学 位 論 文 の 要 旨

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学　位　論　文　名　　　Simplified Method for the Chemical Diagnosis of Organic Aciduria Using GC/MS

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論 文 内 容 の 要 旨

INTRODUCTION

Due to the expanded newborn screening for organic acidemias by electrospray ionization-tandem mass spectrometry (MS/MS), gas chromatograph/ mass spectrometry (GC/MS) has been becoming increasingly important for the chemical diagnosis on infants with positive screening results or high-risk children for organic acid disorders. However, the major disadvantage of the GC/MS method is that the sample preparation is extremely labor-intensive and time-consuming.

We developed a rapid and simple sample preparation method by improving the conventional solvent extraction and trimethylsilyl (TMS) derivatization procedures. The volume of extraction solvent and the number of extraction cycles were extensively reduced and the method which is called "flash-heater derivatization technique" was used. In the flash-heater derivatization method, derivatization reagent together with the extract of sample was injected into the GC/MS. We studied the validation and the clinical effectiveness of this method in chemical diagnosis of organic acidemia, comparing the conventional standard methods.

MATERIALS AND METHODS

1. Methods

Solvent extraction

An aliquot of urine equivalent to 0.02 mg of creatinine (volume of urine is generally between 0.01 - 0.20 ml) was diluted with distilled water to adjust the final volume to 0.20 ml in a 1.5ml-glass vial. To the diluted urine was then added 2 units of urease, and the mixture was incubated for 30 min at $37°C$. Four micro liters of the internal standard solution (0.5 mg/ml each of margaric acid (MGA) and tropic acid) was then added. The mixture was acidified with 35 µl of HCl (6N) and shaken for approximately 30 sec with a vortex mixer. The organic acids were

then extracted with 1.2 ml of ethyl acetate by mixing vigorously for approximately 30 sec with a vortex mixer. The mixture was centrifuged at 2,010 x g for 5 min and the organic layer was treated with anhydrous sodium sulfate (0.5g) prior to GC/MS analysis.

Flash-heater derivatization

The analysis was performed on a gas chromatograph coupled to a quadrupole mass spectrometer equipped with a split/ splitless injection port. The mass spectrometer was operated in selected ion monitoring mode.

The flash-heater derivatization in this method was carried out as follows. A 1 μl aliquot of the extract in ethyl acetate was sandwiched between two plugs of the BSTFA + 1%TMCS in a 10 μl injection syringe by drawing in sequence: 1 μl of BSTFA + 1%TMCS, 0.5 μl of air, 1 μl of the extract, 0.5 μl of air and 0.5 μl of BSTFA + 1%TMCS. The sample was then injected into the injection port heated at 280°C

2. Urine sample analysis

Analysis of urine samples spiked with organic acids

50 μg/ml each of 14 kinds of organic acids (certain representative compounds were chosen from commercially obtainable compounds), and the internal standard solution at 10 μg/ml were spiked to normal urine sample. The solution was analyzed by both the conventional method and the new method in order to validate the new method.

Determination of cut-off values

Urine samples were collected from forty normal control children, and analyzed by the new method to determine the cut-off values. TMS derivatives of methylmalonic acid, propionylglycine, glutaric acid, isovaleryglycine, 3-methylcrotonylglycine, 3-hydroxyglutaric acid, 2-hydroxyglutaric acid, methylcitric acid were quantified as the diagnostic markers for methylmalonic acidemia (MMA), propionic acidemia (PPA), isovaleric acidemia (IVA), glutaric aciduria type I (GAI) and multiple carboxylase deficiency (MCD), respectively. The quantitative results were expressed in the form of the peak area ratios of the target compounds to that of the internal standard (MGA), and the mean values and the standard deviations (SD) were calculated. The cut-off value was tentatively defined as the mean plus three SDs.

Chemical diagnosis by the new method

Urine samples were obtained from 20 patients with the following disorders (previously diagnosed with the conventional method and clinical symptoms): MMA, PPA, IVA, GAI and MCD. The derivatives of these diagnostic markers were quantified and compared with the cut-off values to identify the abnormally increased metabolites. Combination of the abnormal metabolites allowed the chemical diagnosis.

RESULTS AND DISCUSSION

In this study, we simplified the solvent extraction and the TMS derivatization of the conventional method. To simplify the solvent extraction, the volume of urine sample was reduced from 2 ml (the conventional method) to 0.2 ml (the new method), and the volume of extraction solvent (ethyl acetate) was proportionally reduced from 12 ml to 1.2 ml., respectively

The number of solvent extractions was reduced from two to one. To simplify the TMS derivatization, the flash-heater derivatization was applied.

Analysis of urine samples spiked with organic acids

The analysis by the new method of organic acids added to urine sample showed excellent repeatability (%RSD less than 10.8%) for the organic acids which produced only one derivative. 3-Hydroxyisovaleric acid (-monoTMS, -diTMS), 2-hydroxyisovaleric acid (-monoTMS, -diTMS) and phenyllactic acid (-monoTMS, -diTMS) produced two kinds of TMS derivatives, but the repeatabilities of the main TMS derivatives (3-hydroxyisovaleric acid-monoTMS, 2-hydroxyisovaleric acid-diTMS and phenyllactic acid-diTMS) showed less than 7.4%. These results indicate that the evaluated organic acids, including those which produced two kinds of TMS derivatives, were also extracted and derivatized quantitatively.

In the patients' urine analysis, the repeatabilities of the main TMS derivatives of the diagnostic markers were excellent (%RSD less than 12%), and were similar to the analysis of urine sample spiked with representative organic acids. From these results, it is concluded that the new method can be applied to the chemical diagnosis, possibly no less than the conventional method.

Determination of cut-off values

The cut-off values were determined by analysis of urine samples from 40 normal control children by this method. As described above, when multiple TMS derivatives were successfully formed, the main TMS derivatives could be chosen as a quantifying ion (Q-ion) of the TMS derivatives of the diagnostic markers: methylmalonic acid-diTMS for MMA, methylcitric acid-tetraTMS for PPA, isovalerylglycine-monoTMS for IVA, glutaric acid-diTMS and 3-hydroxyglutaric acid-triTMS for GAI, and 3-methylcrotonylglycine-monoTMS and methylcitric acid-tetraTMS for MCD.

Chemical diagnosis by the new method

The chemical diagnostic results of 20 patients with five different types of organic acidemias (MMA, PPA, IVA, GAI and MCD) were similar to those of the conventional method. Although the %RSDs of measured values in the new method are somewhat larger than those in the conventional method, it was suggested that the new method could be applied practically to the chemical diagnosis using GC/MS. Further studies may be required for other diseases, including mild-cases or therapy monitoring.

CONCLUSION

The new method (including simple solvent extraction and flash-heater derivatization technique) which we developed allowed simple, rapid and cost-efficient GC/MS organic acid analysis with a smaller volume of urine sample and reagents. It was successfully applied to 20 patients with the five different types of organic acidemias, and demonstrated that the utility was no less reliable than that of the conventional method. This method could be applied for the chemical diagnosis of organic acidemias in clinical laboratories.