. The acidity of the mobile phase also can affect the conformation of the stationary phase. All of these factors can affect enantioseparations [29].

Considering the pKa values for teicoplanin to be 3.2 and 5.6 [43], the observed trends can be elaborated as follows: In any mobile phase that has an excess of acid, most of ionizable groups are protonated, giving the stationary phase a net positive charge, which results in retaining the carboxylated analytes. By increasing the pH of aqueous solvent up to a certain level (considering the stationary phase and analytes' pKa) and increased ionization of analytes, attractive coloumbic interactions between the carboxylate group of analyte molecules and positive charged groups of the stationary phase (i.e., primary amine) increase and lead to higher retention and selectivity.

Salt concentration effect with formate-containing mobile phases

Additives have been used in the LC reversed phase mode, normal phase mode, and HILIC mode to improve peak shapes and enhance resolution [31, 43–48]. Figure 4 shows that by increasing the concentration of ammonium formate in aqueous solution (at constant pH), retention increased and efficiency and resolution improved. The same general trend was observed in Table S2 in SI, with both acetonitrile and methanol organic modifiers.

The salt concentration (i.e., ionic strength) effects could be attributed to the fact that salt molecules have a shielding effect on electrostatic interactions between analytes and the stationary phase. The salt molecules could shield the analytes from interaction sites of the same charge (repulsive interaction) and result in longer retention as observed in this study [49]. Also, this could be the effect of salting out analytes from the mobile phase. Additionally, the shielding effect could improve the mass transfer of analytes by minimizing secondary interactions like silanol activity, leading to improved peak efficiencies which resulted in better resolutions [49].

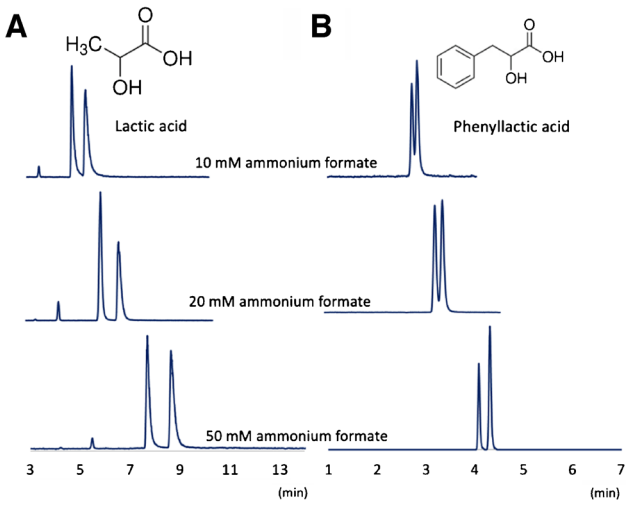


Fig. 4 Total ion chromatograms showing the effect of ammonium formate concentration on enantioseparation of lactic acid and phenyllactic acid. Mobile phase: 85/15 acetonitrile/ammonium formate pH = 5, flow rate 0.3 ml/min, detection: MS, negative ion mode, monitored m/z: **A** lactic acid: 89; **B** phenyllactic acid: 169

Effect of organic modifier and mobile phase flow rate with formate-containing mobile phases

As indicated in Table S2 in the SI, at similar mobile phase compositions, acetonitrile-containing mobile phases provide greater retention and enantioresolution than methanol-containing mobile phases. However, as seen in Table 2, the optimal mobile phase organic modifier was sometimes methanol and sometimes acetonitrile. Clearly, there are tradeoffs when it comes to the optimum condition. When higher salt concentrations were used to improve the separation, retention times increased. Therefore, it was beneficial to use methanol

as the organic modifier to avoid lengthy retention times that occurred with acetonitrile. This was the case for 2-hydroxyglutaric acid, 2-hydroxyisovaleric acid, 2-hydroxystearic acid, and 2-hydroxy-3-methylvaleric acid. For 2-hydroxyglutaric acid, the change in retention by changing the organic modifier was quite pronounced. As shown in Fig. 5, using methanol as an organic modifier and 100 mM ammonium formate concentration resulted in an optimum separation in less than 5 vs. 37 minutes with an acetonitrile-containing mobile phase. As mentioned before, in some cases like lactic acid, lowering the retention times (using methanol as the organic modifier) resulted in coelution with impurities of similar low m/z. Therefore, in this case, the optimal organic modifier was acetonitrile (Table 2).

Figure 6 shows that lowering the flow rate improved the enantioresolution. Since methanol-containing mobile phase resulted in lower retentions and faster separations (vs. acetonitrile-containing mobile phases of the same composition), lower flow rates could be used to increase enantioresolution with methanol-containing mobile phases (Fig. 6). Previous studies have also shown that macrocyclic glycopeptide-based SPP chiral stationary phases had lower van Deemter minimum compared to other columns of the same dimensions [31, 50].

Optimized conditions using UV detection and phosphate-containing mobile phases

Table 3 lists the optimum conditions for separation of all of the AHAs in this study using phosphate-containing mobile phases and UV detection. It should be noted that the analyte concentrations where 1–2 orders of magnitude higher in these studies (with UV detection) than in the aforementioned LC–MS studies given the lower sensitivity of UV vs. MS detection. Also, only acetonitrile organic modifier and a phosphate-based salt could be used at these low detection wavelengths. Interestingly, most of these chiral α-hydroxy acids could be well resolved with both approaches provided optimized conditions were used. Also, as observed with formate-based mobile phases, the S enantiomer always eluted before the R enantiomer for analytes that had single enantiomer standards available. In order to obtain the optimized conditions, effects of salt concentration and the acidity of the mobile phase were investigated as discussed in upcoming sections.

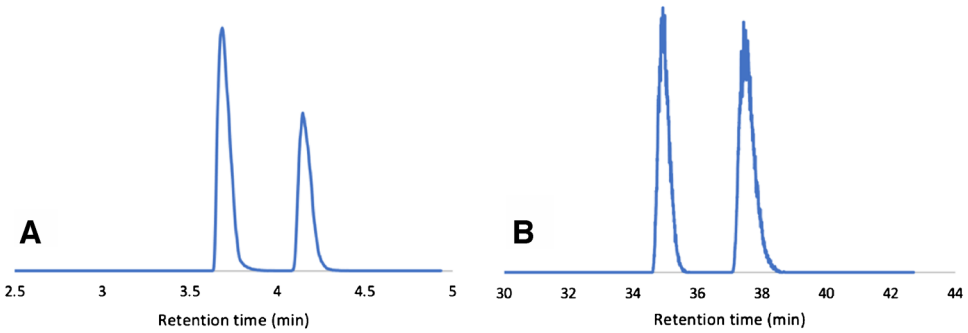


Fig. 5 Effect of organic modifier on enantioseparation of 2-hydroxyglutaric acid. Mobile phase: **A** 90/10, methanol/100 mM ammonium formate pH = 5, flow rate: 0.2 ml/min vs. **B** 85/15, acetonitrile/50 mM ammonium formate pH = 5, flow rate: 0.3 ml/min. Detection: MS, negative ion mode, monitored m/z: 147

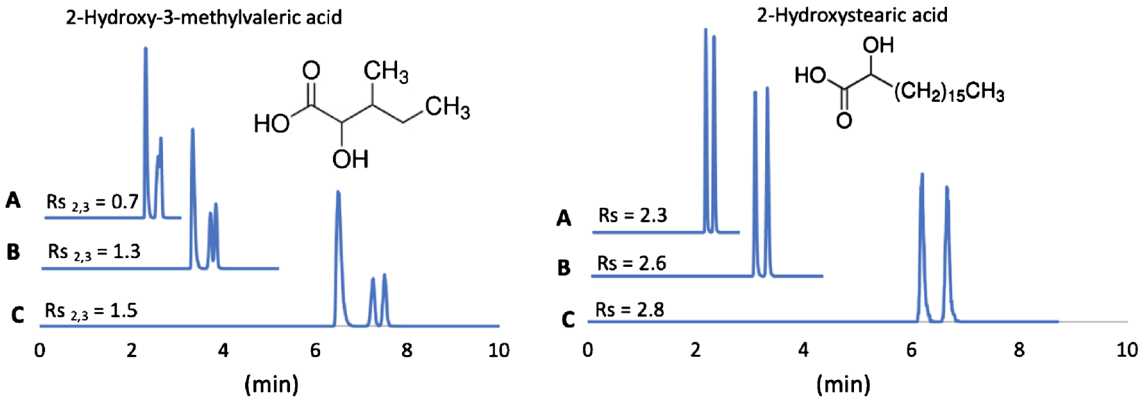


Fig. 6 Effect of mobile phase flow rate on enantioseparation of 2-hydroxystearic acid and 2-hydroxy, 3-methylvaleric acid. Mobile phase: 90/10 methanol/100 mM ammonium formate pH = 5. (A) 0.3 ml/min, (B) 0.2 ml/min, (C) 0.1 ml/min. Detection: MS, negative ion mode, monitored m/z: 299 for 2-hydroxystearic acid and 131 for 2-hydroxy-3-methylvaleric acid

Table 3 Optimum conditions for chiral separation of α-hydroxy acids using UV detection. HPLC column: TeicoShell. 15 cm × 3 mm i.d., 2.7 μm SPP. For information of all investigated mobile phases, see Table S2. k_1, retention; α, selectivity; *Rs*, resolution; *PDP*, potassium dihydrogen phosphate

Name	Optimum mobile phase condition	k_1	α	Rs
Lactic acid	82/18 acetonitrile/PDP, 10 mM, pH = 5, 0.3 ml/min	0.62	1.52	3.3
2-Hydroxyglutaric acid	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	2.14	1.12	2.3
2-Hydroxybutyric acid	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.62	1.20	2.1
2-Hydroxyisovaleric acid	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.38	1.16	1.5
2-Hydroxystearic acid	82/18 acetonitrile/PDP, 20 mM, pH = 6, 0.3 ml/min	−0.12	NA*	1.0
Mandelic acid	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.19	3.98	11.4
2-Hydroxy-3-methylvaleric acid (peaks 1, 2)	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.30	1.20	1.3
2-Hydroxy-3-methylvaleric acid (peaks 2, 3)	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.43	1.22	2.0
Phenyllactic acid	82/18 acetonitrile/PDP, 20 mM, pH = 6, 0.3 ml/min	0.12	1.32	1.2
p-Hydroxyphenyllactic acid	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.35	1.17	1.5
2-Hydroxyisocaproic acid	82/18 acetonitrile/PDP, 20 mM, pH = 5, 0.3 ml/min	0.28	1.36	2.3

*The first enantiomer of this analyte eluted before the dead time. Therefore, the k value was negative and selectivity was not calculated

Effect of the mobile phase acidity with phosphate-containing mobile phases

As observed in Fig. 7, mobile phases that contained potassium dihydrogen phosphate as the additive and UV detection showed analogous trends to formate-containing mobile phases with MS detection (Fig. 3). By increasing the pH of the aqueous solvent, all compounds showed an increase in retention until an aqueous solvent pH of 4 (5 in the case of lactic acid), followed by a decrease. Again, 2-hydroxyglutaric acid was the one exception and showed a continuous increase when increasing the pH of the aqueous solvent (Fig. 7A). Two trends were observed for selectivity. All analytes showed an increase in selectivity with increasing pH of the aqueous solvent from 3 to 6, except 2-hydroxyglutaric acid which showed an increase until pH = 4 and then the selectivity slightly decreased (Fig. 7C). Increasing the pH of the aqueous solvent from 3 to 5 increased resolution for all compounds. However, from pH 5 to pH 6, all analytes showed decreased resolution, except phenyllactic acid which continued to show an increase in resolution. Phenyllactic acid enantiomers did not resolve at pH 3. Clearly, higher pHs (≥ 4) are critical for separation of this particular analyte (Fig. 7B and Table S2). The chromatographic data for retention, selectivity, and resolution of all analytes is listed in SI Table S2.

Salt concentration effect with phosphate-containing mobile phases

As listed in Table S2 of SI, the mobile phase salt concentration effect for potassium dihydrogen phosphate showed the same general trends as observed with ammonium formate–containing mobile phases. Increasing salt concentrations increased retention and improved peak efficiencies and enantiomeric resolution. In the case of mandelic acid, phenyllactic acid, and p-hydroxyphenyllactic acid, in 5 mM KH_2PO_4 concentration, one of the enantiomers eluted before

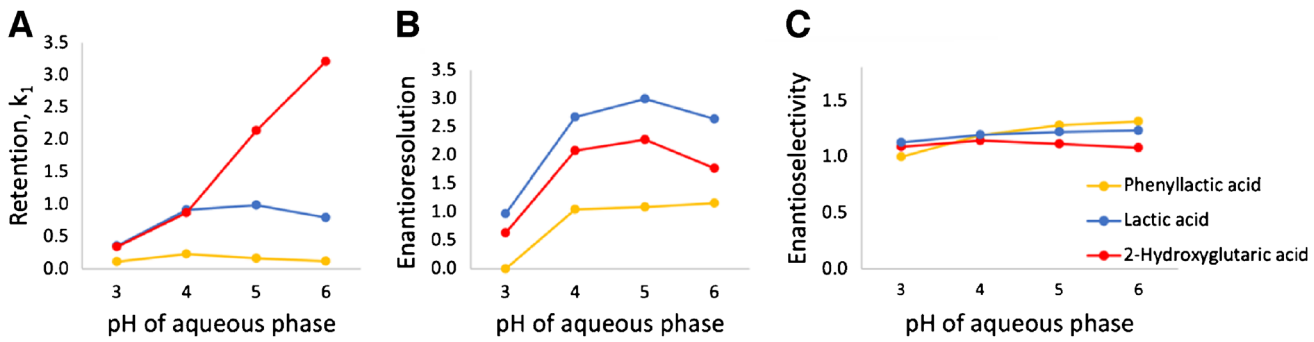


Fig. 7 Observed trends on effect of mobile phase acidity on **A** retention (k_1), **B** enantioresolution, and **C** enantioselectivity. Mobile phase: 82/18 acetonitrile/KH_2PO_4 20 mM, flow rate 0.3 ml/min. Detection: UV at 205 nm

the dead time. By increasing the concentration to 10 and 20 mM, the retention increased and peaks eluted after the dead time (see Fig. 8 and Table S2 in SI). It should be noted that the combination of high salt concentration (i.e., phosphate salt) and high pH of the aqueous solvent could lower the lifetime of the column, especially if high salt concentrations are being used [51]. The reason that one enantiomer eluted before the dead time is the Donnan ion-exclusion effect. By increasing the salt concentration and increasing the concentration of counterions, Donnan potential decreases and the retention time of the analytes increases [34].

Determination of enantiomeric impurities in standards

Chiral small molecules could serve as building blocks in asymmetric synthesis of a variety of compounds [52]. Furthermore, if used in bioanalytical studies, as per the compounds in this report, one must be aware of the presence of enantiomeric impurities in almost all standards.

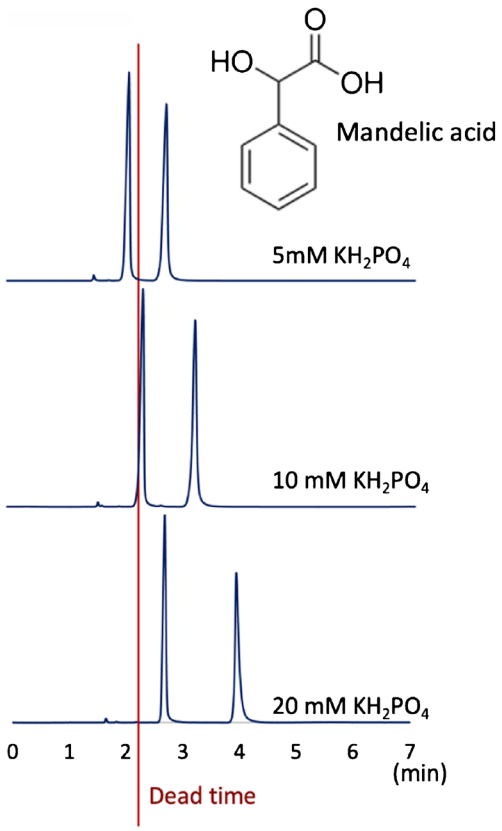


Fig. 8 Effect of salt concentration on retention of mandelic acid. Mobile phase: 82/18 acetonitrile/KH_2PO_4 pH = 5, flow rate 0.3 ml/min, detection: UV at 205 nm, dead time: 2.26 min. Dead times were measured by injection of acetone under each mobile phase condition and the average value was 2.3 ± 0.1 min

Therefore, it is important to analyze the chiral reagents prior to using them to ensure the purity of final products. Previous studies on supposedly pure chiral catalysts, auxiliaries, and synthons showed that they contain various levels of enantiomeric impurities [52–56]. As observed in Fig. 9 and Table 4, the standards of the chiral hydroxy acids in this study all contained enantiomeric impurities.

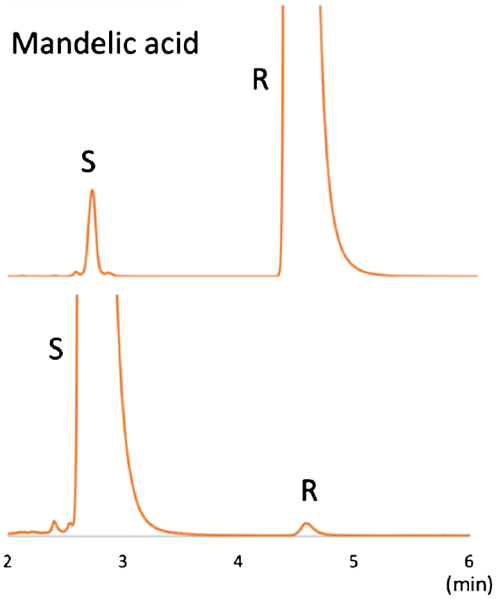


Fig. 9 Enantiomeric impurities in commercial mandelic acid standards. Mobile phase: 82/18 acetonitrile/potassium dihydrogen phosphate, 20 mM, pH = 5, 0.3 ml/min. UV detection at 205 nm

Table 4 Enantiomeric purity of analytes of this study that had singe enantiomer standards available. Mobile phase: 82/18 acetonitrile/potassium dihydrogen phosphate, 20 mM, pH = 5, 0.3 ml/min. UV detection at 205 nm

Name	S enantiomer (± 0.1%)	R enantiomer (± 0.1%)
S-2-Hydroxyglutaric acid (disodium salt)	99.6	0.4
R-2-Hydroxyglutaric acid (disodium salt)	0.1	99.9
S-Lactic acid	98.9	1.1
S-Lactic acid (sodium salt)	99.6	0.4
R-Lactic acid (sodium salt)	0.2	99.8
S-Mandelic acid	99.9	0.1
R-Mandelic acid	2.0	98.0
S-2-Hydroxyisovaleric acid	≥ 99.9	≤ 0.1
R-2-Hydroxyisovaleric acid	1.0	99.0
S-Phenyllactic acid	96.6	3.4
S-2-Hydroxyisocaproic acid	99.3	0.7

Conclusions

This work established that different optimized HPLC separation conditions were needed for enantioseparation of alpha hydroxy acids using MS vs. UV detection using formate and phosphate salts as additives in the mobile phase, respectively. The effect of mobile phase acidity and salt concentration was found to have similar trends for mobile phases with formate and phosphate salts. Increasing the aqueous solvent's pH generally resulted in increased retention, while enantioresolution and enantioselectivity increased until a certain point and remained the same or decreased after. Increasing the salt concentration generally led to increased retention and enantioresolution. The choice of organic modifier affected the enantioseparations since acetonitrile-containing mobile phases produced longer retention times and higher enantioresolution. When high salt concentrations were needed for separation, using methanol as the organic modifier was beneficial since it resulted in significantly lower retention times vs. acetonitrile. For UV detection studies, only phosphate-based salts and acetonitrile-containing mobile phases could be used due to their low background absorption at low wavelengths. It was shown that the standard samples of α -hydroxy acids contained enantiomeric impurities.

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Author contribution Conceptualization: DWA; methodology: SA and DWA; formal analysis: SA and DWA; writing—original draft preparation: SA; writing—review and editing: DWA and SA; funding acquisition: DWA; resources: DWA; supervision: DWA.

Declarations

Conflict of interest The authors declare no competing interests.

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