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Quantification of urinary 5-aminolevulinic acid by gas chromatography–mass spectrometry

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Abstract

In this report, we describe a method for the specific quantification of urinary 5-aminolevulinic acid. It is based on gas chromatographic mass spectrometric measurement of the trimethylsilyl ester of the ethylacetoacetate pyrrole derivative of 5-aminolevulinic acid. Selected ion monitoring (SIM) was used for quantification using 3-hydroxymyristic acid as an internal standard. The detection limit of this method was 0.1 nmol/ml. This method was applied to the determination of urinary 5-aminolevulinic acid from a tyrosinemia type I patient and normal subjects, and 21.4 mmol/mol creatinine and 0.54 ± 0.49 mmol/mol creatinine ($n = 7$), respectively, were detected. Less than 0.2 ml urine was sufficient for the determination of 5-aminolevulinic acid in healthy subjects.

Keyword: 5-Aminolevulinic acid

1. Introduction

Fumarylacetoacetate hydrolase (FAH) deficiency (McKusick 276700, tyrosinemia type I) is a clinically severe inborn metabolic disease that results in liver and kidney damage [1,2]. Biochemically, FAH deficiency is characterized by accumulation of succinylacetone (SA), which play a major role in the pathogenesis of clinical symptoms [3]. The diagnosis of FAH deficiency depends on the detection of SA and other tyrosine metabolites (e.g. 4-hydroxyphenyllactate (4HPLA) and 4-hydroxyphenylpyruvate (4HPPA)) and by the measurement of FAH activity [2,3].

For many years, treatment of FAH deficiency was confined to dietary phenylalanine and tyrosine restriction [4], and the current treatment of this disease is liver transplantation [5]. Recently, a potent inhibitor of 4-hydroxyphenylpyruvate dioxygenase, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedion (NTBC), was reported [6]. This therapeutic agent was reported to provide significant improvement of symptoms in patients [7], reducing tyrosine degradation and production of SA. Therefore, it is very important to diagnose FAH deficiency as early as possible to initiate suitable treatment.

The detection of urinary SA has been shown to be the most important finding for the diagnosis of FAH deficiency. However, SA is an unstable compound and reacts non-enzymatically with various amino acids and proteins [8], and case reports exist of patients with undetectable urinary levels of SA [4,9].

5-Aminolevulunic acid (5ALA) is also excreted at high levels in patients with tyrosinemia type I [10]. This compound appears in urine because SA potently inhibits its metabolism by 5-aminolevulinate dehydratase [11]. Therefore, 5ALA is another important metabolite used for the diagnosis of FAH deficiency. The methods generally used for measuring 5ALA are based on the spectrophotometric assay described by Mauzerall and Granick [12], but this method is relatively non-specific. Sensitive gas–liquid chromatographic (GLC) methods have been reported by Gibbs et al. [13] and Gorchein [14], and these methods are based on the derivatization of 5ALA to pyrrole derivatives followed by detection with flame ionization or electron-capture. Other methods described by Okayama et al. [15] and Minder [16] were based on high-performance liquid chromatography (HPLC) with fluorescence detection. These GLC and HPLC methods, however, are not highly specific and require a very extensive pretreatment of samples, which impairs their

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routine use in laboratories. We therefore have modified Gibbs method and developed alternative rapid and reliable method for measuring 5ALA using gas chromatography mass spectrometry (GC/MS).

2. Experimental procedures

2.1. Chemicals

5-Aminolevulinic acid (5ALA), ethylaceoacetate, 3-hydroxymyristic acid (3HM) and trimethylchlorosilane (TMCS) were purchased from Tokyo-Kasei Kogyo (Tokyo, Japan). *N*,0-Bis(trimethylsilyl)-trifluroacetamide (BSTFA) was obtained from Wako Pure Chemicals (Osaka, Japan). Analytical-grade diethyl ether and methanol were also purchased from Wako Pure Chemicals (Osaka, Japan) and used for sample preparation.

2.2. Urine samples

Urine samples from patients were surplus to the requirements for essential clinical investigation. The urine from patient with tyrosinemia type I was obtained by absorption on filter paper and re-extraction with distilled water.

2.3. Sample preparation

The modified and simplified Gibbs method [13] was used for the derivatization of 5ALA. In a 10 ml glass tube with Teflon screw cap, 200 μl urine was added to 400 μl of ethylacetoacetae and methanol mixture (1:1). The mixture was incubated at 90 °C for 15 min in dry-aluminum block heater. After cooling to room temperature, 1 ml of 0.9% NaCl solution was added to the reaction mixture and was acidified with 0.1 M HCl to pH 4.0. 3-Hydroxymyristic acid (2 nmol) was added as an internal standard. The pyrrol derivative of 5ALA was extracted three times with 3 ml of diethyl ether. Samples were shaken for 10 min with mechanically, and centrifuged at 1500 × *g* for 5 min. The fraction of ether was combined and concentrated to minimum amount (about 0.2 ml) using a vortex concentrator under reduced pressure. The residue was transferred to a 3 ml glass vial and evaporated to near dryness under N_2 at 65 °C. Vials were capped with teflon/silicone disk in a closed screw cap and trimethylsilylated with 100 μl of BSTFA and 10% TMCS at 80 °C for 30 min.

Other urinary metabolites were extracted and analyzed as described in earlier reports [17,18].

2.4. Gas chromatography mass spectrometry

A Shimadzu QP-5000 GC/MS system (Kyoto, Japan) was used for the detection of 5ALA. A fused silica capillary column (J&W DB-5MS, 0.25 μm × 0.25 mm × 30 m, Agilent Technologies, USA) was used for the separation. The column temperature was hold at 220 °C for 2 min initially, and then programmed to 325 °C in increments of 17 °C/min. The selected ion monitoring (SIM) method was used for the detection and quantification of 5ALA. A fragment ion *m/z* 295 [M–$HCOOC_2H_5$] was used for 5ALA–pyrrol–2TMS and *m/z* 233 [$TMSOCHCH_2COOTMS$] for 3HM–2TMS (internal standard). A calibration curve was obtained from the ion peak area ratio of these ions. Other tyrosine metabolites were analyzed at GC/MS condition of former report [17].

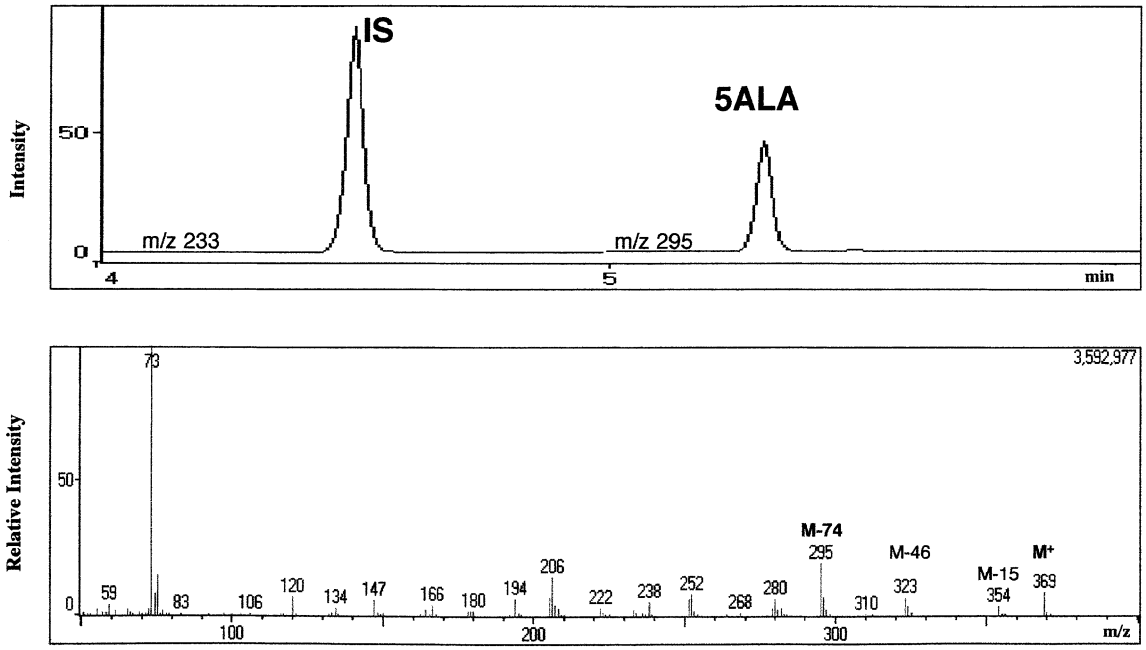

Fig. 1. GC/MS/SIM chromatogram and mass spectrum of 5-aminolevulinate–pyrrole–TMS derivative. IS: 3-hydroxymyristic acid, 5ALA: 5-aminolevulinic acid.

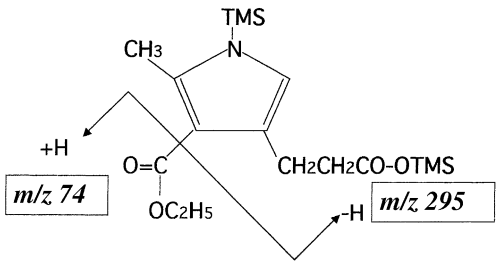

Fig. 2. Structure of 5-aminolevulinate–pyrrole–2TMS (MW: 369).

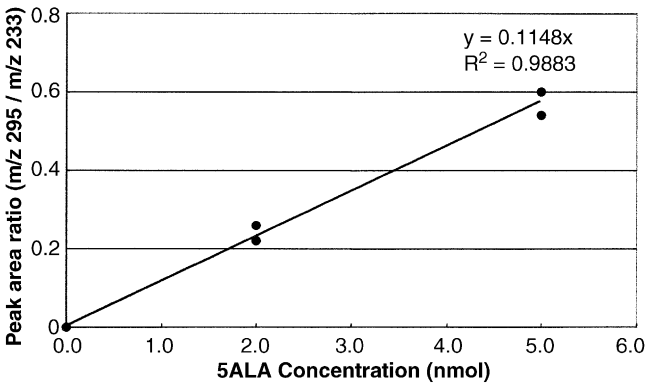

Fig. 3. Calibration curves for 5-aminolevulinic acid.

3. Result and discussion

Many reports on tyrosinemia type I reported that this type of hepatorenal tyrosinemia is one of the leading causes of childhood liver transplantation [1,2]. Detection of SA is the most important evidence for diagnosis of this disease. However, there are case reports of patients with undetectable levels of SA. Succinylacetone is a very reactive compound, and can form adducts with several amino acids and peptides, which may explain why it is undetectable in some patients. Therefore, it is said that urinary organic acid analysis should be repeated several times. High urinary excretion of 5ALA is one of other biochemical abnormalities in tyrosinemia type I. This is due to the fact that SA is a potent inhibitor of 5-aminolevulinate dehydrogenase [19]. Therefore, the detection of abnormally increased 5ALA in urine indicates the possibility of SA accumulation in the patient, and is a useful evidence for the diagnosis of tyrosinemia type I.

We have previously diagnosed tyrosinemia type I by GC/MS detection of urinary SA, 4HPLA, 4HPPA and *N*-acetyltyrosine [20]. In this report, we add to this repertoire of tests with a sensitive and reliable GC/MS assay for 5ALA. 5-Aminolevulinic acid was converted to its pyrrole derivatives by a condensation reaction with ethylacetoacetate (Knorr synthesis). The pyrrole derivative was then trimethylsilylated and analyzed by GC/MS. Our GC/MS method is very simple and much more sensitive compared with Gibbs GLC method [13]. Only 0.2 ml urine was used for the sample preparation. Fig. 1 shows the GC/MS/SIM chromatogram of an authentic sample of 5ALA and its mass spectrum. The 5ALA–pyrrole–2TMS derivative has *m*/*z* of 369 [M^+], 354 [M–15], 337[M–46] and 295 [M–74]. The

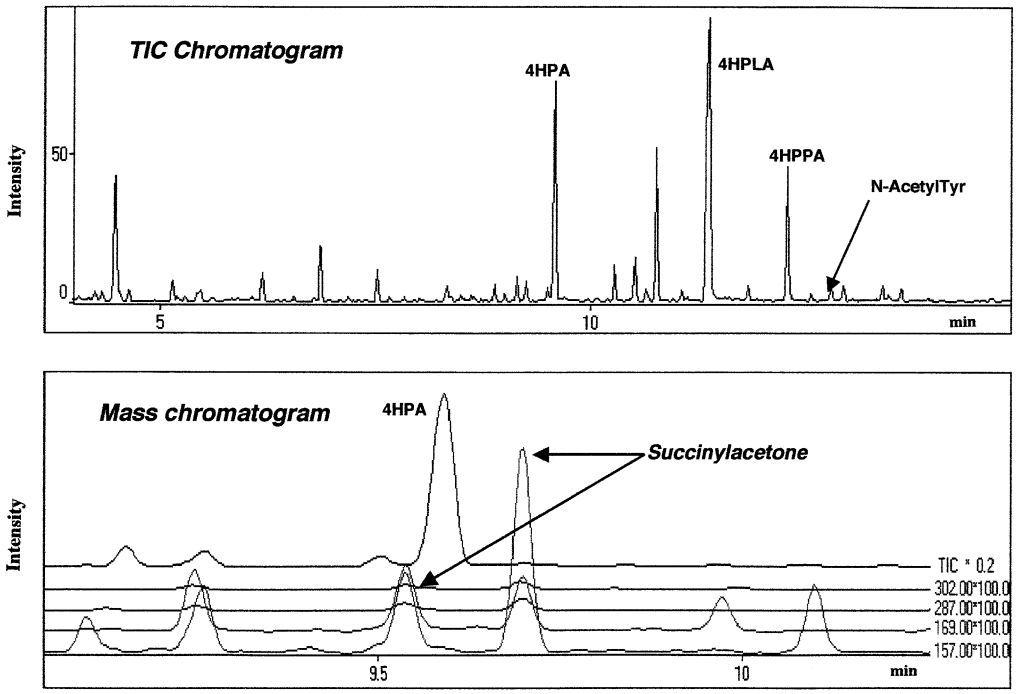

Fig. 4. Total ion chromatogram (TIC) and mass chromatogram of urinary metabolites in a patient with tyrosinemia type I. Diethyl ether extraction method was used for extraction of urinary metabolites. 4HPA: 4-hydroxyphenylacetate, 4HPLA: 4-hydroxyphenyllactate, 4HPPA: 4-hydroxyphenylpyruvate, *N*-acetylTyr: *N*-acetyltyrosine.

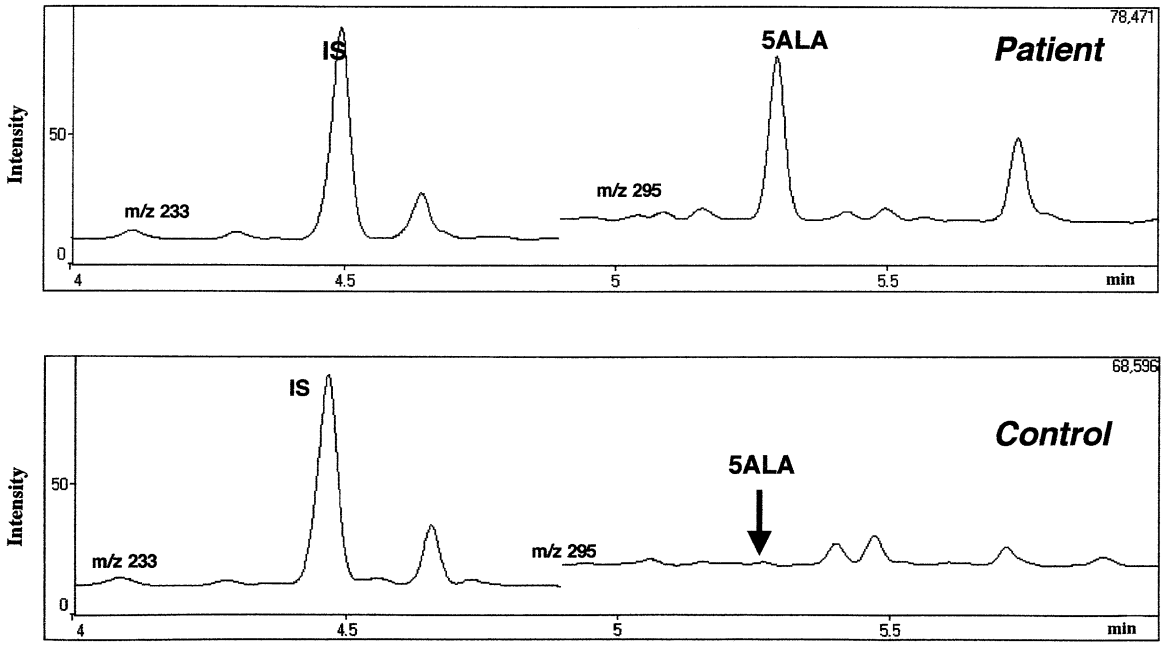

Fig. 5. SIM chromatograms of urinary 5-aminolevulinate in a patient with tyrosinemia type I and control IS: 3-hydroxymyristate, 5ALA: 5-aminolevulinate.

ion *m/z* 295 [M–$HCOOC_2H_5$] was used for the quantification of this pyrrole derivative, and *m/z* 369 $[M]^+$ was also used as reference ion (Fig. 2). Fig. 3 shows the calibration curves for 5ALA derivatized to ethylacetoacetate–pyrrole. With this method, we could detect as little as 0.1 nmol 5ALA/ml urine and CV% at 5 nmol was 11.9%.

Fig. 4 shows the total ion chromatogram (TIC) of urinary organic acids and the mass chromatogram of SA extracted with organic solvent extraction method from the urine of a patient diagnosed as having tyrosinemia type I. 4-Hydroxyphenyllactate, 4-hydroxyphenylpyruvate, and *N*-acetyltyrosine were detected in large amounts. Succinylacetone was also detected by mass chromatography but only in small amounts. Fig. 5 shows the SIM chromatogram for the detection of 5ALA with new quantitative method in this patient. In this assay, 5ALA could be determined quantitatively. The level of 5ALA in this tyrosinemia type I patient was calculated as 21.4 mmol/mol creatinine (control = 0.53 ± 0.49, range = 0.10–1.44 mmol/mol creatinine). Tyrosinemia type I was confirmed in this patient by the presence of increased urine SA and 5ALA. We also analyzed urinary 5ALA in three other patients who had liver transplantation and were doubted tyrosinemia type I. Although these patients have abnormalities in metabolites associated with tyrosine metabolism, SA was not detected in their urine. The levels of 5ALA before liver transplantation for these three patients were 1.4, 2.5, and 3.2 mmol/mol creatinine, respectively, and these values were within the normal range (mean + 5 S.D.). From these results, the possibility of tyrosinemia type I was discounted in these three patients.

Although 3-hydroxymyristic acid was used as an internal standard in this report, more sensitive analysis is expected for 5ALA quantification using its homologue or stable isotope labeled compound as an internal standard. This GC/MS technique is very sensitive and reliable in the quantification of 5ALA, and furthermore, is very convenient for the diagnosis of tyrosinemia type I.

4. Conclusions

We have developed a gas chromatography–mass spectrometric method to determine urinary 5-aminolevulinic acid after conversion to a pyrrole derivative followed trimethylsilylation. The sample preparation is simple and rapid, and this method is highly specific, sensitive, and applicable to routine laboratory use.

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