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Enantiomeric separation and discrimination of 2-hydroxy acids as *O*-trifluoroacetylated (*S*)-(+)-3-methyl-2-butyl esters by achiral dual-capillary column gas chromatography

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Abstract

An efficient method is described for the simultaneous enantiomeric separation of 18 different racemic 2-hydroxy acids for the determination of their absolute configurations. It involves the conversion of each enantiomer into a diastereomeric *O*-trifluoroacetylated (*S*)-(+)-3-methyl-2-butyl ester for the direct separation by achiral dual-capillary column gas chromatography with subsequent identification and determination of its chirality by retention index (*I*) library matching. The enantiomers of each acid were well separated with high resolution values ($R \geq 1.4$) on DB-5 and DB-17 columns of different polarity. When temperature-programmed *I* values of 2-hydroxy acid enantiomers as their diastereomeric derivatives were measured on both columns, the *I* values were characteristic of each enantiomer. Simple *I* matching with the reference values was thus useful in cross-checking each acid enantiomer for the identification and chiral discrimination. When applied to urine samples, the present method allowed positive identification of most of the spiked 2-hydroxy acids from normal urine and for endogenous (*S*)-lactic acid and (*S*)-2-hydroxybutyric acid from a clinical urine specimen. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Derivatization, GC; 2-Hydroxycarboxylic acids

1. Introduction

Among the structurally diverse biogenic organic acids, chiral 2-hydroxycarboxylic acids are known to be important biochemical indicators of inborn errors of metabolism [1–11]. They originate mainly by reduction of α-keto acids that are produced from the metabolism of branched-chain amino acids. The simultaneous detection of various 2-hydroxy acids and accurate determination of their absolute configurations in a single analysis is now of great importance for the study of their biochemical roles, as well as in the systematic diagnosis of metabolic disorders [4,11,14].

In recent years, gas chromatography (GC) in combination with mass spectrometry (MS) is preferentially applied to the multi-component profiling analysis of organic acids for metabolic studies [11–19]. Prior to enantiomeric separation by GC, both

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direct and indirect methods require 2-hydroxy acids to be converted into volatile derivatives. When considering this prerequisite, indirect enantiomeric separation of the racemic 2-hydroxy acids as volatile diastereomeric derivatives using conventional achiral stationary phases with high thermal stability and long-term durability appears to be more preferred over the direct approach. In the literature, indirect separation is widely used because of the simplicity and versatility of the applications [1,3,14,20–22]. Furthermore, it offers a quicker enantioseparation for analytes possessing easily derivatizable hydroxyl and carboxyl functional groups such as 2-hydroxy acids.

The most widely utilized method of diastereomeric reactions is the esterification of carboxyl groups with chiral alcohols [1–3,14], with subsequent acylation of the remaining hydroxyl or phenolic hydroxyl groups. Another commonly employed approach is the acylation of the hydroxyl groups with chiral acid chlorides, or carbonation with chiral chloroformates after esterification of the carboxyl group [14,21]. Another approach is the amidation of carboxyl groups with chiral amines after ethoxymation of the hydroxyl group [20].

The chirality of each separated enantiomer is mainly determined by cochromatography with the enantiomerically pure (*R*)- or (*S*)-acid standards [1–3]. This requires at least a second GC run of real samples, which is a very cumbersome approach in view of the overall analysis time. Accordingly, it is desirable to develop an efficient method for the simultaneous peak identification and chiral configuration. The recent advances in GC technology permit the use of retention index (*I*) sets measured on multiple stationary phases of different polarity for the positive peak identification [12,13,17–19]. However, no attempt has been made to apply *I* comparison to the enantiomeric discrimination to date. In our previous profiling and screening analyses of organic acids as their *tert*.-butyldimethylsilyl (TBDMS) derivatives [17,18], the characteristic retention index (*I*) set of each acid measured on dual-capillary columns of different polarity was found to be useful for the positive peak identification through *I* matching with the reference values in our laboratory-built *I* library that currently contains over 200 organic acid standards as TBDMS derivatives [18]. This GC–*I* matching system has been routinely used to screen for organic acids in our laboratory.

It occurred to us that combining the efficient achiral dual-capillary column GC–*I* matching system with diastereomeric separation could provide not only the chemical identification but also the accurate chirality of each 2-hydroxy acid enantiomer in a single analysis. As the first step towards this goal, the present study was undertaken to examine the usefulness of GC–*I* matching in routine chiral discrimination, when combined with the enantiomeric separation of 18 biologically important 2-hydroxy acids as diastereomeric *O*-trifluoroacetyl (*S*)-(+)-3-methyl-2-butyl esters [2] simultaneously on achiral dual-capillary columns of different polarity [17,18].

2. Experimental

2.1. Materials

The following 18 racemic 2-hydroxy acids were obtained from various vendors such as Sigma–Aldrich (Milwaukee, WI, USA): lactic, 2-hydroxybutyric, 2-hydroxyisovaleric, glyceric, 2-hydroxyvaleric, 2-hydroxyisocaproic, 2-hydroxy-3-methylvaleric, 2-hydroxycaproic, 2-hydroxyoctanoic, mandelic, *m*-hydroxymandelic, 3-phenyllactic, malic, 2-hydroxydecanoic, 2-hydroxyglutaric, *p*-hydroxyphenyllactic, 2-hydroxydodecanoic and 2-hydroxytetradecanoic, including the enantiomerically pure (*S*)-(+)-lactic, (*S*)-(+)-2-hydroxyisovaleric, (*S*)-glyceric, (*S*)-(−)-2-hydroxyisocaproic, (*S*)-2-hydroxy-3-methylvaleric, (*S*)-(+)-mandelic, (*S*)-(−)-3-phenyllactic, (*S*)-(+)-malic and (*S*)-2-hydroxyglutaric acids. (*S*)-(+)-3-Methyl-2-butanol, acetyl chloride and trifluoroacetic anhydride (TFAA) were purchased from Sigma–Aldrich and *n*-hydrocarbon standards (C_{10}–C_{22}, even numbers only) from Polyscience (Niles, IL, USA). Silylating reagent, *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford, IL, USA). Acetonitrile, toluene, isooctane and ethyl acetate of spectroanalyzed grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used as received.

2.2. Acid and hydrocarbon standard solutions

Each standard solution of chiral acids was made

up at 10 μg/μl in acetonitrile as their free acid forms. Hydrocarbon solution containing *n*-hydrocarbons (C_{10}–C_{22}, even numbers only), each at 1.0 μg/μl in isooctane, was used as the external standard solution for the *I* measurement.

2.3. (S)-(+)-3-Methyl-2-butylation and O-trifluoroacetylation

A mixed solution of acids containing 10 μg of each acid was evaporated to dryness under a gentle stream of nitrogen at 40°C. To the residue were added toluene (20 μl), (*S*)-(+)-3-methyl-2-butanol (20 μl) and acetyl chloride (1 μl). The mixture was then heated at 100°C for 1 h to form diastereomeric esters. After the removal of excess reagents (under a nitrogen stream), the concentrate was reacted with TFAA (20 μl) in the presence of acetonitrile (10 μl) at 60°C for 20 min. After the addition of toluene (20 μl) as the keeper, the reaction mixture was evaporated to remove excess of reagents for the direct analysis by GC and GC–MS. In place of TFAA, MBTFA was investigated as the reagent for the trifluoroacetylation. And silylation of the remaining hydroxyl groups using MTBSTFA at 60°C was tested following the (*S*)-(+)-3-methyl-2-butylation.

2.4. Sample preparation

Aliquots (corresponding to 0.25 mg of creatinine) of urine samples without spiking or after spiking with racemic 2-hydroxy acids (20 μg each) were adjusted to pH 1–2 with conc. sulfuric acid, saturated with sodium chloride and then extracted with diethyl ether (3×2 ml). The ethereal extract was dried over magnesium sulfate and evaporated to dryness, and then subjected to (*S*)-(+)-3-methyl-2-butylation with subsequent *O*-trifluoroacetylation as described above.

2.5. Gas chromatography and gas chromatography–mass spectrometry

GC analyses were performed with a Hewlett-Packard HP Model 5890A gas chromatograph series II, equipped with a split/splitless capillary inlet system and two flame ionization detection (FID) systems and interfaced to a HP 3365A GC Chemstation (Hewlett-Packard, Avondale, PA, USA). The injector and detector temperatures were 260 and 280°C, respectively. Samples (ca. 0.5 μl) were injected in the splitless mode with a purge delay time of 0.7 min. The retention index (*I*) measurements were carried out using a dual-capillary column system made of DB-5 (SE-54 bonded phase) and DB-17 (OV-17 bonded phase) fused-silica capillary columns (J&W Scientific, Rancho Corodova, CA, USA; dimensions 30 m×0.25 mm I.D., 0.25 μm film thickness). The two columns were connected to a deactivated fused-silica tubing (1 m×0.25 mm I.D.) as retention gap via a Y-splitter. The inlet pressure of helium as the carrier gas was set to 137.5 kPa. The oven temperature was held at 60°C for 2 min, then programmed to 280°C at a rate of 3°C/min. The GC Chemstation processed the two FID signals simultaneously in dual-channel mode. A standard solution of *n*-hydrocarbons (C_{10}–C_{22}, even numbers only) in isooctane was injected as the external references and temperature-programmed *I* values were computed via a built-in retention index program by linear interpolation between the retention times of adjacent hydrocarbon standards. For the peak identification and chirality determination by computer *I* matching, a database of reference *I* library using *I* sets of 36 chiral acid enantiomers measured on the dual-columns was built into the GC computer system.

GC analyses for optimization of the diastereomeric reaction conditions were performed by a Younglin M600D Model gas chromatograph (Young Lin Instrument, Kyunggido, South Korea) equipped with a split/splitless capillary inlet system and an FID system interfaced to a Younglin Autochro-WIN data acquisition system. The injector and detector temperatures were 260 and 280°C, respectively. Samples (ca. 1.0 μl) were injected in the splitless mode (purge delay time of 0.7 min) and analyzed on an Ultra-2 (SE-54 bonded phase) capillary column (25 m×0.20 mm I.D., 0.33 μm film thickness; Hewlett-Packard) under the oven temperature conditions described above. The inlet pressure of nitrogen used as the carrier gas was set to 28 kPa. All the GC analyses were performed in triplicate.

To obtain mass spectra, a HP 5890A series II gas chromatograph, interfaced to a HP 5970B mass spectrometer (70 eV, electron impact mode), which was on-line to a HP 59940A MS Chemstation was used. Samples were injected into an Ultra-2 (SE-54 bonded phase) capillary column (25 m×0.20 mm

I.D., 0.11 μm film thickness) in the split injection mode (10:1) at 260°C, and the oven temperature was initially 60°C for 2 min and then raised to 280°C at 3°C/min. The interface and ion source temperatures were 280 and ca. 250°C, respectively. The mass range scanned was 50–650 u at a rate of 0.99 scan/s.

3. Results and discussion

In an earlier work, multi-component analysis of 13 different racemic 2-hydroxy acids in a single analysis was first attempted as their diastereomeric (*S*)-(+)-3-methyl-2-butyl esters [(*S*)-MBEs] of *O*-trifluoroacetylated (*O*-TFA) or *O*-trimethylsilylated acids [2]. We adopted this diastereomeric reaction scheme with minor changes for the simultaneous conversion of 18 biologically important 2-hydroxy acids into diastereomeric *O*-TFA/(*S*)-MBE derivatives which were examined for the enantiomeric separation. The temperature-programmed *I* set of each derivative measured on achiral DB-5 and DB-17 dual-capillary columns of different polarity tested whether they are useful in the identification and discrimination of 2-hydroxy acid enantiomers by simple *I* matching with the reference values.

Upon the (*S*)-(+)-3-methyl-2-butylation with subsequent *O*-trifluoroacetylation, all 2-hydroxy acids were converted to their corresponding diastereomeric *O*-TFA/(*S*)-MBE derivatives, yielding a single derivative for each acid enantiomer. The structure of each diastereomer was confirmed by GC–MS. No serious racemization was observed with enantiopure acid standards during the reactions. Toluene was used as a keeper during the evaporation to prevent loss of short-chain hydroxy fatty acids with 3–7 carbons such as lactic acid, which become volatile after the acylation. In preliminary experiments to attempt silylation of the remaining hydroxyl groups with MTBSTFA following the (*S*)-(+)-3-methyl-2-butylation, the chiral ester groups were observed to be substituted by TBDMS groups along with hydroxyl groups.

Under the present GC conditions, the enantiomeric separation of 18 racemic acids as their *O*-TFA/(*S*)-MBE derivatives on the achiral DB-5 and DB-17 dual-capillary columns was feasible in one analytical run within 55 min as shown in Fig. 1. One set of four peaks corresponding to 2-hydroxyglutaric acid (peak 15) and *p*-hydroxyphenyllactic acid (peak 16) were not resolved on the DB-5 column of low polarity, while three sets of four peaks (peaks 10 and 11, peaks 14 and 13, and peaks 16 and 15) were not resolved on the DB-17 column of intermediate polarity. Except for 2-hydroxyglutaric and *p*-hydroxyphenyllactic acids (peaks 15 and 16), the unresolved acids on the DB-17 column were well resolved on the DB-5 column. When the acids were individually analyzed, each enantiomeric pair was baseline resolved displaying a single peak with good peak shape. The high efficiency of the capillary columns allowed small selectivity factors, α, as low as 1.003 to be easily exploited to achieve excellent resolution between the enantiomeric pairs with high resolution factors ($R \geq 1.4$) on both columns. As listed in Table 1, the resolution factors were obtained for most acid enantiomers in the range of 1.4–4.4, while separation factors were in the range of 1.003–1.049.

The elution order of the enantiomers was determined using authentic enantiopure standards of established absolute configurations such as (*S*)-(+)-lactic, (*S*)-(+)-2-hydroxyisovaleric, (*S*)-glyceric, (*S*)-(−)-2-hydroxyisocaproic, (*S*)-2-hydroxy-3-methylvaleric, (*S*)-(+)-mandelic, (*S*)-(−)-3-phenyllactic, (*S*)-(+)-malic and (*S*)-2-hydroxyglutaric acids that were commercially available (Table 1). In all cases (*R*)-enantiomers were eluted ahead of (*S*)-enantiomers on both columns as reported previously [2]. Moreover, it was noticed that the elution orders of most of the acids in racemic form on the two columns were the same with the exception of 2-hydroxydecanoic, malic and *p*-hydroxyphenyllactic acids, but their retention times were very different. Therefore, the temperature-programmed *I* set measured with dual-capillary columns was characteristic for each acid enantiomer (Table 1) and thus useful in cross-checking each organic acid enantiomer by simple *I* matching. This *I* matching was useful especially for the discrimination among the enantiomeric pairs and between the positional isomers since their mass spectral patterns are mostly indistinguishable.

As demonstrated in the typical dual chromatographic profiles of spiked normal urine in comparison with urine blank (Fig. 2), the present dual-

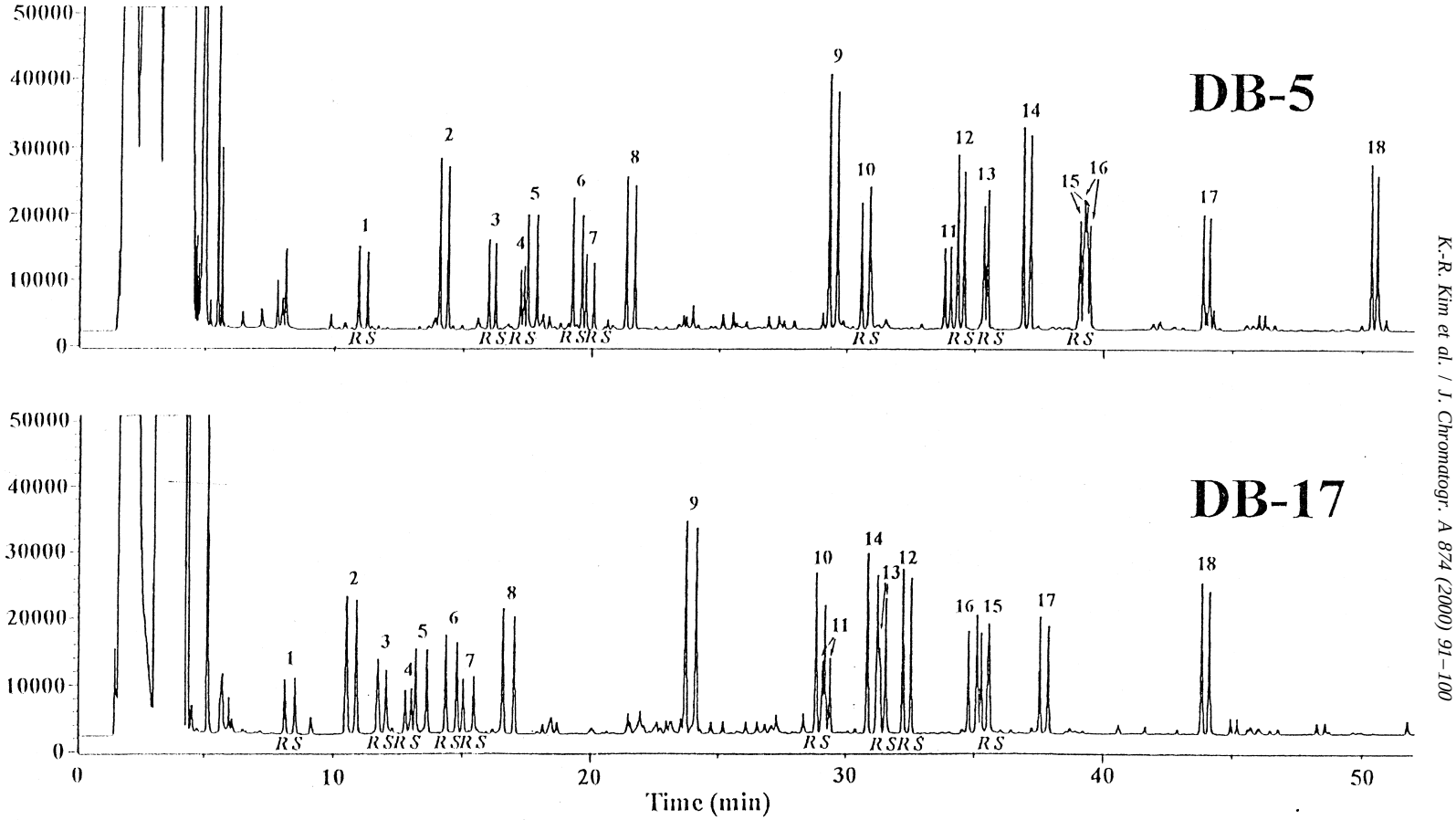

Fig. 1. Dual chromatograms of 2-hydroxy acid enantiomers separated on the DB-5 and DB-17 (both 30 m×0.25 mm I.D., 0.25 μm film thickness) dual-capillary column system. The oven temperature was held at 60°C for 2 min, then programmed to 280°C at a rate of 3°C/min. Samples (ca. 0.5 μl) were injected in the splitless mode with a purge delay time of 0.7 min. Peaks: 1=lactic acid; 2=2-hydroxybutyric acid; 3=2-hydroxyisovaleric acid, 4=glyceric acid 5=2-hydroxyvaleric acid; 6=2-hydroxyisocaproic acid; 7=2-hydroxy-3-methylvaleric acid; 8=2-hydroxycaproic acid; 9=2-hydroxyoctanoic acid; 10=mandelic acid; 11=*m*-hydroxymandelic acid; 12=3-phenyllactic acid; 13=malic acid; 14=2-hydroxydecanoic acid; 15=2-hydroxyglutaric acid; 16=*p*-hydroxyphenyllactic acid; 17=2-hydroxydodecanoic acid; 18=2-hydroxytetradecanoic acid.

Table 1
Gas chromatographic data of 2-hydroxy acids as diastereomeric *O*-trifluoroacetylated (*S*)-(+)-3-methyl-2-butyl esters

No.	Chiral acid	Separation factor (α)[a]		Resolution factor (R)[b]		GC I data set[c]	
		DB-5	DB-17	DB-5	DB-17	DB-5	DB-17
1	(*R*)-(−)-Lactic	1.032	1.049	4.4	3.6	1024.0	1067.3
	(*S*)-(+)-Lactic					1030.9	1078.8
2	2-Hydroxybutyric	1.023	1.037	3.8	2.7	1092.1	1144.7
	2-Hydroxybutyric					1100.1	1156.4
3	(*R*)-(−)-2-Hydroxyisovaleric	1.016	1.028	3.1	1.9	1139.6	1187.7
	(*S*)-(+)-2-Hydroxyisovaleric					1146.2	1199.1
4	(*R*)-Glyceric	1.008	1.018	1.5	1.8	1170.3	1207.2
	(*S*)-Glyceric					1174.2	1211.9
5	2-Hydroxyvaleric	1.021	1.032	4.0	3.4	1179.1	1224.1
	2-Hydroxyvaleric					1188.5	1233.4
6	(*R*)-(+)-2-Hydroxyisocaproic	1.019	1.030	4.0	3.4	1220.2	1250.5
	(*S*)-(−)-2-Hydroxyisocaproic					1227.9	1260.4
7	(*R*)-2-Hydroxy-3-methylvaleric	1.016	1.027	3.6	3.3	1231.7	1267.3
	(*S*)-2-Hydroxy-3-methylvaleric					1238.4	1277.0
8	2-Hydroxycaproic	1.017	1.026	3.7	3.6	1265.4	1303.1
	2-Hydroxycaproic					1273.2	1313.6
9	2-Hydroxyoctanoic	1.011	1.017	3.1	3.4	1448.7	1481.3
	2-Hydroxyoctanoic					1456.2	1491.1
10	(*R*)-(−)-Mandelic	1.010	1.012	2.9	3.1	1479.4	1605.7
	(*S*)-(+)-Mandelic					1486.8	1614.5
11	*m*-Hydroxymandelic	1.007	1.010	2.3	2.7	1559.9	1611.3
	m-Hydroxymandelic					1565.6	1619.3
12	(*R*)-(+)-3-Phenyllactic	1.007	1.010	2.4	2.9	1569.4	1695.4
	(*S*)-(−)-3-Phenyllactic					1575.7	1703.5
13	(*R*)-(−)-Malic	1.003	1.007	1.4	2.3	1595.3	1671.4
	(*S*)-(+)-Malic					1599.4	1677.6
14	2-Hydroxydecanoic	1.008	1.013	2.7	3.0	1633.9	1661.0
	2-Hydroxydecanoic					1641.4	1671.4
15	(*R*)-2-Hydroxyglutaric	1.007	1.006	2.2	2.0	1690.3	1773.1
	(*S*)-2-Hydroxyglutaric					1697.0	1779.2
16	*p*-Hydroxyphenyllactic	1.006	1.009	2.0	2.9	1696.4	1762.8
	p-Hydroxyphenyllactic					1704.0	1771.3
17	2-Hydroxydodecanoic	1.006	1.009	2.5	3.1	1820.5	1842.5
	2-Hydroxydodecanoic					1827.8	1851.9
18	2-Hydroxytetradecanoic	1.005	1.007	2.1	2.6	2009.8	2027.5
	2-Hydroxytetradecanoic					2017.1	2036.7

[a] Separation factor (α) was the ratio of retention times of two peaks.
[b] Resolution factor (R) was the ratio of separation between two peaks to average width of the two peaks.
[c] Retention index (I) measured on DB-5 and DB-17 (30 m×0.25 mm I.D., 0.25 μm film thickness) dual-capillary columns programmed from 60°C (2 min) to 280°C at a rate of 3°C/min.

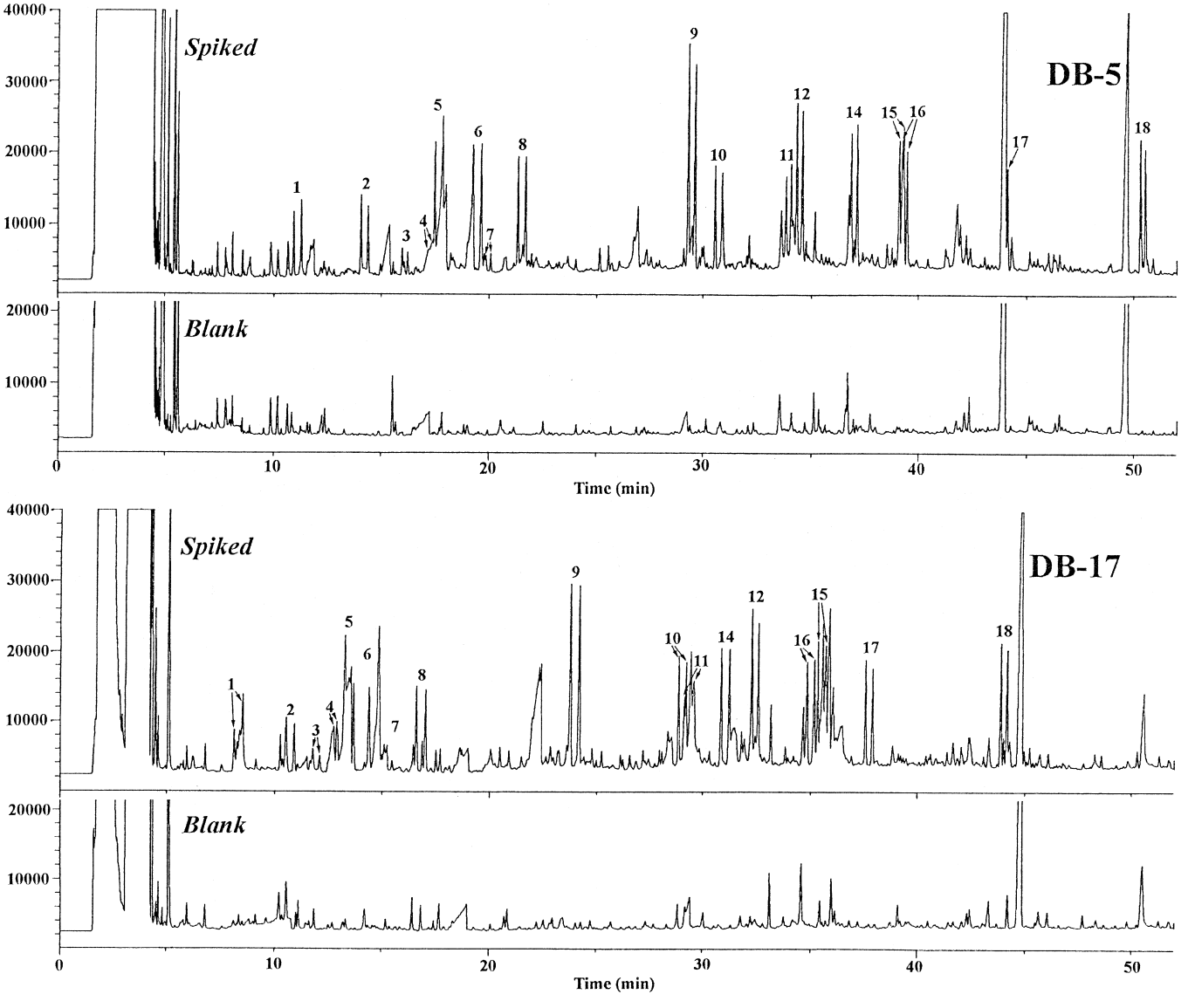

Fig. 2. Dual chromatographic profiles of normal urine specimen without spiking (blank) and after spiking (spiked) with 2-hydroxy acids separated on the DB-5 and DB-17 (both 30 m×0.25 mm I.D., 0.25 μm film thickness) dual-capillary column system. GC conditions and peak numbers as those in the Fig. 1.

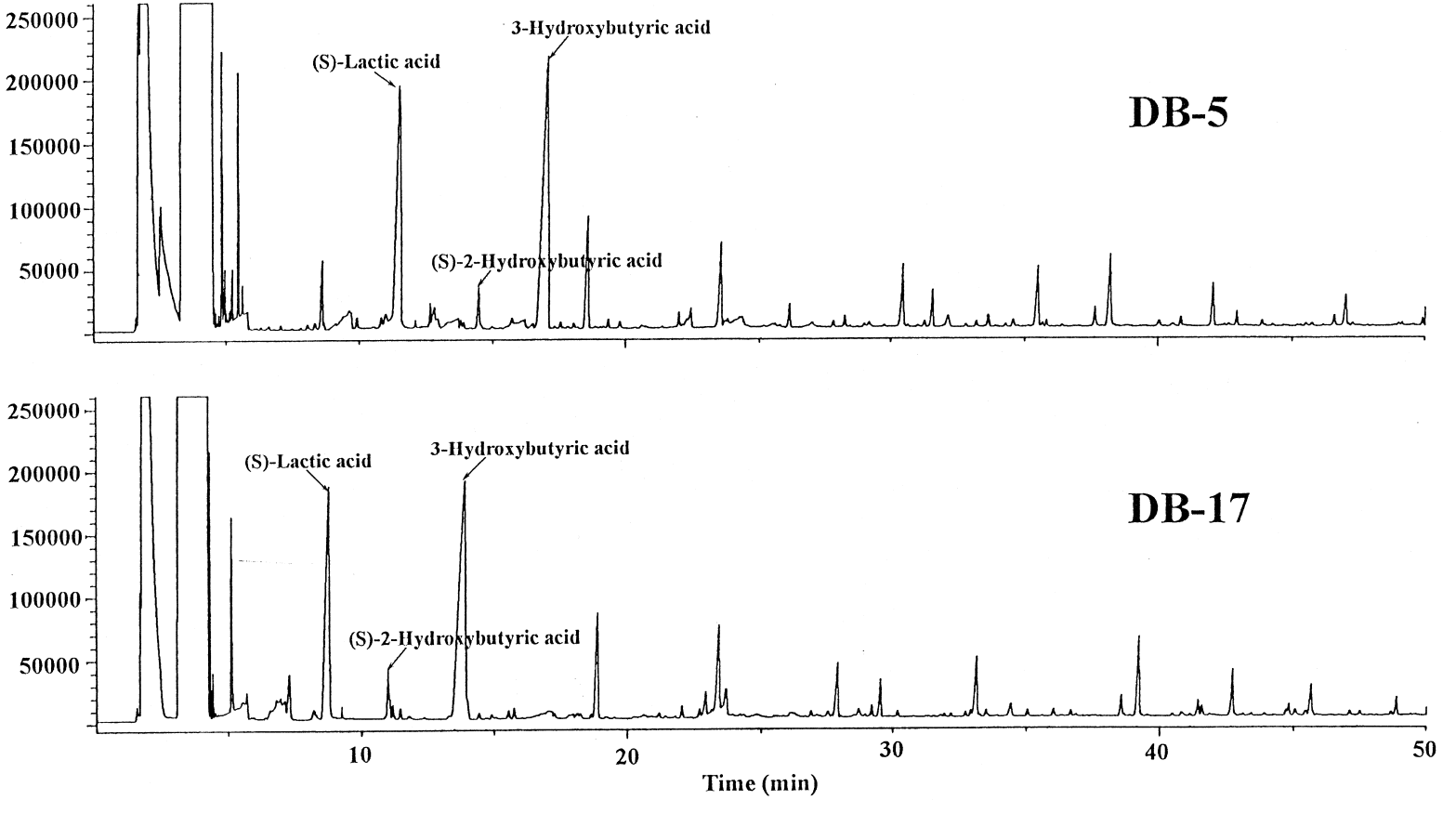

Fig. 3. Dual chromatographic profiles of clinical urine specimen from a patient with respiratory chain disorder separated on the DB-5 and DB-17 (both 30 m×0.25 mm I.D., 0.25 μm film thickness) dual-capillary column system. GC conditions as described in Fig. 1.

capillary column method permitted rapid and accurate chiral discrimination of most of the spiked racemic acids. 2-Hydroxyisovaleric acid (peak 3), glyceric acid (peak 4) and 2-hydroxy-3-methylvaleric acid (peak 7), because of their weak intensity, were prone to being interfered with by the interfering endogenous acids eluted close to them. Malic acid was not observable in the expected positions on both columns. When applied to a clinical urine specimen from a patient with respiratory chain disorder, (*S*)-lactic acid was positively detected in high concentrations along with (*S*)-2-hydroxybutyric acid and 3-hydroxybutyric acid (Fig. 3). The chirality of 3-hydroxybutyric acid excreted at higher concentration could not be determined due to the fact that no resolution of its enantiomers as the present diastereomeric derivatives is achieved. This was in good agreement with the previous findings in the literature [2,4]. Each peak was further confirmed by GC–MS. Presently, our *I* library contains 18 organic acid standards as *O*-TFA/(*S*)-MBE derivatives, which will continue to be expanded to include other 2-hydroxy acids for the identification of unknown acids and their chiral discrimination in clinical urine analysis.

4. Conclusions

The present enantiomeric separation on achiral dual-capillary columns of different polarity was useful for the rapid and accurate chiral discrimination of 18 different 2-hydroxy acid standards as their diastereomeric *O*-TFA/(*S*)-(+)-3-methyl-2-butyl esters based on *I* matching. The identification based on the *I* matching solved the problem of tedious cochromatographic procedure and shortened the overall analysis time. When applied to normal urine samples, chirality of each spiked 2-hydroxy acid enantiomer was accurately determined except for malic acid. From the urine sample of a patient with respiratory chain disorder, endogenous (*S*)-lactic acid and (*S*)-2-hydroxybutyric acid were positively identified along with 3-hydroxybutyric acid of unknown chirality. An extension of the present method to other diastereomeric derivatization procedures is under way to be used as the complementary confirmation tool in the enantiomeric separation and discrimination of diverse hydroxy acids including 3-hydroxybutyric acid.

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References

[1] J.P. Kamerling, G.J. Gerwig, M. Duran, D. Ketting, S.K. Wadman, Clin. Chim. Acta 88 (1978) 183.
[2] W.A. Koenig, I. Benecke, J. Chromatogr. 195 (1980) 292.
[3] J.P. Kamerling, M. Duran, G.J. Gerwig, D. Ketting, L. Bruinvis, J.F.G. Vliegenthart, S.K. Wadman, J. Chromatogr. 222 (1981) 276.
[4] J.P. Kamerling, J.F.G. Vliegenthart, in: H. Jaeger (Ed.), Glass Capillary Chromatography in Clinical Medicine and Pharmacology: Determination of the Absolute Configuration of Chiral Hydroxy Acids By GLC, Marcel Dekker, New York, 1985, pp. 371–391.
[5] W.L. Nyhan, G.D. Shelton, C. Jakobs, B. Holmes, C. Bowe, C.J.R. Curry, C. Vance, M. Duran, L. Sweetman, J. Child Neurol. 10 (1995) 137.
[6] G.F. Hoffmann, C. Jakobs, B. Holmes, L. Mitchell, G. Becker, H.P. Hartung, M.L. Nyhan, J. Inherit. Metab. Dis. 18 (1995) 189.
[7] R.J.A. Wanders, P. Mooger, J. Inherit. Metab. Dis. 18 (1995) 194.
[8] H. Watanabe, S. Yamaguchi, K. Saiki, N. Shimizu, T. Fukao, N. Kondo, T. Orii, Clin. Chim. Acta 238 (1995) 115.
[9] E. Chen, W.L. Nyhan, C. Jakobs, C.M. Greco, A.J. Balkovich, V.A. Cox, S. Packman, J. Inherit. Metab. Dis. 19 (1996) 335.
[10] Y. Geerts, W.O. Renier, J. Bakkeren, J. Dejong, J. Neuro. Sci. 143 (1996) 166.
[11] M. Heil, F. Podebrad, T. Beck, A. Mosandl, A.C. Sewell, H. Boehles, J. Chromatogr. B 714 (1998) 119.
[12] K. Tanaka, D.G. Hine, A.W. Dull, T.B. Lynn, Clin. Chem. 26 (1980) 1839.
[13] M.Y. Tsai, C. Oliphant, M.W. Josephson, J. Chromatogr. 341 (1985) 1.
[14] L. Powers, S.T. Ciraolo, K.C. Agarwal, A. Kumar, C. Bomont, M.V. Soloviev, F. David, S. Desrochers, H. Brunengraber, Anal. Biochem. 221 (1994) 323.
[15] P. Duez, A. Kumps, Y. Mardens, Clin. Chem. 42 (1996) 609.
[16] I.M. Bengtsson, D.C. Lehotay, J. Chromatogr. B 685 (1996) 1.
[17] K.R. Kim, J.H. Kim, D.J. Jeong, D.J. Paek, H.M. Liebich, J. Chromatogr. B 701 (1997) 1.

[18] K.R. Kim, H.G. Park, M.J. Paik, H.S. Ryu, K.S. Oh, S.W. Myung, H.M. Liebich, J. Chromatogr. B 712 (1998) 11.
[19] J.M. Halket, A. Przyborowska, S.E. Stein, W.G. Mallard, S. Down, R.A. Charmers, Rapid Commun. Mass Spectrom. 13 (1999) 279.
[20] A. Carlson, O. Gyllenhaal, J. Chromatogr. 508 (1990) 333.
[21] E.P. Mitchell, L. Evanx, P. Schultz, R. Mudsen, J.W. Yarbro, C.W. Gehrke, K. Kuo, J. Chromatogr. 581 (1992) 31.
[22] N.R. Srinivas, W.C. Shyu, R.H. Barbhaiya, Biomed. Chromatogr. 9 (1995) 1.