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RESOLUTION OF UNDERIVATIZED 2-HYDROXY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for enantiomeric separation of free 2-hydroxy acids is described. Reversed-phase chromatography and copper(II) complexes of N,N-dialkyl-L-amino acids as chiral mobile phases are used for the separation. The detection is performed by post-column derivatization with iron(III) and subsequent visible absorption measurement.

INTRODUCTION

In the last few years there has been great progress in the development of enantiomeric separation, with many problems being solved by the use of gas chromatography (GC) or high-performance liquid chromatography (HPLC). 2-Hydroxy acids have aroused interest because of their apparent importance in metabolism[1], and in certain diseases[2,3]. The first diastereomeric separation of 2-hydroxy acids was achieved by GC[4]. The use of glass capillary columns and later fused-silica columns has enabled efficient enantiomeric separation of a series of 2-hydroxy acids[5–9]. For resolution it is necessary to derivatize the carboxylic and hydroxy groups. However, two procedures have been published[9,10] whereby it is possible to resolve 2-hydroxy acid esters with free hydroxy groups.

Enantiomeric separation by HPLC began with the resolution of underivatized amino acids[11]. Polymers covalently coupled with amino acid enantiomers and mobile phases containing Cu(II) or other metal ions[11,12] were used for these separations. Hare and Gil-Av[13] and Gil-Av *et al.*[14] introduced copper–proline complexes as chiral mobile phases in ion-exchange and later in reversed-phase chromatography. Another approach was to use N,N-dialkylamino acids[15,16] instead of proline.

Similar HPLC techniques were successfully used to separate underivatized mandelic acid[17–20]; UV detection was employed. Copper–L-amino acid complexes were used for separation of a number of underivatized aliphatic and aromatic 2-hydroxy acids[21]. After post-column derivatization with iron(III) perchlorate in perchloric acid, the 2-hydroxy acid–iron complexes were monitored with an absorbance detector at 420 nm.

In this paper the enantiomeric resolution of 2-hydroxy acids using copper–N,N-dialkyl-L-amino acid complexes as chiral mobile phases is described.

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TABLE I

CAPACITY FACTORS OF ALIPHATIC 2-HYDROXY ACIDS

Chiral additive: $Cu(DPA)_2$. k = Capacity factor; k_2 = k of the second enantiomer eluted; k_1 = k of the first enantiomer. Not Sep. = Not separated; not elut. = not eluted; −, not measured under these conditions.

2-Hydroxy acid	*$Cu(DPA)_2$*				*$Cu(DPA)_2$ + 5% CH_3CN*				*$Cu(DPA)_2$ + 10% CH_3CN*				*$Cu(DPA)_2$ + 20% CH_3CN*			
	C_{18}		*RP-8*		*C_{18}*		*RP-8*		*C_{18}*		*RP-8*		*C_{18}*		*RP-8*	
	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1
Lactic acid	2.89	1.75	2.66	1.76	–		0.76	0.50	0.22	0.14	0.20	0.12	Not sep.		–	
2-Hydroxybutyric acid		–	7.98	4.48	–		2.88	1.64	0.79	0.43	0.93	0.52	0.23	0.15	–	
2-Hydroxy-2-methylbutyric acid		–		–			3.44	2.09	0.96	0.60	1.02	0.66	0.31	0.23	–	
2-Hydroxy-*n*-valeric acid		–		–	–			–	2.39	1.41	2.60	1.56	0.65	0.45	–	
2-Hydroxyisovaleric acid		–		–	–		9.04	4.49	2.32	1.23	2.46	1.29	0.64	0.39	–	
2-Hydroxyisocaproic acid		–		–	–			–	5.10	3.50	6.55	3.45	1.29	0.97	–	
2-Hydroxyoctanoic acid		–		–	–			–		–		–	Not elut.		18.38	13.20
Malic acid	4.13	3.26	3.38	3.00	–			–		–		–		–		–
Tartaric acid	Not sep.		Not sep.		–			–		–		–		–		
Glyceric acid	Not sep.		Not sep.		–			–		–		–		–		–

EXPERIMENTAL

The detector was a Model 441 UV/VIS monitor (Waters Assoc., Milford, MA, U.S.A.), operated at 436 nm. The pumps used were Eldex models (Eldex Labs., Menlo Park, CA, U.S.A.) and the injector was a Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop. The stainless-steel columns (25 × 0.46 cm I.D.) were packed by the slurry method[22]. The stationary phases were Nucleosil 5 C_{18} (5 μm) (Macherey & Nagel, Düren, F.R.G.), LiChrosorb RP-8 (10 μm) and RP-2 (10 μm) (Merck, Darmstadt, F.R.G.). The compounds N,N-dimethyl-L-valine and N,N-di-*n*-propyl-L-alanine were prepared according to the method of Bowman and Stroud[23,24]. Aqueous solutions of 8 m*M* N,N-dialkyl-L-amino acid and 4 m*M* of copper(II) acetate were used as mobile phases. Acetonitrile was added to the mobile phases when shorter retention times were desired. Tables I–VII summarize the experimental conditions used.

An aqueous 2.5 m*M* iron(III) salt solution was used as post-column reagent. It was prepared by adding the salt to water adjusted to pH 1.5–2 with 70% (v/v) $HClO_4$. The free 2-hydroxy acid samples (C_3–C_6) were dissolved in water or acetonitrile (C_8). DL-, D- and L-pantolactone were incubated in an alkaline solution in screw-cap vials at 100°C for 1 h in an oven.

RESULTS AND DISCUSSION

The enantiomeric separation of 2-hydroxy acids was performed by using N,N-dimethyl-L-valine or N,N-di-*n*-propyl-L-alanine and copper(II) acetate as chiral mobile phases, and the stationary phases C_{18}, RP-8 and RP-2. The results are presented in Tables I–VII and Figs. 1–4.

A reasonable separation of aliphatic and aromatic 2-hydroxy acids was obtained by the use of an aqueous N,N-dialkyl-L-amino acid solution; however, the retention times were far in excess of those desired of such an analytical method. Addition of acetonitrile to the stock solutions of the chiral phases solved this problem. It is seen that for any 2-hydroxy acid, alteration of the composition of the mobile phase may lead to good resolution. The retention times were also decreased by changing the stationary phase, *e.g.*, from C_{18} to RP-8. In each case the L-enantiomer was the second to be eluted.

In general the retention times increase with increasing chain length of the aliphatic 2-hydroxy acids (Tables I, II) as expected. It was noted that the first enantiomer of a given 2-hydroxy acid was eluted after and even sometimes together with the second enantiomer of a smaller homologue. In the case of 2-substituted 2-hydroxy acids such as 2-hydroxy-2-methylbutyric acid a different elution profile was observed. The first peak of this acid eluted in between the enantiomers of 2-hydroxybutyric acid (Fig. 1).

By using RP-8 as stationary phase, 2-hydroxyoctanoic acid (Tables I, II) could be eluted when 20% acetonitrile was added to $Cu(DPA)_2$ (DPA = monoanion of N,N-di-*n*-propyl-L-alanine) or $Cu(DMV)_2$ (DMV = monoanion of N,N-dimethyl-L-valine). Under the same conditions, 2-hydroxyoctanoic acid was not eluted on C_{18}. The interaction of 2-hydroxyoctanoic acid with the RP-8 stationary phase is not as strong as with C_{18}. However, 2-hydroxyoctanoic acid had very similar retention times on RP-8 and RP-2 (Table VII).

TABLE II

CAPACITY FACTORS OF ALIPHATIC 2-HYDROXY ACIDS

Chiral additive: $Cu(DMV)_2$.

2-Hydroxy acid	*$Cu(DMV)_2$*				*$Cu(DMV)_2$ + 5% CH_3CN*				*$Cu(DMV)_2$ + 10% CH_2CN*				*$Cu(DMV)_2$ + 20% CH_3CN*			
	C_{18}		*RP-8*		*C_{18}*		*RP-8*		*C_{18}*		*RP-8*		*C_{18}*		*RP-8*	
	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1
Lactic acid	1.15	0.4	1.05	0.34	0.27	0.04	0.36	0.19	0.13	0.06	0.33	0.14	Not sep.		–	
2-Hydroxybutyric acid	5.45	1.7	5.47	1.80	1.21	0.41	1.28	0.54	0.47	0.18	0.51	0.28	0.31	0.20	–	
2-Hydroxy-2-methylbutyric acid	5.75	2.37	6.04	2.68	1.41	0.69	1.56	0.86	0.61	0.34	0.60	0.42	0.26	0.19	–	
2-Hydroxy-*n*-valeric acid		–		–	3.77	1.22	4.22	1.59	1.49	0.66	1.57	0.73	0.54	0.32	–	
2-Hydroxyisovaleric acid		–		–	5.51	1.63	4.66	1.60	1.64	0.63	1.37	0.50	0.50	0.26	–	
2-Hydroxyisocaproic acid		–		–	12.63	5.36	11.57	5.15	5.24	2.26	3.71	2.07	0.97	0.66	–	
2-Hydroxyoctanoic acid		–		–		–		–		–		–		–	8.03	5.90
Malic acid	2.57	1.9	1.56	1.13		–		–		–		–		–		–
Tartaric acid		Not sep.		Not sep.		–		–		–		–		–		–
Glyceric acid		Not sep.		Not sep.		–		–		–		–		–		–

TABLE III

CAPACITY FACTORS OF AROMATIC 2-HYDROXY ACIDS

Chiral additive: $Cu(DPA)_2$.

2-Hydroxy acid	*$Cu(DPA)_2$ + 10% CH_3CN*				*$Cu(DPA)_2$ + 20% CH_3CN*			
	C_{18}		*RP-8*		C_{18}		*RP-8*	
	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1
Mandelic acid		–	5.63	3.61	1.50	1.15	1.30	0.97
2-Methylmandelic acid		–	–		2.35	1.86	2.20	1.74
4-Hydroxymandelic acid		–	1.01	0.63	Shoulder		–	
4-Hydroxy-3-methoxymandelic acid		–	1.50	1.00	Shoulder		–	
3-Hydroxy-4-methoxymandelic acid		–	2.05	1.32	Not sep.		–	
Atrolactic acid		–	–		1.64	1.25	–	
3-Phenyllactic acid		–	–		2.66	1.96	–	

Similar observations can generally be made for aromatic 2-hydroxy acids (Tables III, IV). With atrolactic acid, which is a 2-methyl-substituted 2-hydroxy acid, a similar retention behaviour was observed as in the case of 2-hydroxy-2-methylbutyric acid. It had a much shorter retention time than its isomer 3-phenyllactic acid and overlaps with the homologue of 3-phenyllactic acid, mandelic acid. The behaviour of the mandelic acid derivatives is of interest. A methyl group in the *ortho*-position of the phenyl ring leads to a longer retention time than that of mandelic acid, but again shorter than that of 3-phenyllactic acid. Substitutions in the *meta*- and *para*-positions lead to a sharp decrease in the retention times. Not only the positions of the substituents, but also their nature (free hydroxy group or methoxy group) may cause this decrease. Such a substitution may hinder the interaction with the stationary phase containing the copper complex.

TABLE IV

CAPACITY FACTORS OF AROMATIC 2-HYDROXY ACIDS

Chiral additive: $Cu(DMV)_2$.

2-Hydroxy acid	*$Cu(DMV)_2$ + 5% CH_3CN*				*$Cu(DMV)_2$ + 10% CH_3CN*				*$Cu(DMV)_2$ + 20% CH_3CN*			
	C_{18}		*RP-8*		C_{18}		*RP-8*		C_{18}		*RP-8*	
	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1
Mandelic acid		–	6.90	2.28		–	2.00	0.94	1.00	0.74		–
4-Hydroxymandelic acid		–	0.81	0.43		–	0.30	0.20		–		–
4-Hydroxy-3-methoxy-mandelic acid		–	1.16	0.66		–	0.36	0.25		–		–
3-Hydroxy-4-methoxy-mandelic acid		–	1.80	0.97		–	0.61	0.41	Not sep.			–
Atrolactic acid		–		–		–	2.60	1.69	1.41	1.13		–
3-Phenyllactic acid		–		–		–	9.82	5.73	2.86	1.81		–

TABLE V

SEPARATION FACTORS (α) OF ALIPHATIC 2-HYDROXY ACIDS

2-Hydroxy acid	*Cu(DPA)$_2$*		*Cu(DMV)$_2$*		*Cu(DPA)$_2$ + 5% CH$_3$CN*		*Cu(DMV)$_2$ + 5% CH$_3$CN*		*Cu(DPA)$_2$ + 10% CH$_3$CN*		*Cu(DMV)$_2$ + 10% CH$_3$CN*		*Cu(DPA)$_2$ + 20% CH$_3$CN*		*Cu(DMV)$_2$ + 20% CH$_3$CN*	
	C_{18}	*RP-8*	*C_{18}*	*RP-8*	*C_{18}*	*RP-8*	*C_{18}*	*RP-8*	*C_{18}*	*RP-8*	*C_{18}*	*RP-8*	*C_{18}*	*RP-8*	*C_{18}*	*RP-8*
Lactic acid	1.65	1.51	2.91	3.08	–	1.52	2.85	1.87	1.58	1.64	2.08	sh.	1.00	–	1.00	–
2-Hydroxybutyric acid	–	1.78	3.21	3.04	–	1.74	2.93	2.38	1.83	1.79	2.63	1.83	1.53	–	1.52	–
2-Hydroxy-2-methyl-butyric acid	–	–	2.43	2.26	–	1.64	2.04	1.82	1.61	1.56	1.77	1.42	1.33	–	1.33	–
2-Hydroxy-*n*-valeric acid	–	–	–	–	–	–	3.10	2.66	1.70	1.67	2.26	2.15	1.44	–	1.68	–
2-Hydroxyiso-valeric acid	–	–	–	–	–	2.02	3.39	2.92	1.88	1.91	2.61	2.78	1.62	–	1.88	–
2-Hydroxyiso-caproic acid	–	–	–	–	–	–	2.36	2.25	1.46	1.50	2.31	1.79	1.33	–	1.48	–
2-Hydroxy-octanoic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	1.38	–	1.36
Malic acid	1.25	1.13	1.36	1.39	–	–	–	–	–	–	–	–	–	–	–	–
Tartaric acid	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–	–
Glyceric acid	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–	–	–	–	–

sh. = Shoulder.

TABLE VI

SEPARATION FACTORS (α) OF AROMATIC 2-HYDROXY ACIDS

sh. = Shoulder.

2-Hydroxy acid	*Cu(DMV)$_2$ + 5% CH$_3$CN*		*Cu(DPA)$_2$ + 10% CH$_3$CN*		*Cu(DMV)$_2$ + 10% CH$_3$CN*		*Cu(DPA)$_2$ + 20% CH$_3$CN*		*Cu(DMV)$_2$ + 20% CH$_3$CN*	
	C_{18}	*RP-8*	C_{18}	*RP-8*	C_{18}	*RP-8*	C_{18}	*RP-8*	C_{18}	*RP-8*
Mandelic acid	–	3.03	–	1.56	–	2.13	1.30	1.34	1.36	–
2-Methylmandelic acid	–	–	–	–	–	–	1.26	1.26	–	–
4-Hydroxymandelic acid	–	1.89	–	1.60	–	1.50	sh.	–	–	–
4-Hydroxy-3-methoxymandelic acid	–	1.76	–	1.50	–	1.44	sh.	–	–	–
3-Hydroxy-4-methoxymandelic acid	–	1.85	–	1.56	–	1.50	1.00	–	1.00	–
Atrolactic acid	–	–	–	–	–	1.54	1.32	–	1.25	–
3-Phenyllactic acid	–	–	–	–	–	1.72	1.36	–	1.58	–

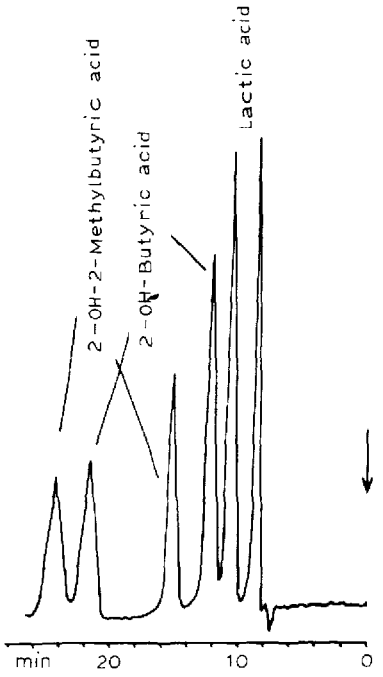
For hydroxy acids such as tartaric and glyceric acids no resolution was achieved (Tables I, II). Malic acid was completely separated, but it was necessary to use the mobile phase without added acetonitrile (Tables I, II). This suggests that the interaction with the copper complex present in the stationary phase is not as strong as in the case of the previous 2-hydroxy acids and also that the retention times of these compounds may not be long enough to obtain resolution. This may be explained by the presence of a β-hydroxy group which appears to hinder resolution, even though an α-hydroxy group is also present, as demonstrated in the case of glyceric and tartaric acids. A similar conclusion may also be drawn for compounds possessing a second carboxylic group. Depending on which carboxylic group is considered, malic acid can be envisaged as an α- and β-hydroxy acid. The case of malic acid indicates that the absence of a second hydroxy group is a necessary condition for separation.

A comparison of the α values (Table V) shows smaller values on RP-8 than on C_{18} under the same conditions. When comparing the α values in the different mobile phases Cu(DPA)$_2$ and Cu(DMV)$_2$, again under the same conditions, the latter appears to be more efficient than the former. The ease with which N,N-dimethyl-L-valine may be prepared also favours the use of Cu(DMV)$_2$ over Cu(DPA)$_2$. 2-Hydroxy acids substituted at C-2 have smaller α values. In general, the α value increases with carbon number up to 2-hydroxyisovaleric acid and then decreases again. In the

TABLE VII

RESULTS ON RP-2

2-Hydroxy acid	*Cu(DMV)$_2$ + 20% CH$_3$CN*		α
	k_2	k_1	
2-Hydroxyisocaproic acid	0.78	0.63	1.28
2-Hydroxyoctanoic acid	5.04	3.95	1.27



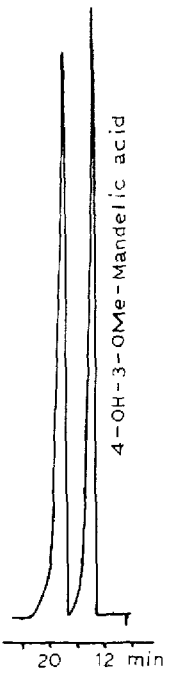
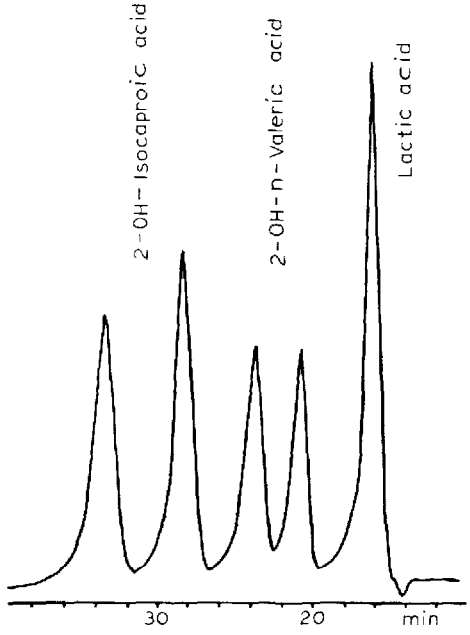


Fig. 1. Enantiomeric separation of three 2-hydroxy acids. Conditions: column, LiChrosorb RP-8, 25 × 0.46 cm I.D., ambient temperature; mobile phase, 4 m*M* copper acetate and 8 m*M* N,N-dimethyl-L-valine (DMV) acetonitrile (95:5), flow-rate 0.5 ml/min; detection, post-column derivatization, 2.5 m*M* iron(III) salt in 0.06 *M* $HClO_4$, flow-rate 0.5 ml/min, absorption in the visible range at 436 nm.

Fig. 2. Enantiomeric separation of 4-hydroxy-3-methoxymandelic acid (VMA). For conditions see Fig. 1.



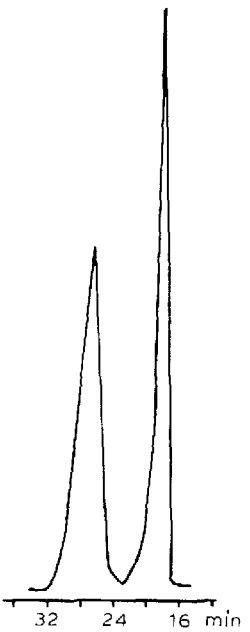
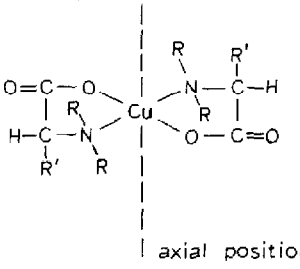


Fig. 3. Enantiomeric separation of 2-hydroxy acids. Conditions: column, Nucleosil 5 C_{18}, 25 × 0.46 cm I.D., ambient temperature; mobile phase, 4 m*M* copper acetate and 8 m*M* N,N-di-*n*-propyl-L-alanine (DPA)–acetonitrile (80:20), flow-rate 0.3 ml/min; detection, see Fig. 1.

Fig. 4. Separation of DL-2,4-dihydroxy-3,3-dimethylbutyric acid after alkaline hydrolysis of DL-pantolactone. Conditions: see Fig. 1; mobile phase flow-rate, 0.3 ml/min. $k_2/k_1 = 3.06/1.80$; $\alpha = 1.7$.

case of the aromatic hydroxy acids investigated (Table VI), mandelic acid shows the largest α value using $Cu(DMV)_2$ as chiral phase (on RP-8). Using $Cu(DPA)_2$ in conjunction with C_{18}, 3-phenyllactic acid has the largest α value. The interaction of $Cu(DMV)_2$ with the 2-hydroxy acids may be easier. The methyl groups of N,N-dimethyl-L-valine appear not to hinder the interaction, in contrast to the *n*-propyl groups of N,N-di-*n*-propyl-L-alanine.



DMV: $R = CH_3$, $R' = CH(CH_3)_2$
DPA: $R = CH_2{-}CH_2{-}CH_3$, $R' = CH_3$

DL-2-Hydroxy acids form diastereomeric complexes with Cu(N,N-dialkyl-L-amino acid anion)$_2$ which may vary in either their stability, solubility or adsorption. For amino acid separation it has been suggested that a ligand exchange of the form $CuL_2 + L' \rightleftharpoons CuLL' + L$ and $CuL_2 + D' \rightleftharpoons CuLD' + L$ may take place[14,16]. However, an outer sphere complex may also lead to the formation of diastereomeric complexes[25]. It is not unlikely that both inner and outer sphere coordination may work together. In an outer sphere complex, interaction, *e.g.*, electrostatic interaction, may occur via the axial position. In the case of the 2-hydroxy acids an outer sphere complex may play an important rôle. Evidence for this comes from the fact that the stability constants for copper–amino acid complexes are larger than those for copper–2-hydroxy acid complexes[26]. Should such an interaction proceed via the axial position then the *n*-propyl groups may interfere more than methyl groups.

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