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Short communication

Liquid chromatographic–atmospheric pressure chemical ionization mass spectrometric analysis of glycine conjugates and urinary isovalerylglycine in isovaleric acidemia

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Abstract

n-Acetylglycine, *n*-propionylglycine, *n*-butyrylglycine, isobutyrylglycine, *n*-valerylglycine, isovalerylglycine, heptanoylglycine, phenylacetylglycine and isovalerylglucuronide were identified based on their liquid chromatographic–atmospheric pressure chemical ionization mass spectra (LC–APCI-MS). We were able to detect the presence of urinary isovalerylglycine in two cases of isovaleric acidemia using LC–APCI-MS. Membrane-filtered urine samples were injected into the LC–APCI-MS system in the negative-ion mode without any further pretreatment, and large amounts of isovalerylglycine were detected as the $[M-H]^-$ ion. The urinary excretion of isovalerylglycine appeared to increase after L-carnitine therapy. This analytical method is quick and easy and it may be a useful tool in understanding dysfunctional conditions in isovaleric acidemia.

1. Introduction

In a number of inborn errors related to organic acid or amino acid metabolism, many abnormal metabolites have been recognized which are derived through the alternative pathway of acylconjugation. This pathway, which includes glycine, glucuronide and carnitine conjugates, plays an important detoxification role. In patients with isovaleric acidemia, which is an inherited disorder of leucine metabolism due to a deficiency of isovaleryl-CoA dehydrogenase [1,2], the supplementation of glycine and/or carnitine has proven to be an effective therapy [3–6]. Hence, in addition to the ordinary analysis of organic acids, it is very useful for treatment evaluation purposes to analyze acylconjugates of glycine and carnitine which are present in the urine.

Gas chromatography–mass spectrometry (GC–MS) is widely used [7,8] in the analysis of many types of organic acids and acyl compounds.

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Although this useful and established method offers excellent sensitivity and identification capabilities, it requires a specific pretreatment procedure for analyzing high-polarity and heat-unstable substances.

In contrast, the liquid chromatographic–atmospheric pressure chemical ionization mass spectrometric (LC–APCI-MS) method is useful for analyzing high-polarity and heat-unstable substances in conventional HPLC conditions. Moreover, this method does not always require derivatization and is easy to use [9]. In this study, we identified some glycine and glucuronide conjugates on the basis of their LC–APCI-MS spectra and then developed a new method for analyzing urinary isovalerylglycine using LC–APCI-MS. With this method, we were able to determine the effect of L-carnitine supplementation on glycine metabolism in two children with isovaleric acidemia.

2. Experimental

2.1. Materials and patients

N-Acetylglycine, *n*-propionylglycine, *n*-butyrylglycine, isobutyrylglycine, *n*-valerylglycine, isovalerylglycine, heptanoylglycine and phenylacetylglycine were synthesized according to the methods described in Ref. [10].

Isovalerylglucuronide was synthesized from the reaction of benzyl 2,3,4-tri-O-benzyl-D-glucopyranuronate and isovaleric chloride, followed by hydrogenolytic debenzylation, according to the methods described in Ref. [11]. Urinary creatinine was measured by the Jaffé method (Creatinine-Test Wako, Wako Pure Chemical, Osaka, Japan).

All other chemicals used were of analytical grade.

Patients: case 1 was a four-year-old girl and case 2 was a five-year-old boy, with isovaleric acidemia. They were both chemically diagnosed in the neonatal period and were in good condition with protein restriction treatment (1.6–1.7 g kg^{-1} day^{-1}), and oral glycine (40–50 mg kg^{-1} day^{-1}) and L-carnitine (40 mg kg^{-1} day^{-1}) supplementation. They were not related to each other and had no family history of consanguinity. Informed consent was obtained from their parents in advance.

2.2. Carnitine loading tests

Intravenous L-carnitine loading (50 mg/kg; 1-h drip) was first performed three days after discontinuing L-carnitine administration. Urine samples were serially collected as shown in Fig. 4. Next, following three carnitine-free days, oral loading of carnitine was started with an initial dose of 25 mg kg^{-1} day^{-1} and raised in 25 mg kg^{-1} day^{-1} increments every four days up to 100 mg kg^{-1} day^{-1}. After an overnight fast, morning urine samples were obtained on the last two days of each dosage and stored at −20°C.

2.3. Sample preparation

All standards were dissolved in distilled water. Urine samples were filtered through a 0.22-μm centrifugal membrane filter (Ultra free C3-GV, Millipore, Bedford, MA, USA) to avoid clogging of the column. Finally, 5 or 10 μl of the samples were directly injected into the system without further pretreatment.

2.4. LC–APCI-MS analysis

A HPLC pump (Hitachi L-6200) combined with an APCI quadrupole mass spectrometer (Hitachi M-1000S) was equipped with a Shim-Pack CLC-ODS column (150 × 6 mm I.D., Shimadzu, Kyoto, Japan) at room temperature. The mobile phase was an ammonium acetate buffer (50 m*M* ammonium acetate adjusted to pH 3.5 with acetate) with 4.6% (v/v) acetonitrile at a flow-rate of 0.8 ml/min.

Mass spectra were recorded using negative-ion (NI) APCI in the cyclic scan mode (scan range: m/z 50–500). Isovalerylglucuronide analysis of patient urine was also conducted in the selected-ion monitoring (SIM) mode at m/z 277, m/z 175 and m/z 101. The vaporizer temperature was 200°C and the desolvator temperature was 399°C. The drift voltage was −40 V and/or −80 V.

To quantify isovalerylglycine in the urine samples, a calibration curve was obtained with mass chromatograms using standard solutions. The peak height of m/z 158 at 8.9 min was measured, and the calibration line was obtained with a correlation coefficient of 0.989 in a range of 5–80 μg. The coefficient of variation using LC–APCI-MS was 0.98% within a one-day analysis (n = 200).

3. Results

Fig. 1 shows the NI-APCI mass spectra of synthesized glycine conjugates and isovalerylglucuronide in a flow injection with a drift voltage of −40 V. The $[M-H]^-$ ions of each acylglycine were detected without fragment ions. With a drift voltage of −80 V, the $[M-H]^-$ ions had some fragments, i.e. $[M-COOH]^-$ and $NH_2CH_2COO^-$ as shown in Fig. 2.

Standards of n-valerylglycine, isovalerylglycine and isovalerylglucuronide, injected into the column system, showed the same mass spectra as that of the flow injection. Their retention times were 10.3, 8.9 and 6.5 min, respectively.

The mass chromatogram and mass spectra of the stable-state isovaleric acidemia urine sample are shown in Fig. 3. A total ion chromatogram peak was observed at the same retention time as

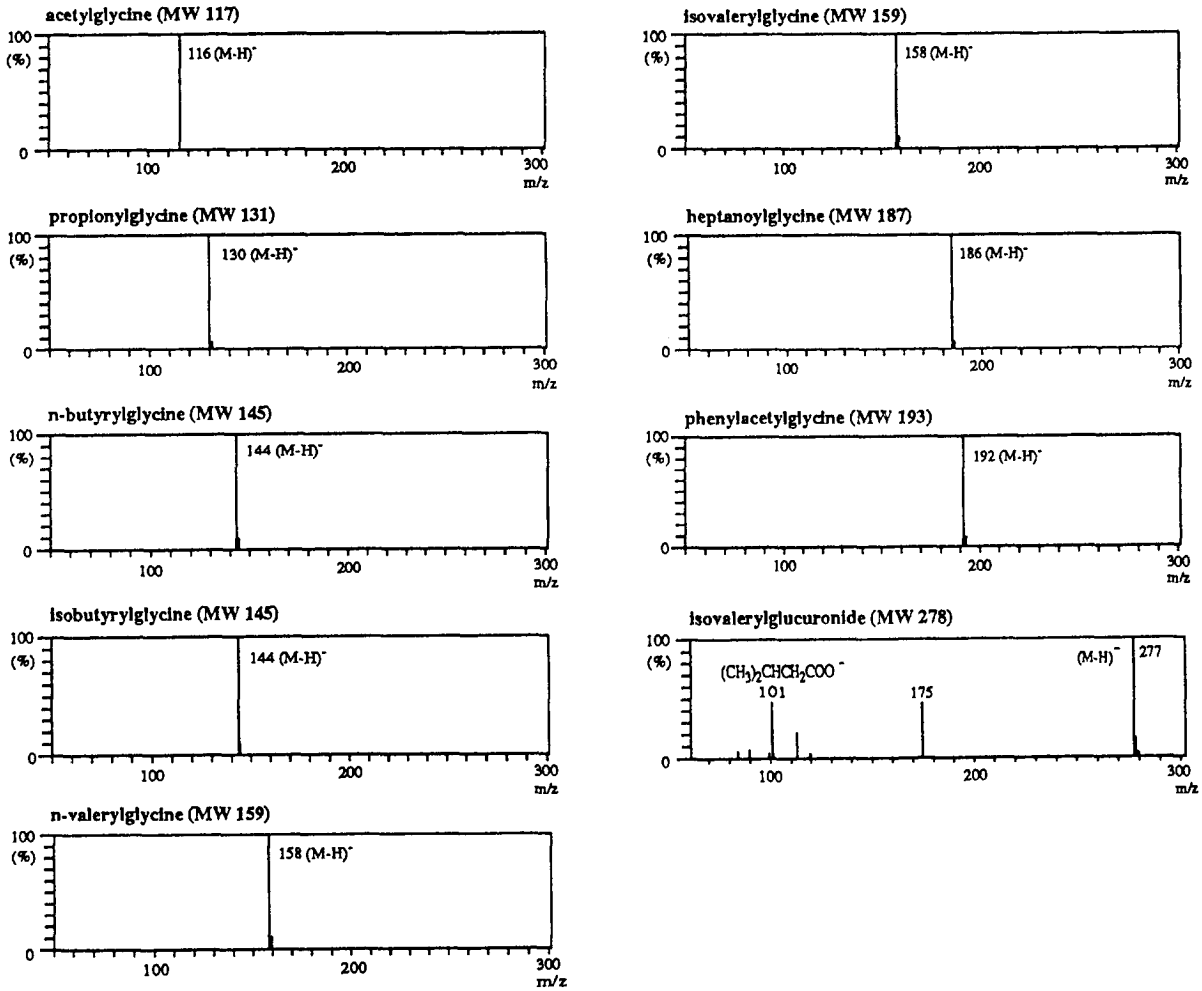

Fig. 1. NI-APCI mass spectra of glycine conjugates and isovalerylglucuronide with a drift voltage of −40 V.

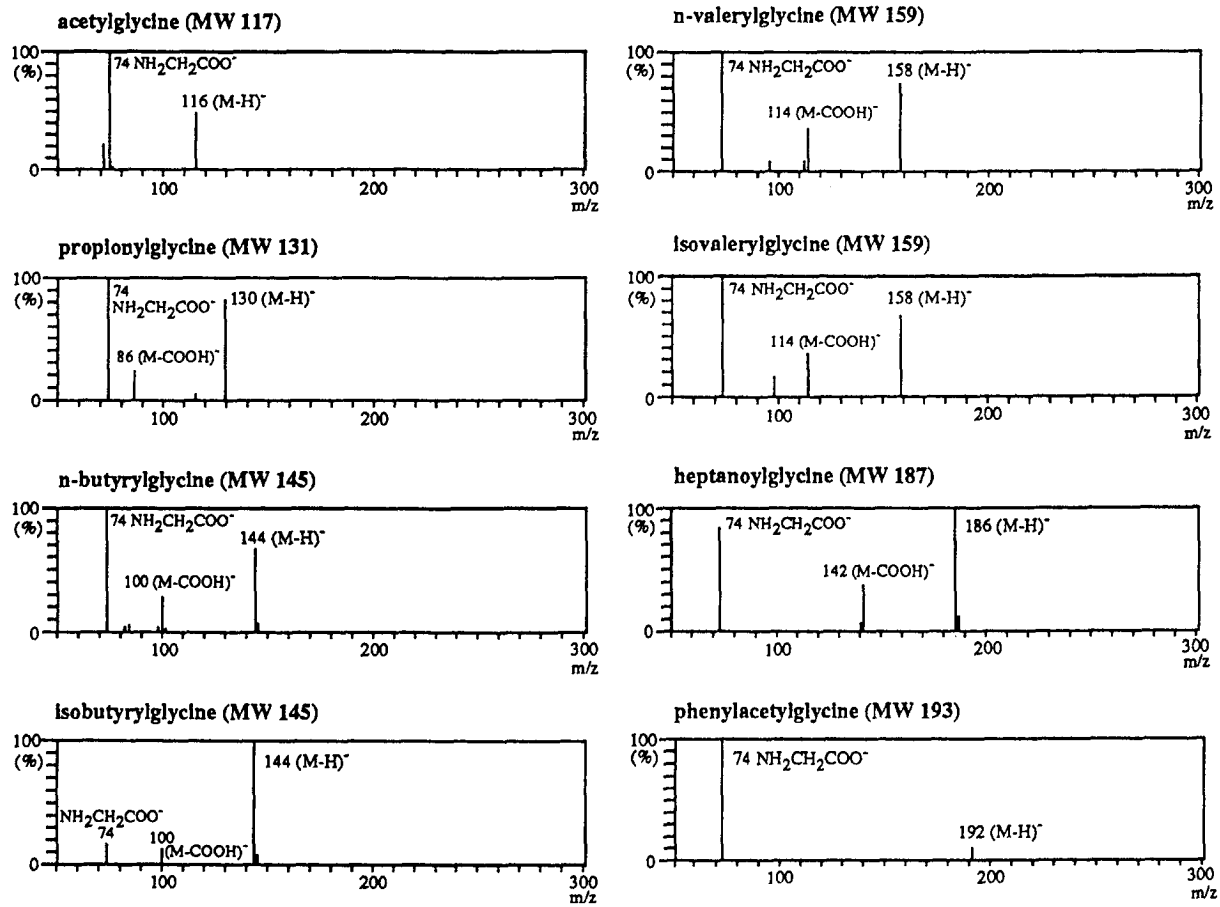

Fig. 2. NI-APCI mass spectra of glycine conjugates with a drift voltage of −80 V.

that of the isovalerylglycine standard. The mass spectrum of this peak at a drift voltage of −40 V showed an ion reading of only m/z 158. An analysis of this peak at a drift voltage of −80 V showed a similar mass spectral pattern of isovalerylglycine. This peak was therefore confirmed as isovalerylglycine. The sample from a healthy child showed no peak of m/z 158 at a retention time of 8.9 min. Cyclic scan and SIM revealed no peaks of m/z 277 (corresponding to the $[M-H]^-$ ion of isovalerylglucuronide) in urine samples from either healthy persons or patients. The minimum detection levels of the standard solution in this system, which was evaluated with a peak/background ratio of 2, were about 1 μg of isovalerylglycine with cyclic scan and about 0.05 μg of isovalerylglucuronide with SIM.

3.1. Carnitine loading test

Intravenous loading of L-carnitine caused remarkable alterations in the urine excretions of isovalerylglycine which were similar in both cases. Shortly after the infusion, the isovalerylglycine excretion level decreased first and gradually increased to ca. twice its initial level after 24 h.

During oral supplementation, the increased L-carnitine dose caused a gradual increase in urinary excretion of isovalerylglycine (Fig. 4).

4. Discussion

In this study we identified urinary isovalerylglycine, other authentic glycine conjugates

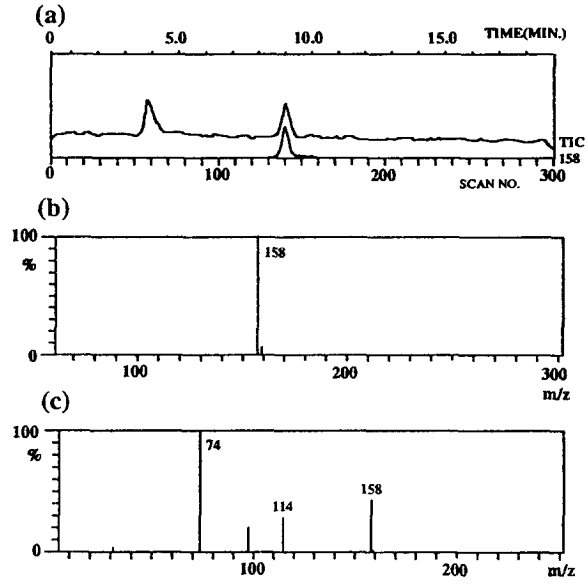

Fig. 3. NI-APCI mass chromatogram (a) and (b–c) mass spectra of a urinary sample from a patient with isovaleric acidemia: (b) drift voltage −40 V; (c) drift voltage −80 V.

and isovalerylglucuronide on the basis of their LC–APCI-MS spectra. Identification of isovalerylglycine with the LC–APCI-MS method was very simple due to the minor pretreatment procedures, and confirmation was easy due to the use of various drift voltage conditions to provide more information about the substances.

Measuring the acyl compounds of glycine and glucuronide is a good way to understand the pathophysiologic condition of certain inborn errors related to organic acid and amino acid metabolism. Isovaleric acidemia, a deficiency of isovaleryl-CoA dehydrogenase, is an inborn error of leucine catabolism [1,2]. In this disease, accumulated isovaleryl-CoA combines with glycine, carnitine, glucuronide, etc. and is excreted into the urine as a detoxifying mechanism [3–6]. Glycine and carnitine conjugates, in particular, are major detoxifying products, and their administration is used as a means of treatment [12–14]. Therefore, analysis of isovalerylglycine and isovalerylcarnitine in isovaleric acidemia is

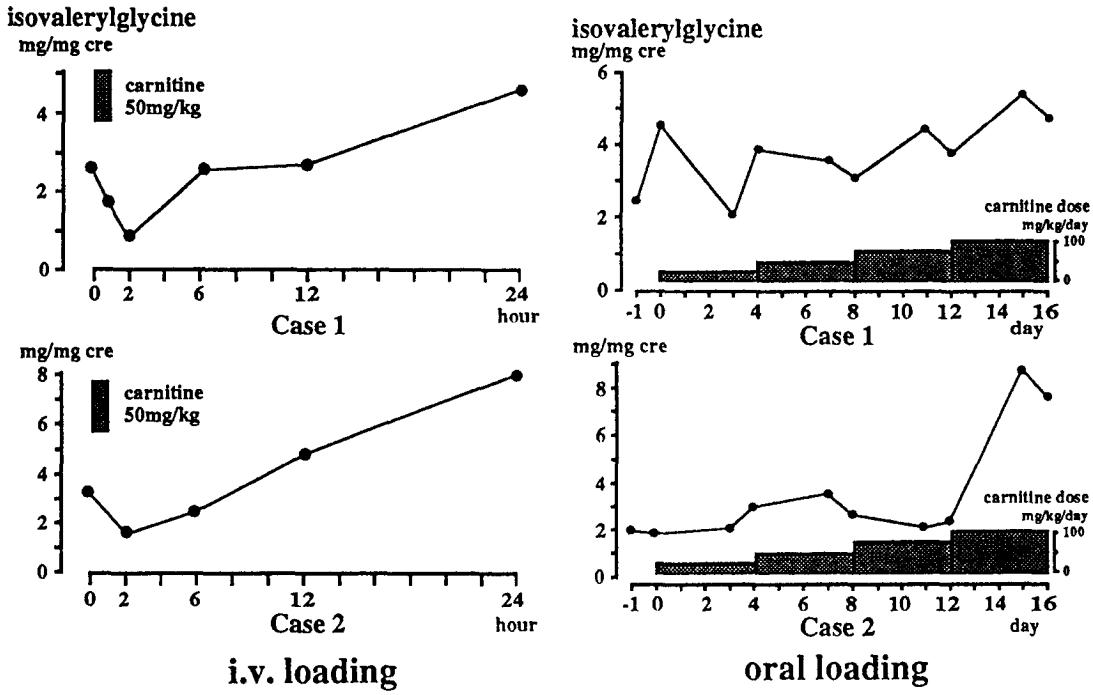

Fig. 4. Effect of intravenous and oral administration of carnitine on urinary isovalerylglycine excretion in two patients with isovaleric acidemia.

an important factor in the evaluation of their pathological state and therapeutic effect.

By connecting an ODS column to the LC–APCI-MS system, we were able to detect a large amount of isovalerylglycine in the urine from the two patients with isovaleric acidemia. Here, the samples were injected directly into the system without any pretreatment other than filtration. No isovalerylglucuronide excretion was detectable either with cyclic scan or SIM. This may be due to the low sensitivity of this system or the patients' good condition during the study [6].

L-Carnitine loading by intravenous injection caused an initial decrease of urinary isovalerylglycine excretion, and thereafter a gradual increase in the excretion level was observed similar to that of oral loading. This excretion level nearly doubled the preloading level in spite of a constant protein intake and glycine dosage. At the stage of decreased isovalerylglycine excretion at intravenous loading, we observed increased urinary excretion of isovalerylcarnitine in another experiment with the same samples [15]. Carnitine is known to provide free CoA and to modulate intramitochondrial acyl-CoA/CoA ratio imbalances [16,17] that occur in isovaleric acidemia. Therefore, supplementary carnitine is likely to supply free CoA by the formation of isovalerylcarnitine and promote the production of isovalerylglycine.

In addition to the organic acid analysis with GC–MS, the speed and ease of this LC–APCI-MS method makes it a useful tool for assessing the precise pathophysiologic condition, thereby enabling therapy tailored specifically to each patient's needs.

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