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High-performance liquid chromatographic measurements of urinary hydroxycarboxylic acids as an index of the metabolic control in non-insulin-dependent diabetic patients

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Abstract

Hydroxycarboxylic acids in urine of patients with non-insulin-dependent diabetes mellitus and of healthy subjects are analyzed as 2-nitrophenylhydrazides by an improved high-performance liquid chromatographic method which has advantages with respect to resolution and analysis time. Variations in levels of hydroxycarboxylic acids, originated from the metabolism of valine, leucine and isoleucine, have been described in the diabetic patients who have good and poor metabolic controls. The sum of the hydroxycarboxylic acids in both groups of diabetic patients was significantly increased compared with the values of the healthy subjects. Statistically significant difference was present between the two groups. In the whole group of diabetic patients, the sum of the hydroxycarboxylic acids correlated with fasting plasma glucose or hemoglobin A_1c ($r=0.548$, $P<0.01$ and $r=0.629$, $P<0.01$, respectively). These results suggest that the relevance of these abnormalities may be used as an index of metabolic control in diabetic patients.

Keywords: Hydroxycarboxylic acid

1. Introduction

Increased concentrations of the branched-chain amino acids (BCAA), such as valine, leucine and isoleucine, have been observed in the plasma of patients with insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) [1]. However, plasma concentrations of the BCAA are more prominently affected than those of other amino acids when human subjects are given either a protein, carbohydrate, or fat meal [2]. Hydroxycarboxylic acids (HAs) in urine are metabolites of the BCAA or products of ketogenesis. Elevated excretions of the HAs have been described in ketoacidosis due to DM or other etiology [3–10]. Therefore, profiling analyses of the HAs are particularly suited to biochemical and diagnostic studies of the disease because of the variety of metabolic changes in DM.

In the investigation of urinary HAs by GC-based methods extraction and prefractionation of the total mixture of organic acids are needed because of the complexity of the mixture of acidic compounds in urine [5,6,8–10]. Therefore, the GC-based methods

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are of minor value for routine clinical applications, and as a result detailed informations on ranges and concentrations in different pathophysiological situations are still incomplete. It is therefore desirable to establish an alternative method with a simple procedure for reliable and accurate results.

We have already described the determination of thirteen HAs in urine by high-performance liquid chromatography (HPLC) after derivatization with 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) to their hydrazides [11]. Our previous HPLC method has several advantages with respect to simplicity and rapidity for the sample preparation over GC-based methods, but unfortunately sometimes there was interference in the detection of 3-hydroxy-2-ethylpropionic acid caused by a few components in the urine matrix during the HPLC analysis. The aims of the present study are to develop an improved separation for the HPLC analysis of urinary HAs originated from the BCAA and to examine its analytical utility in the assessment of the metabolic control in patients with NIDDM.

2. Experimental

2.1. Chemicals and reagents

All HA solutions in ethanol were obtained from Yamamura Chemical Laboratories (Kyoto, Japan). 3-Hydroxyisobutyric acid was synthesised according to the literature [4]. A 2-NPH·HCl (Tokyo Chemical, Tokyo, Japan) solution (0.02 *M*) was prepared by dissolving the reagent in 0.1 *M* HCl–ethanol (1:1, v/v). A 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1-EDC·HCl) (Sigma, St. Louis, MO, USA) solution (0.25 *M*) was prepared by dissolving the reagent in a solution of pyridine (3%, w/v) in ethanol. A KOH solution (10%, w/v) was prepared by dissolving the reagent in methanol–water (1:1, v/v). All the reagent solutions were stable at least three months when kept below 5°C, and were commercially available from Yamamura Chemical. All other chemicals were reagent grade.

2.2. HPLC analysis

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an on-line degasser ERC-3310 (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV-Vis detector. The detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 30°C using a Shimadzu GTO-6A column oven.

The separation was performed with a J'sphere ODS-M 80 column (particle size 4 μm, 150×6 mm I.D.), packed at Yamamura Chemical.

All analyses were carried out isocratically using methanol–acetonitrile–water (32:8:60, v/v) as the eluent at a flow-rate of 2.0 ml/min. The pH of the eluent was maintained at 4–5 by adding 0.1 *M* HCl. The eluent was filtered through a Nucleopore filter (pore size 0.2 μm; Nomura Micro Science, Osaka, Japan).

2.3. Subjects

Studies were carried out on 27 patients with NIDDM (aged 54.0±9.6 years, mean±S.D.) after receiving their informed consent. The ranges of fasting plasma glucose (FPG) and hemoglobin A_1c (HbA_1c) of the patients were $100 \leq FPG \leq 250$ mg/dl and $6.0 \leq HbA_1c \leq 13.5\%$, respectively. The patients were classified in two groups namely good and poor control groups using the parameters of FPG and/or HbA_1c. The good control group ($FPG \leq 130$ mg/dl or $HbA_1c \leq 7.0\%$) consisted of 15 patients (aged 53.3±8.0 years). The poor control group comprised other 12 patients (aged 54.9±11.6 years). Seven healthy subjects (aged 43±11 years, $FPG \leq 90$ mg/dl and $HbA_1c \leq 5.8\%$) were also studied as controls. Urine samples were obtained as the second urine after an overnight fast from NIDDM patients and healthy subjects.

The measurements of FPG and HbA_1c were carried out by Automated Glucose Analyzer Glucoroder-MK II (A&T, Tokyo, Japan) and Auto A_1c HA-8110 A (Kyoto Daiichi, Kyoto, Japan), respectively.

2.4. Assay procedure

Urinary HAs were converted into their hydrazides as previously described method with a slightly modification [11]. Briefly, to 200 μl of urine sample, 200 μl of ethanol containing 100 nmol of 2-hy-

droxy-2-methylbutyric acid as the internal standard and 1 ml of 0.2 *M* HCl–ethanol (1:9, v/v) were added. After the centrifugation, the supernatant solution was taken and evaporated with a stream of nitrogen at room temperature. To the residue, 100 μl of water, 200 μl of ethanol, 200 μl of 2-NPH·HCl solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of 10% (w/v) KOH solution, the mixture was further heated at 80°C for 5 min and then cooled.

The resulting hydrazide mixture was neutralized by adding 4 ml of 0.033 *M* phosphate buffer (pH 6.4)–0.5 *M* HCl (7:1, v/v) and washed twice with 4 ml of *n*-hexane. The aqueous layer was taken and the HA hydrazides were extracted twice 4 ml of diethyl ether. The combined ether layer was dried over anhydrous Na_2SO_4 and evaporated with a stream of nitrogen at room temperature. The residue was redissolved in 100 μl of methanol and an aliquot (4–20 μl) was injected into the chromatograph. All centrifugations were carried out at 450 *g* for 5 min.

2.5. Calculation

HA concentrations have been calculated by using calibration curves. In order to correct changes of HA concentrations by urine quantity, the values of HA were measured as displacement on nmol/(mg Cr)·10^{-2} using urine creatinine (Cr). Cr was measured by an Automated Creatinine Analyzer Hitachi 736-40 (Hitachi, Tokyo, Japan).

3. Results and discussion

3.1. Derivatization and HPLC analysis

We first tried to determine the HAs originated from the BCAA in urine from NIDDM patients and healthy subjects by our previously published HPLC method [11], but unfortunately the detection of 3-hydroxy-2-ethylpropionic acid hydrazide was sometimes interfered with by a few components in the urine matrix. This problem is settled by introducing the newly developed J'sphere ODS-M80 column with a simple isocratic elution system, and the separation of thirteen HA hydrazides has progressed remarkably in retention times. However, 3-hydroxyisobutyric acid, originating from the metabolism of valine, could not be separated from 3-hydroxybutyric acid in all instances. Fig. 1 shows a typical separation of the fourteen HA hydrazides by HPLC analysis with methanol–acetonitrile–water (32:8:60, v/v) as the eluent and with detection in the visible region.

Another investigation was needed to decrease the derivatization period required. The derivatization rate gradually increased with increasing temperature, but the detector response decreased with reproducible yields. By decreasing the retention times of the individual HA hydrazides, on the other side, the observed detector signals were increased. Therefore, using an optimum treatment of 5 min at 8°C, the HAs studied here were rapidly converted to their hydrazides and a decrease of 25 min compared to the previous derivatization period [11] was obtained.

3.2. Quantitative analysis

Calibration curves were constructed by derivatizing increasing amounts of the HAs in the presence of 2-hydroxy-2-methylbutyric acid as the internal standard and analysing as described above. The calibration test was replicated five times. From the chromatograms obtained, the relationships between the ratio of the peak heights of the HA hydrazides to that of the internal standard and the concentrations of the HAs were calculated by the least-squares method. The calibration curves were linear at least between 2 pmol and 5 nmol per injection with correlation coefficients of 0.999–1.000. The limits of detection, based on a signal-to-noise ratio of 2, were 1–2 pmol per injection.

3.3. Recovery and precision

Known amounts (50 and 200 nmol) of mixtures of the HAs were added to pooled urine sample to examine the precision of HA measurements across a broad range of HA concentrations and to test the efficiency of HA recovery in the assay procedure. Each aliquot was analyzed by nine separate measurements for the HA contents. The recoveries are corrected for the initial presence of HA in the urine sample. In this experiments, the recoveries of the HAs were in the range of 96.4–103.2% (C.V.=1.0–

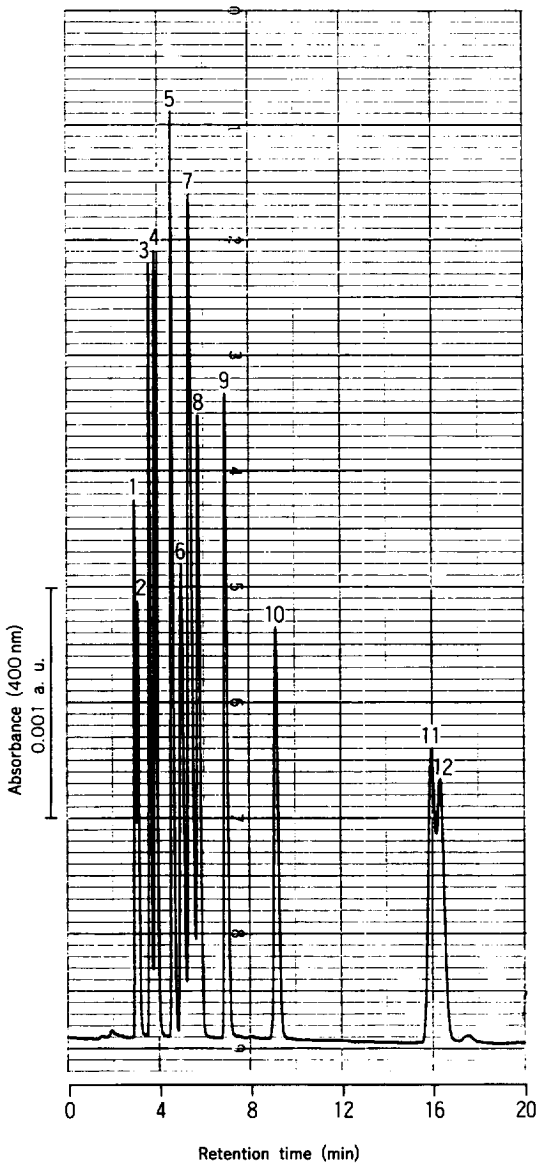

Fig. 1. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 14 hydroxycarboxylic acids obtained with visible detection. Peaks: 1=glycolic; 2=3-hydroxypropionic; 3=lactic; 4=3-hydroxybutyric and 3=hydroxyisobutyric; 5=2-hydroxyisobutyric; 6=3-hydroxy-2-methylbutyric; 7=2-hydroxybutyric and 3=hydroxyisovaleric; 8=3-hydroxy-2-ethylpropionic; 9=2-hydroxy-2-methylbutyric (I.S.); 10=2-hydroxyisovaleric; 11=2-hydroxyisocaproic; 12=2-hydroxy-3-methylvaleric acid hydrazide. The sample size varied from 100 to 300 pmol of each acid.

3.8%) and 97.8–104.1% (C.V.=0.8–4.2%), respectively.

The intra-assay precision was evaluated by assaying six times the same urine sample. The inter-assay precision was determined by analyzing spiked urine sample on different days over one week ($n=6$). The intra- and inter-assay precision ranged from 0.6–3.6% and 0.9–4.4%, respectively.

3.4. Applicability

The present HPLC method applied to the determination of the HAs originated from the BCAA in urine from NIDDM patients and healthy subjects. The HA profiles of urine samples from a healthy subject and a poorly controlled diabetic patient are shown in Fig. 2. In the chromatograms monitored by visible absorbance some unknown peaks were appeared, but the HA hydrazides in the samples were identified by comparison of the retention times with those of standards and also cochromatography of the standards and urine samples with a different elution system. Liebich and Forst [6] and our previous report [11] indicated that 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid were present in very low amounts and were often below the detection limit in normal urine, while the other HAs were regularly found. The present study demonstrate that in urine from diabetic patients 2-hydroxyiso-valeric acid is identified as a measurable peak, but the latter two acids can not detect even in poorly controlled diabetic patients. Other BCAA metabolites, such as 3-hydroxy-2-methylbutyric acid, 3-hydroxyisovaleric acid and 3-hydroxy-2-ethylpropionic acid, are found in all the urine samples from diabetic patients and healthy subjects. 3-Hydroxyisobutyric acid, which is a major component among the HAs, may be closely eluted with 3-hydroxybutyric acid.

At first, a correlation between the HAs and any of the existing parameters such as FPG or HbA_1c was investigated. In 27 NIDDM patients significant correlations were demonstrated between the sum of the HAs and FPG and HbA_1c ($r=0.548$, $P<0.01$ and $r=0.629$, $P<0.01$, respectively). From these results, it is thought that the sum of the HAs has both characteristics of a short-term control parameter like FPG and a long-term control parameter like HbA_1c.

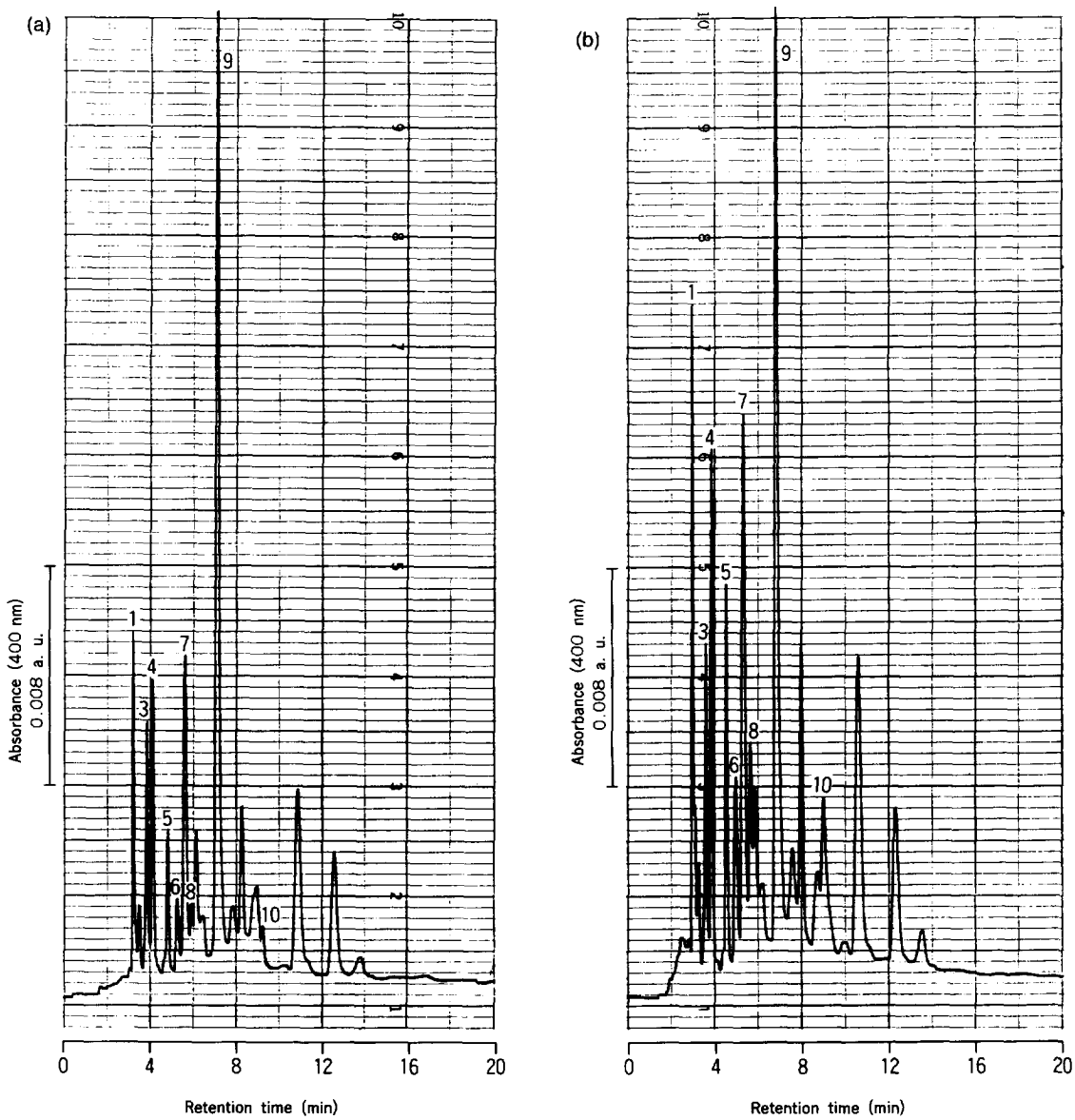

Fig. 2. Chromatograms of the derivatized hydroxycarboxylic acids in urine samples from a healthy subjects (a) and a poorly controlled patients with NIDDM (b). Each peak number corresponds to the number in Fig. 1.

In addition to examine whether the HA has reflected metabolic control of the NIDDM patients, the patients were classified into two groups namely good and poor control groups using the parameters of FPG and/or HbA_1c. The mean values of the individual and sum of the four HAs in urine from NIDDM patients and healthy subjects are listed in Table 1. Elevated excretions of individual HAs were observed in urine from diabetic patients. Significant differences were present in the sum of the HAs and 3-hydroxyiso-valeric acid between poor and good control groups. In both groups, the sum of the HAs and 2-hydroxyisovaleric acid were significantly higher when compared with healthy subjects. These results suggest that the sum of the HA levels may become markers of metabolic control in NIDDM

Table 1
Indices of diabetic control and urinary hydroxycarboxylic acids in diabetic and healthy subjects

Group	Hydroxycarboxylic acid (nmol/(mg Cr)·10^{-2})				
	Peak 6	Peak 7	Peak 8	Peak 10	Total
Healthy subjects (n=7)	0.26±0.06	1.38±0.53	0.43±0.11	0.01±0.006	2.08±0.53
Diabetic subjects (n=27)	0.31±0.06	2.62±0.23[a]	0.46±0.06	0.54[d]±0.38	3.92±0.25[e]
Good control (n=15)	0.30±0.08	2.16±0.24	0.38±0.05	0.40[c]±0.11	3.23±0.23[a]
Poor control (n=12)	0.33±0.09	3.19±0.36[b]	0.53±0.12	0.72±0.56	4.78±0.36[f]

Each peak number corresponds to the number in Fig. 1. Total indicates the sum of each peak. Data are expressed as mean±S.E.
[a]Significantly different from healthy subjects; $P<0.05$.
[b]Significantly different from good control in diabetic subjects; $P<0.05$.
[c]Significantly different from healthy subjects; $P<0.005$.
[d]Significantly different from healthy subjects; $P<0.001$.
[e]Significantly different from healthy subjects; $P<0.01$.
[f]Significantly different from good control in diabetic subjects; $P<0.005$.

because of the positively association with other indices such as FPG and HbA_1c. One possible explanation for our observation is that in NIDDM insulin resistance or inappropriately low serum insulin levels [1,12] decrease muscle uptake of the BCAA, and accelerate the BCAA catabolism in kidney. Further study is needed to clarify the metabolic pathway involved in the formation of the HAs and the clinical significance of the abnormalities in diabetic patients.

4. Conclusion

This study provides a notable HPLC method for the analysis of the HAs originated from the BCAA. Without complicated isolation steps, the use of precolumn derivatization in conjunction with HPLC to analyze urine samples from NIDDM patients and healthy subjects has an unquestionable advantage over the previously published methods. Thus, the present method may be used for biomedical investigation and therapeutic monitoring in diabetes mellitus because it gives reliable and accurate results with reducing overall analysis time, cost and labor.

References

[1] P. Vannini, G. Marchesini, G. Forlani, A. Angiolini, A. Ciavarella, M. Zoli and E. Pisi, Diabetologia, 22 (1982) 217.
[2] S.A. Adibi, Metabolism, 25 (1976) 1287.
[3] S. Landaas, J. Chromatogr., 54 (1974) 39.
[4] S. Landaas, J. Chromatogr., 64 (1975) 143.
[5] J. Greter, S. Lindstedt, H. Seeman and G. Steen, Clin. Chem., 26 (1980) 261.
[6] H.M. Liebich and C. Forst, J. Chromatogr., 309 (1984) 225.
[7] T. Niwa, K. Yamada, T. Ohki and H. Furukawa, J. Chromatogr., 337 (1985) 1.
[8] T. Niwa, J. Chromatogr., 379 (1986) 313.
[9] H.M. Liebich, J. Chromatogr., 379 (1986) 347.
[10] H.M. Liebich, J. Chromatogr., 468 (1989) 157.
[11] H. Miwa, M. Yamamoto and T. Asano, Anal. Biochem., 185 (1990) 17.
[12] P. Felig, E. Marliss and G.F. Cahill, N. Engl. J. Med., 281 (1969) 811.