Note

Assay for Urinary Methylmalonic Acid by High-pressure Liquid Chromatography

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An assay for urinary methylmalonic acid by high-pressure liquid chromatography was devised. Methylmalonic acid could be assayed over a range of 5-80 μg by this method, which is one of the most convenient and useful assays for the acid in urine as an index of cobalamin deficiency.

Mammalian livers contain 5'-deoxyadenosylcobalamin-dependent methylmalonyl-CoA mutase (EC 5.4.99.2) catalyzing the isomerization of 1-methylmalonyl-CoA to succinyl-CoA during the degradation of various metabolites of the Krebs cycle. Cobalamin-(Cbl) deficiency and metabolic diseases result in methylmalonic aciduria. The elevated levels of urinary and/or serum methylmalonic acid (MMA) have been used as an index of Cbl deficiency.

MMA in urine and/or serum of Cbl-deficient patients and experimental animals has been assayed by gas chromatography mass spectrometry, which is sufficiently sensitive and specific for assay of MMA in screening tests for Cbl deficiency. However, it is difficult for most scientists working on the nutritional and biochemical studies of Cbl deficiency to obtain this expensive apparatus only to assay urinary or serum MMA.

In this paper, we devised an assay method for urinary MMA by high-pressure liquid chromatography (HPLC), which is easily accessible in most laboratories. We also discuss the availability of this method in assaying MMA in urine of normal and Cbl-deficient rats.

Ten male weanling Wistar rats (3-wk-old, 40 ± 5 g), born to 14-wk-old parents fed a Cbl-deprived diet for 8 wk, were used. Six-wk-old parent rats were obtained from Clea Inc. (Tokyo, Japan).

The Cbl-deprivation diet was prepared as described previously. The diet was identical to the Cbl-deprivation diet, except that 50 μg of cyanocobalamin per kg diet was included. The 3-wk-old weanling rats were housed in individual metabolic cages at 23°C in a room with a 12-h light:dark cycle. They were fed the control or Cbl-deprivation diet ad libitum and had free access to tap water for 11 wk. Body weight (105.26 ± 5.4 g) of the 14-wk-old Cbl-deficient rats were <35% of those of the control rats and had <96% lower hepatic Cbl content (41.30 ± 0.7 ng/g tissue), indicating that the rats fed the Cbl-deprivation diet in our experiments develop a severe Cbl deficiency.

Urines of rats were sampled for 24 h in individual metabolic cages. To a sample (2.0 ml), an equal amount of 7% (v/v) perchloric acid (Nacalai Tesque, Kyoto, Japan) was added, mixed vigorously, and centrifuged at 1000 x g for 10 min at 4°C. The supernatant was used a perchloric acid extract. In the case of samples containing a large amount of MMA, the extracts were diluted several times with 7% (v/v) perchloric acid and used. The perchloric acid extracts (3.0 ml) were put on a column (10 × 50 mm) of Dowex 50 × 4 (H+ form) (Muromachi Kagaku Kogyo, Ltd., Tokyo, Japan) and eluted with 7% (v/v) perchloric acid. The unabsorbed fraction (5.0 ml) was collected and analyzed by HPLC. More than 85.3 ± 6.5% MMA was recovered in the unabsorbed fraction when a known amount of authentic MMA was added to urine and extracted by this method.

The samples obtained were analyzed on an ion exchange HPLC column with a Jasco (Japan Spectroscopic Co., Ltd., Tokyo, Japan) HPLC apparatus (880-PU Pump, 870-UV Spectrophotometer and 807-IT Chromato-data Processor) and a column oven (CS-600, Chromato Science Co., Ltd., Tokyo, Japan). The sample (100 μl) was put on an ion exchange column (Shodex Ionpak C-811, 8 × 500 mm; Showa-denko Co., Ltd., Tokyo, Japan) equilibrated with 3 μm HClO4 at 60°C and isocratically eluted at the same temperature at flow rate of 1.0 ml/min. The eluate from the HPLC column was mixed with 15 mm Na2HPO4 solution containing 0.2 mm bromothymol blue (Nacalai Tesque) as a pH indicator at a flow rate of 1.5 ml/min by the use of a Jasco HPLC pump (880-PU) and a mixer (895-51). MMA was monitored by measurement of the absorbance of 445 nm. Retention time of authentic MMA was 13.0 min.

The peak area for each standard MMA was plotted with the amount of MMA on the x-axis and peak area on the y-axis, and then a best-fit straight line between the plotted points was drawn (Fig. 1).

When the extracts of urines of the 14-wk-old control rats with (Fig. 2A) or without (Fig. 2B) a known amount of authentic MMA, and that of the Cbl-deficient rats (Fig. 2C) were analyzed by the HPLC system, MMA was separated completely from other organic acids in each sample. The identical result was also obtained.

![Fig. 1. Effects of Concentration of Standard MMA on Peak Area of MMA.](image-url)

Detailed procedures of HPLC are described in the text.

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Fig. 2. Chromatograms of HPLC of Urinary Extract of Control and Cbl-Deficient Rats.

The extracts of urines of the 14-wk-old control rats with (A) or without (B) 10 µg of authentic MMA, and that of the Cbl-deficient rats (C) were analyzed by the HPLC system. Arrow represented peak of MMA with retention time of 13.0 min. Detailed procedures of the MMA extraction and HPLC analysis were described in the text.

in extracts of serum, liver, and kidney of the rats. These results indicate that MMA in biological samples can be measured with the plot over a range of 5–80 µg.

Urinary MMA levels of the 14-wk-old control and Cbl-deficient rats were 0.1 ± 0.0 and 15.6 ± 1.7 mg MMA/mg creatinine, respectively, by this HPLC system. Severe Cbl-deficient animals have been reported to excrete about 20 mg MMA/mg creatine,8) and 25–86 mg/d29) in urine and to show a hepatic accumulation of MMA (333 nmol/g wet weight),10) which is assayable by this method.

Normal level of urinary MMA in human has been reported as less than 5.0 µg MMA/mg creatinine and they are increased several times by Cbl deficiency.41) Although the elevated urinary MMA level (10–25 µg/mg creatinine) is significantly lower than that of the Cbl-deficient experimental animals, our HPLC method is sufficiently sensitive for assay of urinary MMA in screening test for Cbl deficiency in humans.

The results presented here indicate that the proposed method is very convenient and useful for assaying MMA in urine of normal and Cbl-deficient patients or animals.

References