Research Paper

Importance of blood succinylacetone measurement using liquid chromatography-tandem mass spectrometry in high-risk screening for hereditary tyrosinemia type I

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Abstract Newborn screening for hereditary tyrosinemia type I (HT1) is performed worldwide by measuring succinylacetone (SA) in dried blood spots using flow-injection tandem mass spectrometry with cut-off values for SA ranging from 1.29 to 10 μM. In Japan, HT1 is not a target disorder for newborn screening, and high-risk screening is performed only after disease onset. Blood SA concentrations in patients with HT1 who develop liver cirrhosis have been reported to be lower than those in the neonatal period, and there is an urgent need for a reliable diagnostic method. Therefore, in this study, we developed a highly sensitive method for SA analysis using liquid chromatography-mass spectrometry. This method allows quantification of SA concentrations at 0.005 μM and enables measurement of SA concentrations not only in patients with HT1 who have liver cirrhosis but also in asymptomatic patients or patients undergoing drug therapy.

Key words: succinylacetone, newborn screening, hypertyrosinemia type I, liquid chromatography-tandem mass spectrometry, diagnosis

1. Introduction

Hereditary tyrosinemia type I (HT1; McKusick 27670) is an autosomal recessive disorder caused by a deficiency in fumarylacetoacetate hydrolase in the tyrosine degradation pathway[1)]. This disease can cause severe liver disease and renal tubular dysfunction via the accumulation of fumarylacetoacetate (FAA) and maleylacetoacetate (MAA), which are readily converted to succinylacetone (SA). The diagnosis of HT1 relies on the detection of increased levels of SA in the blood and/or urine. Treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione has now become the standard treatment for the management of HT1 and provides significant improvement of symptoms in patients by reducing the levels of FAA, MAA, and SA in biological fluids.

Newborn screening for HT1 in Europe using SA as a screening marker was first reported in 2007[2)]. In the initial study, screening was performed using a non-kit method; SA in dried blood spots (DSB) was derivatized into the oxime form. Then, flow injection-tandem mass spectrometry (FI-MS/MS) analysis was performed with a cutoff value of 10 μM. A review paper published in 2017[3)] reported that the methods used by screening laboratories included both non-kit and kit methods and that the cutoff values of these methods were not consistent, ranging from 1.29 to 10 μM. These discrepancies raised concerns about the accuracy of screening.

In Japan, HT1 has not been a target disorder for newborn screening due to the low frequency of patients with the disease and difficulties with the derivatization method. Instead, urinary organic acid analysis was performed to detect increased SA excretion for infants with symptoms of liver failure together

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with high tyrosine levels. However, we encountered a patient with HT1 who had mildly elevated serum SA levels without pathological increases in urinary SA due to advanced liver cirrhosis[4]. In this case, the serum SA levels measured by liquid chromatography (LC)-MS/MS were lower than the cutoff values for newborn screening using FI-MS/MS in the review paper mentioned above. Notably, serum SA levels in patients with asymptomatic HT1 and treated patients with HT1 measured by gas chromatography (GC)-MS were much lower than those of untreated patients with HT1[5].

In the current study, we improved our LC-MS/MS method to measure serum SA levels in patients with HT1, as described above, and examined whether this approach could be useful in clinical applications.

2. Materials and Methods

2.1. Materials

2.1.1. Biological samples

DBS samples from two patients with HT1 prepared during the newborn period for mass screening using MS/MS were stored in a refrigerator in screening laboratories and then transported to our laboratory at the University of Fukui and measured using LC-MS/MS after obtaining permission from the parents of each patient. DBS and serum and urine samples from some patients with HT1 were collected at the time of disease onset. Three patients with HT1 had liver cirrhosis at diagnosis, and diagnoses of HT1 were confirmed by gene analysis. Informed consent was obtained from all subjects involved in the study.

2.1.2. Chemicals

A NeoSMAAT kit for MS/MS newborn screening, which contained labeled acyl-carnitines and amino acids, was purchased from Sekisui Medical Co. (Tokyo, Japan). Succinylacetone was purchased from Tokyo Chemical Industry (Tokyo, Japan), and succinylacetone-$^{13}C_5$ was purchased from the VU Medical Center Metabolic Laboratory (Amsterdam, The Netherlands).

2.2. Methods

For routine LC-MS/MS analysis of serum SA, 6 μL serum was placed in a small test tube with a punched piece of unused filter paper for newborn screening, and 0.22 mL of acetonitrile solution containing succinylacetone-$^{13}C_5$ (0.2 nmol/mL), 0.1% hydrazine H_2O, and 0.1% formic acid was added. The tube was shaken slowly for 45 min. The supernatant, which contained oxime derivatives of SA (SA-oxime) and SA-$^{13}C_5$, was obtained by centrifugation, dried, and then redissolved in 60 μL of 2% formic acid/methanol. For the analysis using 120 μL serum, the combined supernatant obtained from ten test tubes with 12 μL serum and 0.44 mL reaction mixture was dried and then redissolved in 2% formic acid/methanol.

To determine the linearity, we analyzed non-patient serum spiked with SA to a final concentration of 0.005−10 μM.

For the analysis of SA in DBS, a 3-mm-diameter punch of DBS was used instead of a serum sample.

An aliquot (10 μL) of the redissolved solution was introduced into the LC mobile phase flow (flow rate: 0.4 mL/min) using a 150 mm×3.0 mm Scherzo SM-C18 column (Imtakt, Portland, OR, USA). Gradient elution of the analytes was achieved using a program with mobile phase A as aqueous 0.5% formic acid and mobile phase B as 0.5 M ammonium formate and 0.5 M NH_4OH (9:1)/methanol (1:9).

A Triple Quad 4500 LC-MS/MS system (Sciex, Tokyo, Japan) was used for LC-MS/MS. The MS/MS analyses were performed in multiple reaction monitoring (MRM) mode using transitions at *m/z* 155/137 for SA-oxime and *m/z* 160/142 for $^{13}C_5$-SA-oxime. Suitable measurement conditions for the designated transitions for SA-oxime and $^{13}C_5$-SA-oxime were identified with the automatic tune function in Analyst software. For quantification, the recorded peak areas of the designated MRM ion set were used.

3. Results

No measurable SA peak was observed on the chromatogram of non-patient serum without spiked SA (n=15). In the analysis of non-patient DBS (n=6), the SA concentration ranged from less than 0.05 to 0.14 μM (Table 1). When non-patient serum containing spiked SA (SA concentration: 0.05−10.0 μM) was prepared and analyzed using our routine method, good linearity (R^2=0.9995) was obtained and intra- and interday assay coefficients of variation (CVs) for SA at a concentration of 0.05 μM were 3.5% and 5.1%, respectively.

In the analysis using 120 μL serum (SA concentration: 0.005−1.0 μM), good linearity (R^2=0.9995) was obtained, and intra- and interday assay CVs for SA at a concentration of 0.005 μM were 4.1% and 5.4%, respectively (Fig. 1). Several unknown peaks in MRM chromatograms for the transition at *m/z* 155/137 (representing SA-oxime) were observed, together with an apparent SA-oxime peak (Fig. 2).

In HT1 patient A, the SA concentrations in DBS were

Table 1. Succinylacetone levels in serum samples, DBSs, and urine samples from patients with HT1

HT1 patient	Age at diagnosis	Measurement at diagnosis			Newborn screening
		SA in urine (mmol/molCr)	SA in DBS (μM)	SA in serum (μM)	SA in stored DBS (μM)
A	5 months	103	2.6	—	28.7
B	2 years 6 months	<4	1.6	1.1	—
C	2 months	<4	—	0.78	5.5
Controls		<4	<0.05–0.14	<0.05	<0.05–0.14

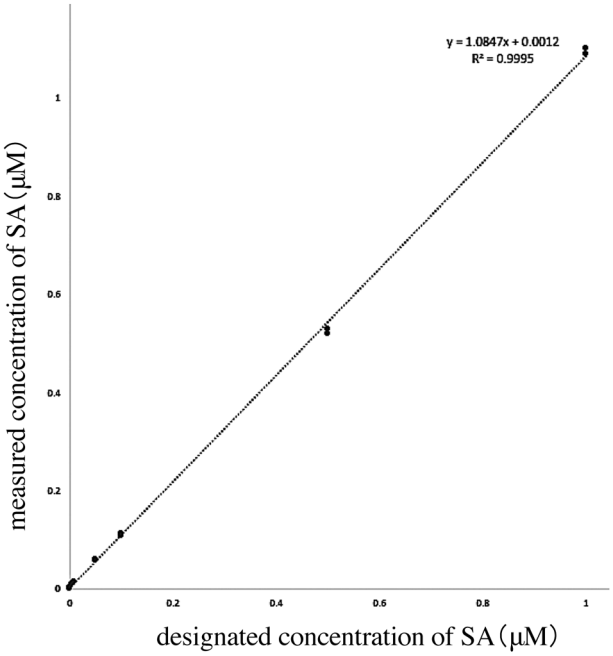

Fig. 1. Regression line of measured SA versus designated serum concentrations of SA in our analysis using 120 μL serum samples.

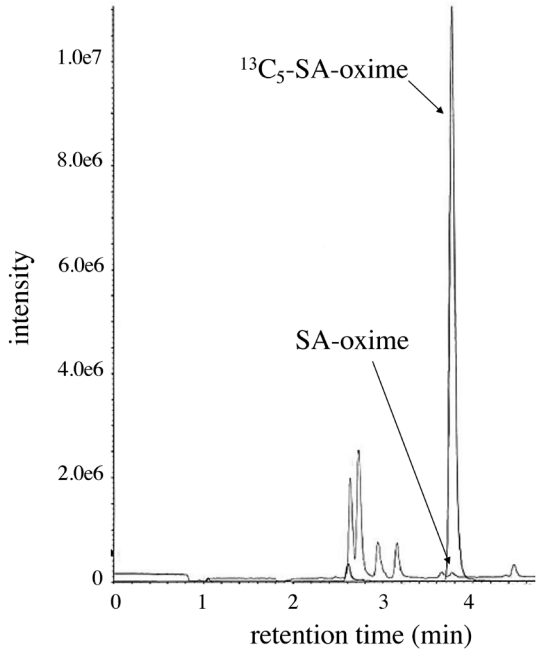

Fig. 2. MRM chromatograms for SA-oxime (*m/z* 155/137) and $^{13}C_5$-SA-oxime (*m/z* 160/142), showing an SA-oxime peak of 0.01 μM together with several unknown peaks.

28.7 μM in the neonatal period and 2.6 μM at the time of diagnosis (5 months of age). In patient B, the SA concentrations at 2.5 years of age were 1.6 μM in DSB, 1.1 μM in serum, and less than 4 mmol/mol Cr in urine. In patient C, the serum SA concentrations at 2 months of age were 0.78 μM and less than 4 mmol/mol Cr in urine, whereas the SA concentration in newborn DBS was 5.5 μM (Table 1).

4. Discussion

According to a review paper on HT1 screening in Europe and the United States[3], the cutoff value for SA in neonatal DBS by FI-MS/MS ranges from 1.29 to 10 μM. The uncertainty regarding test accuracy is assumed to be related to differences in sample preparation methods. Many of the laboratories surveyed in the review paper used non-kit methods, although SA was derivatized in different ways in both laboratories using kit and non-kit methods. A recent report from a screening facility using the Neobase2 kit[6] showed that the SA cutoff value was 1.5 μM and that the non-patient control value was 0.60±0.07 μM. However, according to a report from the 2020 CDC Newborn Screening Quality Assurance Program (NSQAP)[7], the cutoff value for the SA concentration in seven screening facilities using the Neobase2 kit ranged from 0.9 to 2.8 μM. They also reported large differences in SA concentrations determined using the FI-MS/MS method at various facilities. In addition, the detection limit for SA using FI-MS/MS may be affected by the presence of nonspecific compounds, which could appear as unknown peaks, as shown in our MRM chromatogram (*m/z* 155/137) for SA-oxime.

In an analysis of SA in blood samples using LC-MS/MS

and SA derivatives of butyl ester and oxime obtained when using dansylhydrazine[8], the SA concentrations in neonatal DBS from patients with HT1 were reported to range from 3.3 to 65 μM, and the lower limit of measurement was 0.2 μM. In present study, the lower limit of measurement was 0.05 μM, although our derivatization method using only hydrazine was simpler than that of the above report.

In an analysis of plasma SA concentrations using GC-MS (lower limit of measurement: 0.024 μM)[5], the plasma SA concentrations were reported to be 0.11−0.14 μM in asymptomatic HT1 cases, 0.021−0.056 μM in treated patients with HT1, and 0.003−0.021 μM in controls, although 0.5 mL of sample was required for measurement. In addition, complex derivatization using fluoride compounds was performed for GC-MS analysis, resulting in four isomer peaks for SA; this seems to be disadvantageous in terms of quantitation. In our LC-MS/MS method, the sample volume required to obtain the same sensitivity as in the above GC-MS was just 0.12 mL, and the simple derivatization procedure was advantageous in terms of accuracy.

Using LC-MS/MS, we found that blood SA concentrations in patients with HT1 with liver cirrhosis were markedly lower than those collected from patients during the neonatal period. Regarding the difference between serum and DBS in our SA analysis, SA concentrations in DBS were higher than that in the serum sample from patient B, as shown in Table 1. These findings suggest that SA may accumulate more markedly in blood cells than in the liquid component of biological fluids from patients with HT1.

It is difficult to obtain consistent quantitative values using the FI-MS/MS method owing to the ion suppression phenomenon[9]. In addition, as shown in Fig. 2, a few unknown peaks appeared in the MRM chromatogram close to the peak for SA-oxime. These peaks may significantly influence SA quantification using FI-MS/MS. Thus, measurement of blood SA concentrations by LC-MS/MS may be essential for the chemical diagnosis of patients with HT1 with a variety of conditions.

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Institutional Review Board Statement

This study was approved by the Institutional Ethics Committee at the University of Fukui (#20130055, #20180029).

Conflict of Interest

The authors declare no conflicts of interest.

References

1) St. Louis M, Tanguay RM: Mutations in the fumarylacetoacetate hydrolase gene causing hereditary tyrosinemia type I: Overview. *Hum Mutat* 9: 291−299, 1997.
2) Turgeon C, Magera MJ, Allard P, Tortorelli S, Gavrilov D, et al: Combined newborn screening for succinylacetone, amino acids, and acylcarnitines in dried blood spots. *Clin Chem* 54: 657−664, 2008.
3) Stinton C, Geppert J, Freeman K, Clarke A, Johnson S, et al: Newborn screening for Tyrosinemia type 1 using succinylacetone—A systematic review of test accuracy. *Orphanet J Rare Dis* 12: 48, 2017.
4) Mori J, Furukawa T, Kodo K, Nakajima H, Yuasa M, et al: A patient with urinary succinylacetone-negative hereditary tyrosinemia type 1. *Pediatr Int* 65(1): e15644, 2023.
5) Cyr D, Giguere R, Villain G, Lemieux B, Drouin R: A GC/MS validated method for the nanomolar range determination of succinylacetone in amniotic fluid and plasma: An analytical tool for tyrosinemia type I. *J Chromatogr B* 832: 24−29, 2006.
6) Lee B, Heo WY, Kim JA, Lee HS: Comprehensive evaluation of the NeoBase 2 non-derivatized MSMS assay and exploration of analytes with significantly different concentrations between term and preterm neonates. *Ann Lab Med* 43: 153−166, 2023.
7) Centers for Disease Control and Prevention (CDC). Newborn Screening Quality Assurance Program (NSQAP) 2020 Annual Summary Report. https://www.cdc.gov/labstandards/pdf/nsqap/NSQAP_Annual_Summary_2020-508.pdf (updated Aug 2020).
8) Al-Dirbashi OY, Rashed MS, Ten Brink HJ, Jacobs C, Filimban N, et al: Determination of succinylacetone in dried blood spots and liquid urine as a dansylhydrazone by liquid chromatography tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 831: 274−280, 2006.
9) Annesley T: Ion suppression in mass spectrometry. *Clin Chem* 49: 1041−1044, 2003.