Journal of Chromatography, 278 (1983) 133—138
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1819

Note

Volatile carboxylic acid profiling in physiological fluids

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(First received March 14th, 1983; revised manuscript received June 3rd, 1983)

Many inborn errors of metabolism are characterized by an increase in the concentrations of various carboxylic acids in blood and urine. For example, the metabolic disorders propionic acidemia [1], methylmalonic aciduria [2,3], maple syrup urine disease [4], isovaleric acidemia [4] and glutaric aciduria type II [5,6] show increased amounts of volatile carboxylic acids in blood or urine. Procedures for the profiling and/or quantitation of these carboxylic acids require their isolation from blood or urine by vacuum distillation [6], extraction [7,8], silicic acid absorption [9] or ion-exchange chromatography [10] prior to gas chromatographic (GC) or gas chromatographic—mass spectrometric (GC—MS) analysis. These procedures are time-consuming and may result in losses of the more volatile acids. We have developed a rapid procedure for the profiling of volatile carboxylic acids in blood and urine that involves neither extraction or derivatization of the carboxylic acids prior to GC analysis.

EXPERIMENTAL

Materials

All reference carboxylic acids were purchased either from Sigma (St. Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.) either as the free acid or as the sodium salt and were used as received. The 10% SP-1200—1% H_3PO_4 on 80—100 Chromosorb W AW (1.83 m × 2 mm I.D.) and the 10% SP-1000—1% H_3PO_4 on 100—200 Chromosorb W AW (1.83 m × 2 mm I.D.) glass GC columns were purchased from Supelco (Bellefonte, PA, U.S.A.). A Varian 3700 gas chromatograph (Palo Alto, CA, U.S.A.) with a flame ionization detector was used for the volatile carboxylic acid profiling. The profiles were recorded on a Varian 9176 recorder.

0378-4347/83/$03.00

Analytical procedure

GC columns were conditioned at 190°C with a nitrogen flow-rate of 30 ml/min. Periodic injection of a 1% formic acid solution was performed until a stable baseline resulted (1—2 days).

Urine profiling. To 1.00 ml of urine were added 10 μl of a 0.05 mmol/ml trimethylacetic acid (internal standard) [9] solution. The sample was acidified by the addition of 3—5 drops of 1 *M* hydrochloric acid. A 2-μl aliquot was injected onto the SP=1200 column. Column temperature, 90°C for 4 min then 5°/min to 200°C; injector temperature, 230°C; detector temperature, 230°C; nitrogen flow-rate, 30 ml/min.

Urine samples exhibiting an abnormal profile were analyzed on the SP-1000 column. A 1-μl aliquot of the sample was injected onto the SP-1000 column. Column temperature, 150°C; injector temperature, 230°C; detector temperature, 230°C; nitrogen flow-rate, 30 ml/min.

Peak identification was by the peak relative retention time (RRT) on each column and peak enhancement when the urine sample was spiked with the authentic reference compound.

Between sample injections, 10 μl of a 1% formic acid solution were injected onto the column to remove ghost peaks.

RESULTS AND DISCUSSION

Blood or urine samples are profiled initially on an SP-1200 column [11] using a temperature gradient. Volatile carboxylic acids that can be separated in this manner are shown in Fig. 1A. Table I lists the retention time and the RRT (relative to the internal standard) of each carboxylic acid. Butanoic acid appears as a shoulder on the internal standard peak (RRT slightly greater than 1.0) while 2-methylbutanoic and 3-methylbutanoic acids are not separated.

In Fig. 1B is shown the profile of an abnormal urine sample (due to valproic acid therapy) on the SP-1200 column. At this sensitivity, the profile of a normal sample would show only the internal standard peak. Abnormal samples detected by the first method are then analyzed isothermally (150°C) on an SP-1000 GC column [11] which permits resolution of a greater number of volatile carboxylic acids than can be separated under the previous conditions. These separations are shown in Fig. 2. In Table II are listed the retention time and RRT (relative to the internal standard) of each carboxylic acid on the SP-1000 column.

Fig. 3A shows the urinary volatile carboxylic acid profile from an infant with methylmalonic aciduria and Fig. 3B shows the plasma volatile carboxylic acid profile from the same infant. Under the GC conditions of the profiling procedure, methylmalonic acid quantitatively undergoes a thermal decarboxylation to propanoic acid [12]. Confirmation that the large propanoic acid peak in these profiles was due to methylmalonic acid and not propanoic acid was provided by a positive (purple) reaction of the urine and plasma samples with tetrazotized *o*-dianisidine (Fast Blue B) [13]. Methylmalonic acid reacts with tetrazotized *o*-dianisidine while propanoic acid does not react.

Without access to a gas chromatograph—mass spectrometer we are not able to confirm the identity of each peak in the chromatogram. Thermally labile

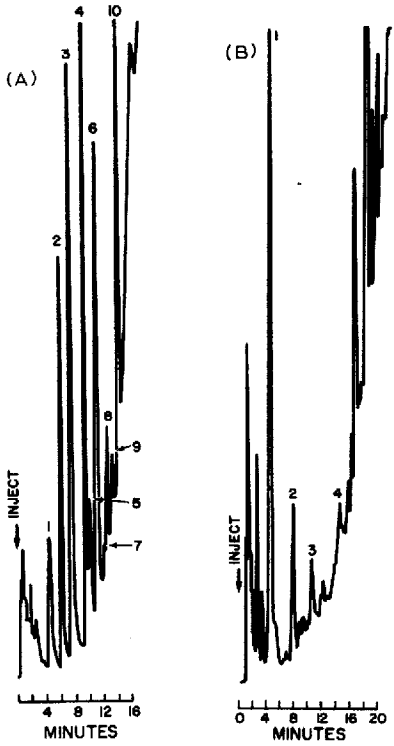

Fig. 1. A, Profile of the standard volatile carboxylic acid mixture on the SP-1200 column using a temperature program: 90°C for 4 min then 5°C/min to 200°C. Peaks: 1 = propanoic acid; 2 = isobutyric acid; 3 = internal standard, trimethylacetic acid; 4 = 2-methylbutanoic and 3-methylbutanoic acids; 5 = crotonic acid; 6 = pentanoic acid; 7 = β-methylcrotonic acid; 8 = tiglic acid; 9 = isocaproic acid; 10 = hexanoic acid. The concentration of each acid was 3 nmol/ml. B, Profile of an abnormal urine sample on the SP-1200 column (temperature program: 90°C for 4 min then 5°C/min to 200°C). Peaks: 1 = 3-heptanone (from valproic acid); 2 = internal standard, trimethylacetic acid; 3 = pentanoic acid; 4 = hexanoic acid and several unidentified peaks.

TABLE I

RETENTION TIMES (t_R) AND RRT OF THE VOLATILE CARBOXYLIC ACIDS ON THE SP-1200 COLUMN (90—200°C TEMPERATURE GRADIENT)

Organic acid	t_R (min)	RRT
Propanoic	4.0	0.58
Isobutyric	5.8	0.81
Internal standard	6.6— 7.2	1.00
Butanoic	6.6— 7.2	1.00
2-Methylbutanoic	8.6— 9.2	1.28—1.30
3-Methylbutanoic	8.6— 9.2	1.28—1.30
Crotonic	9.0—10.4	1.36—1.39
Pentanoic	10.2—11.0	1.53—1.55
β-Methylcrotonic	11.4—12.2	1.69—1.73
Tiglic	11.8—12.6	1.75—1.79
Isocaproic	12.6—13.2	1.83—1.91
Hexanoic	13.2—14.2	1.97—2.00

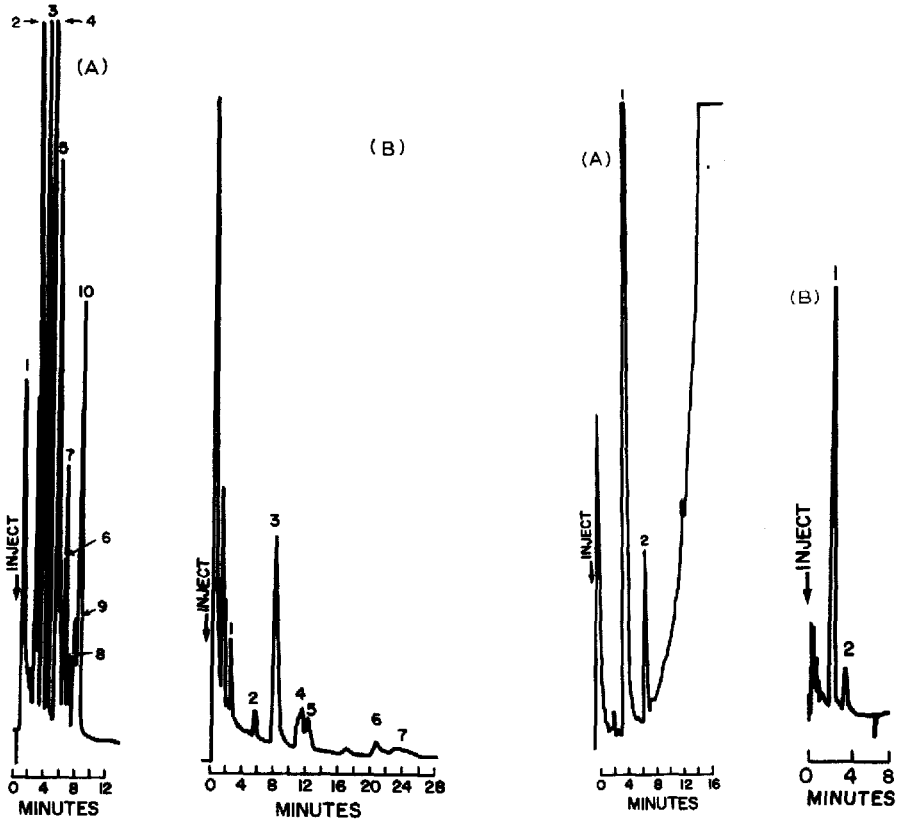

Fig. 2. A, Profile of the standard volatile carboxylic acid mixture on the SP-1000 column isothermal at 150°C. Peaks: 1 = propanoic and isobutyric acids; 2 = internal standard, trimethylacetic acid; 3 = butanoic acid; 4 = 2-methylbutanoic and 3-methylbutanoic acids; 5 = crotonic acid; 6 = pentanoic acid; 7 = β-methylcrotonic acid; 8 = isocaproic acid; 9 = tiglic acid; 10 = hexanoic acid. The concentration of each acid was 3 nmol/ml. B, Profile of the standard volatile α-ketocarboxylic acid mixture isothermally (150°C) on the SP-1000 column. Peaks: 1 = internal standard; 2 = pyruvic acid; 3 = α-ketoisovaleric acid and α-ketobutyric acid; 4 = α-ketovaleric acid and α-keto-β-methylvaleric acid; 5 = α-ketoisocaproic acid; 6 = succinic acid; and 7 = lactic acid. The concentration of each acid was 0.4 μmol/ml.

Fig. 3. A, Profile of a urine sample from an infant who had methylmalonic aciduria (SP-1200 column). Peaks: 1 = propanoic acid (11.0 μmol/ml) from the thermal decarboxylation of methylmalonic acid; 2 = internal standard, trimethylacetic acid. B, Profile of a plasma sample from an infant who had methylmalonic aciduria (SP-1200 column). Peaks: 1 = propanoic acid (17.4 μmol/ml) from the thermal decarboxylation of methylmalonic acid; 2 = internal standard, trimethylacetic acid. Attenuation $16 \cdot 10^{-11}$.

carboxylic acids may decompose under these chromatographic conditions, thus altering peak identification. Each carboxylic acid, however, did afford a single peak with a reproducible relative retention time when analyzed by GC. Definitive identification of each peak should be determined by GC—MS.

TABLE II

RETENTION TIMES (t_R) AND RRT OF THE VOLATILE CARBOXYLIC ACIDS ON THE SP-1000 COLUMN (ISOTHERMAL AT 150°C)

Carboxylic acid	t_R (min)	RRT
Propanoic	2.0—2.2	0.83—0.92
Isobutyric	7.0—2.2	0.83—0.92
Internal standard	2.4	1.00
Butanoic	7.5—3.0	1.25
2-Methylbutanoic	3.6—3.8	1.50—1.58
3-Methylbutanoic	3.6—3.8	1.50—1.58
Crotonic	4.6—4.8	1.92—2.00
Pentanoic	5.0—5.4	2.08—2.25
β-Methylcrotonic	5.8—6.0	2.42—2.50
Isocaproic	6.4	2.67
Tiglic	7.2	3.00
Hexanoic	7.6	3.17
Pyruvic	4.6—4.8	1.92—2.00
α-Ketoisovaleric	8.0	3.08
α-Ketobutyric	8.0	3.08
α-Keto-β-methylvaleric	11.2	4.31
α-Ketovaleric	11.2	4.31
α-Ketoisocaproic	12.4	4.77
α-Ketocaproic	17.0	6.54
Succinic	20.6	7.92
Lactic	23.4	9.00

Ghost peaks [11] can be a problem on both the SP-1200 and the SP-1000 columns. We were able to minimize the appearance of ghost peaks in our profiles by the injection of 10 μl of a 1% formic acid solution onto the column at the end of each profile.

With these procedures, the average column lifetime is 6 months for the SP-1200 column and 8—12 months for the SP-1000 column. Over the course of daily use, the retention time of the volatile acids on the SP-1200 column steadily decreases over the 6-months lifetime of the column. For example, the retention time for the internal standard on a new column was 6.6 min, after 3 months of use the internal standard retention time was 5.4 min and after 5 months the retention time was 4.0 min. The resolution of the volatile acids was not changed over the column lifetime. There is a similar but less rapid change in the volatile acid retention times over the lifetime of the SP-1000 column.

We have found that this profiling procedure permits the rapid identification of abnormal volatile fatty acid concentrations in the plasma and urine of ill newborns suspected of having inborn errors of metabolism. Recently this method permitted positive identification of methylmalonic aciduria in an infant only 30 min after the sample was received in the laboratory. Since early institution of therapy is crucial for a favorable outcome in these circumstances, we recommend this method for early identification of the volatile carboxylic acids in body fluids.

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