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Journal of Chromatography B, 823 (2005) 44–46
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Stability of 5-aminolevulinic acid on dried urine filter paper for a diagnostic marker of tyrosinemia type I

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Received 30 September 2004; accepted 10 February 2005
Available online 5 March 2005

Abstract

The chemical diagnosis of tyrosinemia type I generally involves the detection of succinylacetone (SA) in patient urine. However, 5-aminolevulinate (5ALA), which accumulates due to succinylacetone's inhibition of porphyrin synthesis, can also be used as diagnostic metabolites. Here we examined the stabilities of these markers on dried urine filter paper. After two weeks at room temperature, the succinylacetone was 10% of its original level, but over 80% of 5-aminolevulinate remained. Thus, although insufficient succinylacetone was recovered from dried urine filter paper to diagnose tyrosinemia type I, 5-aminolevulinate was readily detected, permitting the diagnosis.

Keywords: 5-Aminolevulinate; GC/MS analysis; Tyrosinemia type I; Dried urine filter paper

1. Introduction

Tyrosinemia type I (fumarylacetoacetate hydratase deficiency McKsic 276700) is a disease causing severe hepato-renal dysfunction that usually develops into carcinoma, and eventually requires liver transplantation [1,2]. Currently, this disease is successfully treated with 2-(2-nitro-4-trifluromethylbenzoyl)-1,3-cyclohexanedione (NTBC) [3]. This medicine inhibits the formation of succinylacetone (SA) and prevents liver damage [4]. Therefore, to initiate treatment before there is significant liver damage, it is very important to diagnose tyrosinemia type I as early as possible.

In tyrosinemia type I, succinylacetoacetate accumulates due to an enzyme defect, but this compound is very unstable and quickly decompose into SA. Therefore, tyrosinemia type I is mainly confirmed by detecting SA in patient urine [1,5]. However, SA is unstable during storage and freeze-thaw treatment, and as a result, it sometimes goes undetected in tyrosinemia type I patients [6]. This is a serious problem for diagnosis, and urine analysis often must be performed several times to detect the disease.

SA is a potent inhibitor of 5-aminolevulinate dehydratase. The accumulation of SA causes an increase in 5-aminolevulinate (5ALA) in the body; therefore, 5ALA is found in large amounts in the urine of patients with tyrosinemia type I [7–9] and is another important marker for the diagnosis of this disease. The level of 5ALA can be determined spectrophotometrically [10,11]. In addition, very recently, we reported the development of a sensitive and reliable procedure for quantifying 5ALA using gas chromatography-mass spectrometry (GC/MS) [12].

Neonatal screening of inborn errors of metabolism is performed using dried blood or urine filter paper, and a practical screening system for inherited metabolic diseases using dried urine filter paper combined with a urease digestion method has been reported [13]. However, tyrosinemia type I is difficult to diagnose using dried urine filter paper because the SA on filter paper may decompose during storage and transit. In this report, we examined the stability on filter paper of 5ALA, as another diagnostic marker of tyrosinemia type I.

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doi:10.1016/j.jchromb.2005.02.002

2. Experimental

2.1. Reagents

5ALA, 4HPLA, 2-oxoglutarate (2OG), 3-hydroxymyristate (3HM), ethylacetoacetate, and *O*-methylhydroxylamine hydrochloride were purchased from Tokyo-Kasei Kogyo (Tokyo, Japan). SA was obtained from Sigma (St. Louis, MO, USA). *N*,*O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) with 10% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). Analytical-grade diethylether, ethylacetate, and methanol were used for sample preparation.

2.2. Sample preparations

To examine the stabilities of metabolites, dried urine filter paper was prepared as follows. Control urine was spiked with a mixture of 5ALA, SA, and 4HPLA (approximately, 500 nmol/ml) and mixed well. One milliliter of this reconstructed urine was applied to filter paper (3 cm × 7 cm, Test Paper UA-5, ADVANTEC, Tokyo, Japan) and dried at room temperature. The filter papers were kept at room temperature or at −20 °C until analysis. The urine on the filter paper was re-extracted by the following method. The filter paper was wetted with 1 ml distilled water and allowed to stand for 30 min at room temperature. The urine was then collected by centrifugation for 10 min at 2000 × *g* and 4 °C. A 0.2-ml portion of the re-extracted urine was used for sample preparation.

Urine specimens from patients with tyrosinemia type I were surplus samples collected by other hospital (Mahidol University, Thailand) for a separate clinical investigation. Samples were applied to filter paper as soon as possible after they were collected from patients, and dried urine filter paper were obtained by airmail and re-extracting them with distilled water.

Urinary 5ALA was analyzed as previously described [12]. SA was converted to its methoxime-TMS derivative by a modification of the reported method [14]. In addition, 4HPLA and 2OG were extracted from the methoxime preparation, and their TMS or methoxime-TMS derivatives were concurrently analyzed by GC/MS.

2.3. GC/MS

A Shimadzu QP-5050A 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, J&W Scientific, Folsom, CA, USA) was used for the separation. The GC/MS conditions were the same as previously described [12,15]. The selected ion monitoring method was used for the detection and quantification of 5ALA. A fragment ion of *m*/*z* 295 [M–$HCOOC_2H_5$] was used for 5ALA-pyrrole-2TMS (MW = 369), and a fragment ion of m/z 233 [TMSO=$CHCH_2COOTMS$] was used for 3HM–2TMS (internal standard, MW = 388). A calibration curve was obtained from the ion peak area ratio of these ions. Fragment ions at *m*/*z* 181, 179, 198, and 229 were used for the quantification of SA-methoxime-TMS (MW = 288), 4HPLA-3TMS (MW = 398), 2OG-methoxime-2TMS (MW = 319), and 2-hydroxyundecanoate-2TMS (internal standard; MW = 346), respectively.

3. Results and discussion

Urine dried onto filter paper can be conveniently sent over distances and is useful for research or clinical tests, as long as the metabolic marker compounds are stable on the filter paper. We therefore examined the stability of 5ALA and SA on filter paper by GC/MS. 5ALA was analyzed by a newly developed analytical GC/MS method that involves derivatization to a pyrrole ester followed by trimethylsilation [12]. This method is very simple, reliable, reproducible, and sensitive. The equation of the linear calibration curve in the practical quantification range, 1.0–10.0 nmol/ml, was $y = 0.0988x - 0.0066$ ($R^2 = 0.9994$). The estimated detection limit was 0.10 nmol/ml urine. The intra-sample precision (CV%) determined at 1 nmol/ml ($n = 5$) and 10 nmol/ml ($n = 5$) was 2.3 and 3.2%, respectively. The inter-sample precision was 10.9% at 5 nmol/ml ($n = 5$). To investigate the stability of SA, its level was assessed by analyzing its methoxime-TMS derivative following derivatization with methylhydroxylamine and the trimethylsilation reagent [14]. Similarly, 4HPLA, a general diagnostic marker of tyrosinemia, was analyzed after conversion to its TMS derivative. 2OG was also examined as the reference keto-acid. All compounds were quantified by the selected ion monitoring method or mass chromatography.

Fig. 1 shows the changes of the target compound concentrations on dried urine filter paper. When the samples were stored at room temperature, SA decreased to 10% of its initial level within two weeks and to less than 5% after 1 month. This finding agrees with the results obtained by Fu et al. [16], who showed that SA is very unstable on filter paper. They concluded that urine absorbed on filter paper is not suitable for the diagnosis of tyrosinemia type I. In contrast, we found that 5ALA was relatively stable on the filter paper. The recovery of this compound after the storage of the filter papers for one week at room temperature was more than 90%, and after two weeks the recovery was 80%. 5ALA was even more stable on the filter paper at −20 °C (no decomposition was observed after 2 months of storage). In addition, 4HPLA was similarly stable on dried urine filter paper. We conclude that tyrosinemia type I can be diagnosed by detecting 5ALA and 4HPLA without sufficient succinylacetone in urine recovered from filter paper. Indeed, we found abnormal amounts of 5ALA and only a trace of SA in dried urine filter papers from tyrosinemia type I patients that were sent to us by airmail from a foreign country (patients KS, MA, and PN), and we were able to diagnose tyrosinemia type I correctly (Table 1).

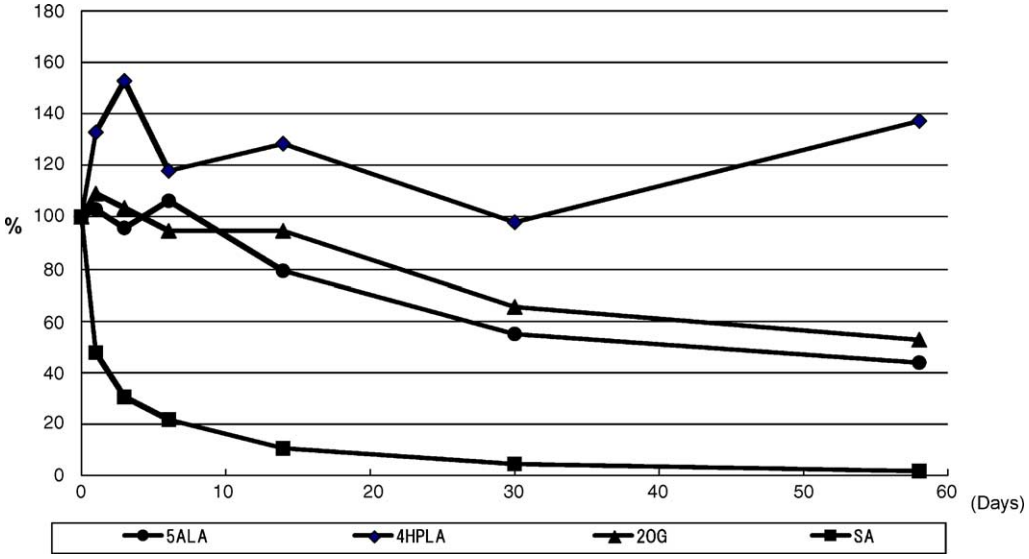

Fig. 1. Stability of compounds in filter paper specimens. The stability is represented as % change from the level on day 0. Abbreviations are the same as in the text.

Table 1
Concentration of 5-aminolevulinate in the urine of patients with tyrosinemia type I

Patient	5ALA concentration (mmol/mol creatinine)	Detection of succinylacetone	Urine origine
Tvrosinemia type I			
KS (3Y, male)	13.6	(++)	Filter paper urine
MA (3Y, female)	23.2	(+)	Filter paper urine
PN (2M, male)	76.1	Trace	Filter paper urine
Other tyrosinosis patient			
Patient 1 (11M, male)	1.4	(−)	Filter paper urine
Patient 2 (2M, male)	4.5	(−)	Frozen urine
Control (neonate, $n = 10$)	0.51 ± 0.46	(−)	Filter paper urine
Control (1M∼1.6Y, $n = 12$)	0.69 ± 0.55	(−)	Frozen urine

4. Conclusion

The measurement of 5ALA concomitant with 4HPLA from dried urine filter paper can be used to diagnose tyrosinemia type I. Tyrosinemia type I can be rapidly and reliably differentiated from hepatic diseases that exhibit tyrosinosis by the detection of urinary 5ALA using a newly developed method for measuring 5ALA.

Acknowledgements

The authors wish to thank Dr. P. Wasant and Dr. S. Liammongkolkul for the tyrosinemia type I urine samples.

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