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Modifications in electrospray tandem mass spectrometry for a neonatal-screening pilot study in Japan

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Abstract

In a neonatal-screening pilot study for inherited disorders in organic acid and amino acid metabolism, we analyzed butyrated acylcarnitines and amino acids in blood spots of more than 20 000 newborns by electrospray tandem mass spectrometry. In order to screen urea cycle disorders, we performed multiple scanning functions with additional stable isotope-labelled internal standards, since such reported functions as neutral loss of m/z 102 or 109 for butyrated amino acids were not sufficient. Arginine levels were measured with arginine-$^{13}C_6$. Hypocitrullinemia for the screening of some urea cycle disorders was detectable by measurement with synthesized citrulline-d_6, although we did not find any such disorders. In the acylcarnitine analysis, we found a patient with propionic acidemia, who has been treated effectively. The increasing false positive rate due to the use of pivalic acid-containing antibiotics in the diagnosis of isovaleric acidemia was a problem in Japan. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Acylcarnitine; Amino acids

1. Introduction

Newborn screening programs for inherited metabolic and endocrine disorders are well established among most developed countries, although target disorders for screening are limited. The limitations exists partly because the frequency of many inherited disorders is thought to be very low, and partly because the addition of traditional tests to diagnose the respective disorders may increase costs [1]. Recent advances in tandem mass spectrometry (MS–MS), however, have indicated the capability of this technique in diagnosing a large group of inherited metabolic disorders simultaneously, using blood spots on Guthrie cards [2–12]. Another powerful tool for newborn screening is a gas chromatography–mass spectrometric method using urease-treated urine [13].

The advantages of the MS–MS method over the gas chromatography–mass spectrometric method are the usage of Guthrie cards as well as the capability in large-scale sample analysis and in the diagnosis of hyperphenylalaninemia and fatty acid oxidation disorders. On the other hand, by measuring urinary orotic acid and uracil levels, the latter method can be used in diagnosing ornithine transcarbamylase (OTC) deficiency, the frequency of which may be

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higher than that of hyperphenylalaninemia or fatty acid oxidation disorders in Japan.

It has been reported, however, that hypocitrullinemia could be a marker for OTC deficiency, carbamyl phosphate synthetase I (CPS-I) deficiency [14], and some mitochondrial disorders [15], which suggests that hypocitrullinemia may be used in ESI–MS–MS screening for these disorders. In such disorders, plasma levels of glutamine, lysine, alanine, and glycine may be additional markers [16]. In MS–MS analysis, there are some difficulties in measuring these amino acids, since some have the same molecular weight or show low product-ion intensities through reported neutral loss of m/z 102 or 119. In addition, for accurate measurements of arginine and citrulline in MS–MS, we should use stable isotope-labelled internal standards, which are not commercially available.

Modifying the MS–MS methods, we have been conducting a neonatal-screening pilot study using electrospray (ESI) MS–MS in Japan for 1.7 years. In this paper we present the results of our attempts to improve ESI–MS–MS screening methods in Japan.

2. Experimental

2.1. Samples

The blood samples used in this pilot study were collected on the fifth or sixth day of life of the newborns, with the informed consent of their parents, using the same filter paper (Toyo Roshi, Tokyo, Japan) as that used in general neonatal screening in Japan.

2.2. Materials

Stable isotope-labelled acylcarnitines used in this study were synthesized in our laboratories [17]. Citrulline-3,3,4,4,5,5-d_6 was synthesized using ornithine-3,3,4,4,5,5-d_6, following the reported methods [18]. Ornithine-3,3,4,4,5,5-d_6 and methionine-d_4 were purchased from CDN isotopes (Miamisburg, OH, USA), and glycine-d_2, alanine-d_3, valine-d_8, leucine-d_{10}, glutamine-d_5, phenylalanine-d_5, tyrosine-d_2, and Arginine-$^{13}C_6$ from Cambridge Isotope Laboratories (Andover, MA, USA). Butanolic HCl (10%), HPLC-grade acetonitrile, methanol, and distilled water were purchased from Nacalai Tesque (Kyoto, Japan).

2.3. Sample preparation

We performed a microplate sample process. One 1/8-inch circle (0.31 cm) from each blood spot (3 μl) was punched out manually into the individual wells of a 96-well microplate. To the blood-spot punch in each well, we added a methanol solution (110 μl) containing known concentrations of stable isotope-labelled standards of the following compounds: 2 nmol of glycine, 1.5 nmol each of alanine, valine and leucine, 0.5 nmol each of methionine, phenylalanine, ornithine and arginine, 0.8 nmol of tyrosine, 0.2 nmol of citrulline, 3 nmol of glutamine, 100/3 pmol of acetylcarnitine, 50/3 pmol each of propionylcarnitine and glutarylcarnitine, 10 pmol of butyrylcarnitine, and 20/3 pmol of octanoylcarnitine. The microplate was covered and the samples were mixed using a model Micro Mixer MX-4 (Sanko Junyaku, Tokyo, Japan) for 20 min. The extracts were transferred to the designated wells in a U-bottomed microplate, and dried under a nitrogen stream. The residue in each well was derivatized with 30 μl of 10% butanolic HCl by heating at 65°C for 15 min. After removing the excess butanolic HCl in each well, the derivatized residue was dissolved in 80 μl of 50% acetonitrile solution, and the plate was sealed with a sheet of plastic wrap and placed in the autoinjector tray for ESI–MS–MS analysis.

2.4. Mass spectrometry

ESI–MS–MS analysis was performed using a model TSQ7000 triple stage mass spectrometer (ThermoQuest, Tokyo, Japan), equipped with a model LC10 HPLC system and a model SIL-10ADVP autoinjector (Shimadzu, Kyoto, Japan).

Using the autoinjector, we injected derivatized samples (10 μl), at interval of 2.5 min, into a flow (15 $\mu l\ min^{-1}$) of 50% acetonitrile, which was introduced into the ESI interface. Flow injection analysis was done. The flow-rate was increased to 500 $\mu l\ min^{-1}$ for 4 s at the end of each data acquisition period to wash the sample loop and ESI.

Table 1
Scan functions in ESI–MS–MS analysis of butyrated acylcarnitines and amino acids in blood spots of the newborns

Order	Scan function	Collision energy (eV)	Scan range (m/z)	Scan time (s)	Target analytes
1	precursor ions of m/z 85	−30	250–500	2.0	acylcarnitines
2	product ions of m/z 459	−30	140–174	0.5	argininosuccinic acid
3	neutral loss of m/z 161	−26	231	0.1	arginine
4	neutral loss of m/z 163	−26	237	0.1	arginine-$^{13}C_6$
5	neutral loss of m/z 102	−18	140–242	1.0	amino acids[a]
6	neutral loss of m/z 56	−12	128–136	0.3	glycine
7	neutral loss of m/z 119	−18	185–197	0.3	ornitine
8	neutral loss of m/z 162	−26	228–240	0.3	citrulline

[a] Alanine, valine, leucine, glutamine, phenylalanine, tyrosine.

Each MS–MS measurement of the samples was initiated by a signal from the autoinjector. The data were collected in the multiple-channel acquisition mode for 1.5 min after every sample injection. The temperature of the heated capillary was set to 200°C. The instrument was operated in positive ion mode. The pressure of argon as a collision gas was set to 2.1–2.3 mTorr, and the collision energy for the respective scanning function was tested to obtained the maximum intensity of the product ion monitored. Scanning functions for the analysis of acylcarnitines and amino acids are listed in Table 1. Raw spectrum data of each sample, which were recorded as a separate data file with each sample's name, were processed through a program to provide a list of spectral peaks displaying the masses and averaged intensities of 15 scans. The intensity data were transferred to Microsoft Excel spreadsheets, and the analyte concentrations were calculated.

3. Results

Mass chromatograms in MS–MS analysis of a blood sample using the multiple scanning functions and averaged spectra of the functions are shown in Fig. 1.

For the measurement of arginine, we used arginine-$^{13}C_6$ as an internal standard, and two adjacent scanning functions of neutral loss of m/z 161 and 163 were performed (Table 1). Product ion scan spectra of butyrated arginine and arginine-$^{13}C_6$ are shown in Fig. 2. The calibration curve was linear in the range of 5–500 nmol ml^{-1} of blood (r^2=0.993), and the intra-day and inter-day coefficient of variation (%; n=7) in the measurement of a control blood spot were 4.6 and 7.0, respectively. Arginine concentration in control newborns was 52±19 (mean±SD) nmol ml^{-1}.

For the measurement of citrulline, we synthesized citrulline-d_6 and performed MS–MS analysis of a neutral loss of m/z 162 with the collision energy of −26 eV. The intensities of m/z 232 in the measurement of butyrated citrulline-d_6 with the scan function mentioned above were less than 1.0% of those of m/z 238. The calibration curve was linear in the range of 2–200 nmol ml^{-1} of blood (r^2=0.991), and the intra-day and inter-day coefficients of variation (%; n=7) at the concentration of 5 nmol ml^{-1} were 8.6 and 10.1, respectively. Citrulline concentration in control newborns was 18.3±4.7 nmol ml^{-1}.

For the measurement of glutamine, we adopted a neutral loss of m/z 102 with a collision energy of −18 eV, since glutamine and lysine have the same molecular weight and similar fragmentation in product ion scan spectra, as shown in Fig. 3. In the measurements with scan functions other than a neutral loss of m/z 102, the contribution of lysine to the monitored intensities was much greater than that of glutamine. In the measurement with a neutral loss of m/z 102, the mean intensity of m/z 203 derived from lysine, which was corrected by that of m/z 208 of glutamine-d_5, was 43% of that derived from glutamine (Fig. 3C). The calibration curve was linear in the range of 50–2000 nmol ml^{-1} of glutamine (r^2=0.989), and the intra-day and inter-day coefficient of variation (%; n=7) in the measurement of a control blood spot was 18.9 and 25.4, respectively.

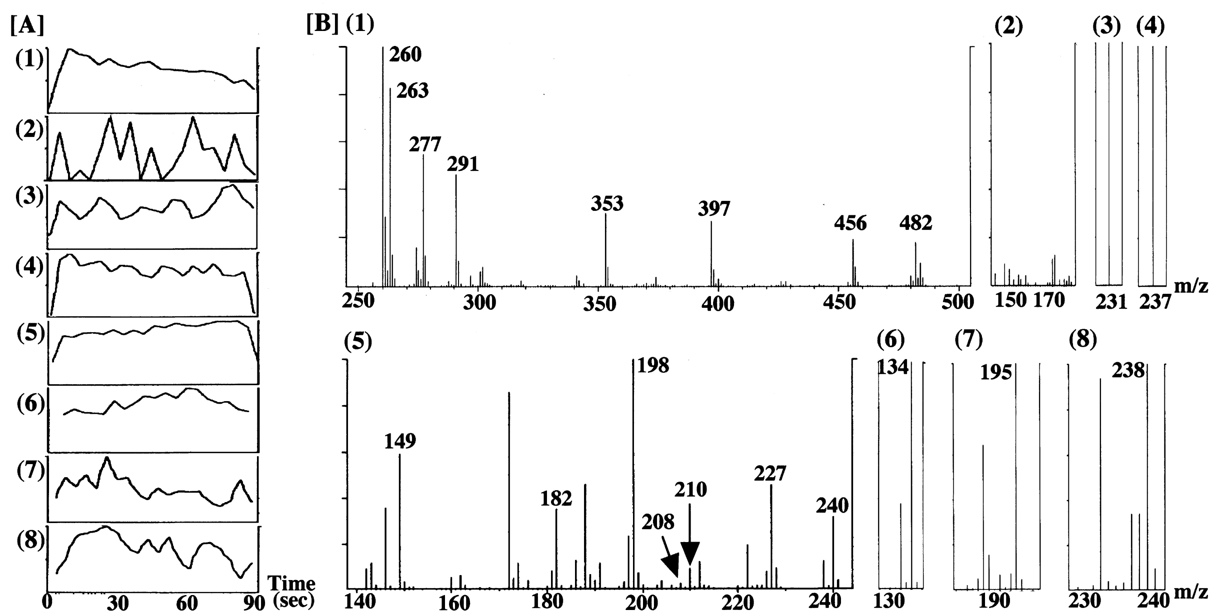

Fig. 1. Mass chromatograms in MS–MS analysis of a blood sample using the multiple scanning functions [A] and averaged mass spectra of each function [B]. Channel and spectrum numbers (1–6) in both figures correspond to the scan function numbers in Table 1. Channel number (7) is a combined display in scan function number 7 and 8. Mass spectra (7-1) and (7-2) in [B] correspond to scan function number 7 and 8, respectively. Intensity axis in mass spectrum was standardized using the highest ion intensity. The peaks in [B]-(1) are the molecular ions of the following acylcarnitine butyryl esters: 260; acetylcarnitine, 263; acetylcarnitine-d_3, 277; propionylcarnitine-d_3, 291; butyrylcarnitine-d_3, 353; octanoylcarnitine-d_9, 397; glutarylcarnitine-d_9, 456; palmitoylcarnitine, 482; oleylcarnitine. The peaks in [B]-(5) to (7-2) are the molecular ions of the following amino acid butyryl esters: 149; alanine-d_3, 182; valine-d_8, 198; leucine-d_{10}, 208; glutamine-d_5, 210; methionine-d_3, 227; phenylalanine-d_5, 240; tyrosine-d_2, 132; glycine, 134; glycine-d_2, 189; ornithine+asparagine, 195; ornithine-d_6, 232; citrulline, 238; citrulline-d_6.

For the measurement of argininosuccinic acid, we used a product ion scan of m/z 459 with a collision energy of −30 eV. We monitored the intensities of m/z 144 and 172, both of which were corrected with the intensity of m/z 227 of arginine-$^{13}C_6$. The detection limit in this measurement of blood spots was about 2 nmol ml^{-1}, and the argininosuccinic acid concentration in control newborns was less than 5 nmol ml^{-1}. The argininosuccinic acid level in a patient with argininosuccinic aciduria under treatment was 82 nmol ml^{-1}, as shown in Fig. 4.

After screening of 23 000 newborns, however, no patient with amino acid disorders was found. We found one patient with propionic acidemia, who has been treated with a protein-restricted diet and L-carnitine and has been growing uneventfully. A false positive rate in the diagnosis of isovaleric acidemia due to the use of pivalic acid-containing antibiotics was 0.37% in the last 10 000 measurement.

4. Discussion

The principal methodology of MS–MS for newborn screening was reported by Millington et al. [8], and then by Rashed et al. [5], although several differences in the methods, including the instrumentation and the target disorders, exist among laboratories in the different countries [2,3,9–12,19]. Most recently, Rashed et al. [2] reported a fully automated analytical system, called CAMPA, using a microplate sample process, which gave the laboratory unit a high-throughput capacity. We have developed a similar microplate sample process independently; such a process can reduce the tedious burden of laboratory personnel and dramatically increase the number of processed samples in our laboratories.

Nevertheless, the screening of urea cycle disorders has not been performed to the full extent, partly

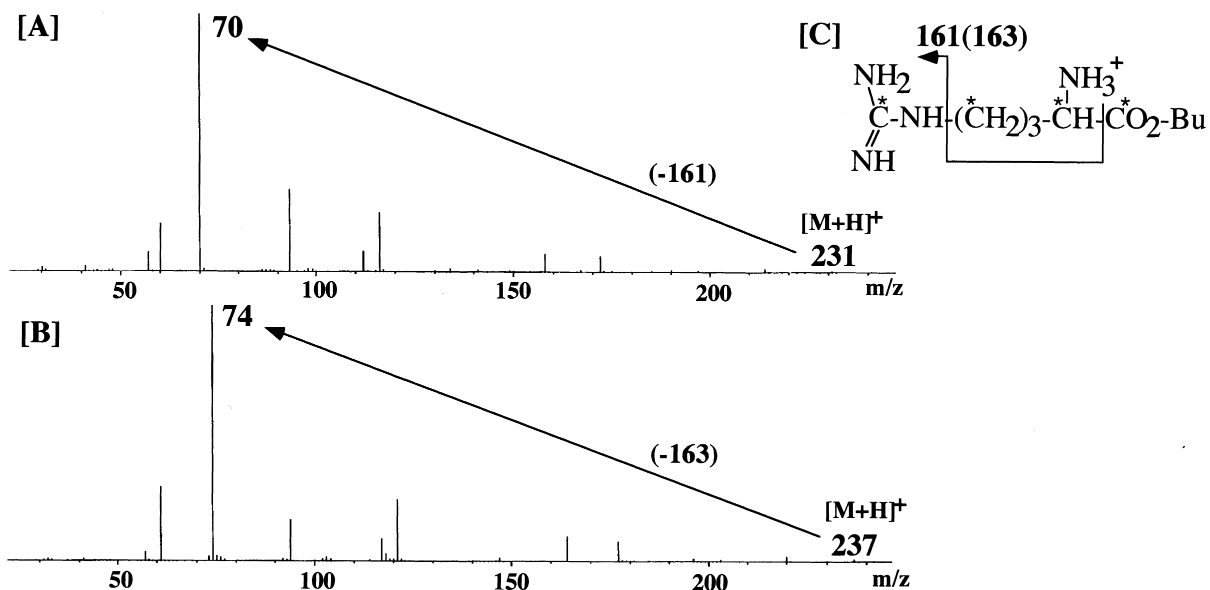

Fig. 2. Product ion scan mass spectra of precursor ion m/z 231 ($[M+H]^+$ of arginine butyryl ester) [A], and of m/z 237 ($[M+H]^+$ of arginine-$^{13}C_6$ butyryl ester) [B] with collision energy of −26 eV. Designated fragmentation is illustrated in the formula of ionized arginine butyryl ester (Bu) [C].

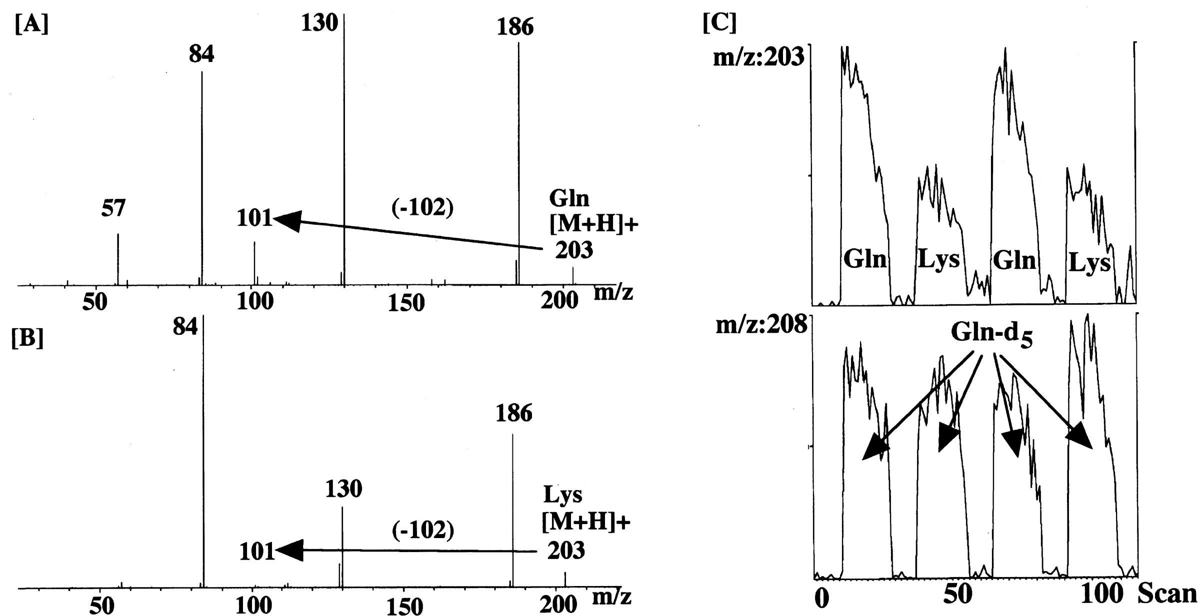

Fig. 3. Product ion scan mass spectra of precursor ion m/z 203 ($[M+H]^+$ of glutamine (Gln) butyryl ester) [A], and of m/z 203 ($[M+H]^+$ of lysine (Lys) butyryl ester) [B]: product ions formed through neutral loss of m/z 102 are indicated in each spectrum. In mass chromatograms in a neutral loss scan of m/z 102 [C], a mixture of glutamine and glutamine-d_5 and that of lysine and glutamine-d_5, the amounts of which were the same, were injected alternatively to determine the relative contribution of glutamine and lysine to the intensities of m/z 203.

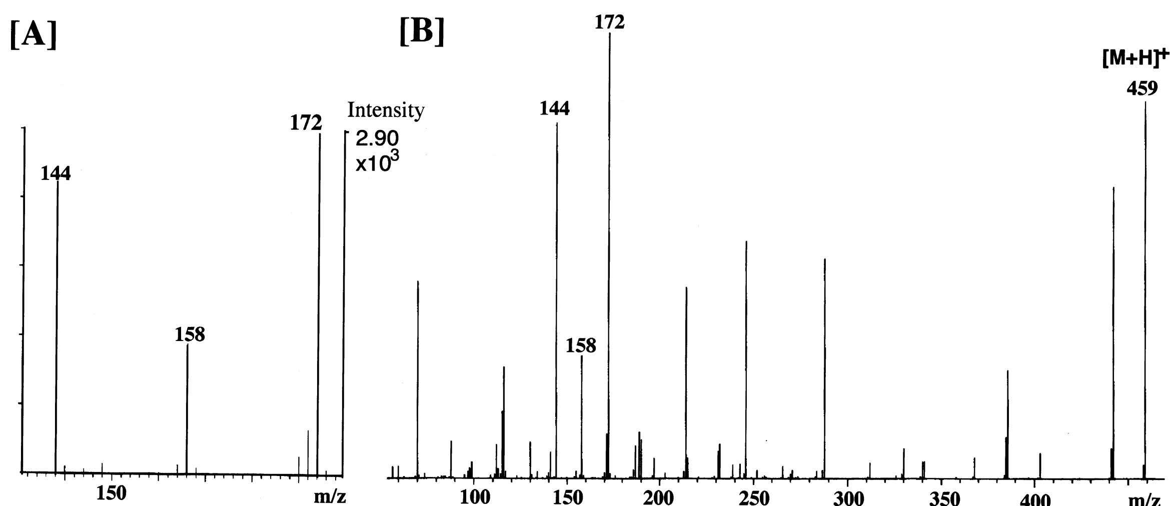

Fig. 4. Product ion scan mass spectra of precursor ion m/z 459 ($[M+H]^+$ of argininosuccinic acid butyryl ester) in the measurement of the blood spot of a patient with argininosuccinic aciduria [A], and of the standard [B].

because of methodological difficulties in MS–MS. Chace et al. [19] reported arginine measurement using a neutral loss of m/z 161 with arginine-d_7, which is not commercially available, as an internal standard, while Rashed et al. [2,5] did not mention arginine measurement. We have shown that arginine measurement with commercially available arginine-$^{13}C_6$ is practical and precise, and it may be an important tool for screening newborns with arginase deficiency in Japan.

The citrulline concentrations of newborn blood spot in this study were less than the values reported by Rabier et al. [15], who defined the citrulline concentration in hypocitrullinemia as below 10 nmol ml^{-1}. Such a discrepancy may be due to differences in the subjects' ages since the values of newborns that Pohlandt [20] reported were similar to our data. Thus, we used a lower cutoff value of citrulline of 7.0 nmol ml^{-1}. Our tentative lower cutoff value caused a 0.4% false positive rate in the last about 4000 measurements, although we did not find any patients with OTC deficiency or CPT-I deficiency.

There are many difficulties in the measurement of glutamine, including its deamination during storage and sample processing. The use of glutamine-d_5 as an internal standard caused the interference with the ion m/z 191 of leucine-d_3 by the deaminated product in neutral loss of m/z 102, and prompted us to use leucine-d_{10}, instead. In this study, we monitored the value of the mixture of glutamine and lysine, the major component of which is glutamine. It has been reported that the lysine levels were elevated, together with those of glutamine, in OTC deficiency or CPT-I deficiency [16], and the value of the mixture may be diagnostic.

In the measurement of acylcarnitines by ESI–MS–MS, few modifications seem to be needed. Although patients with medium-chain acyl-CoA deficiency have not been diagnosed in Japan, we recently found two cases with a late-onset type of very-long-chain acyl-CoA dehydrogenase deficiency. In this regard, a recent report [21] that retrospective analysis of the newborn blood spot of a patient with this disorder showed the typical acylcarnitine abnormalities is encouraging. As reported by Abdenur et al. [12], the increasing use of pivalic acid-containing antibiotics in neonatal medicine is a major problem for ESI–MS–MS screening in Japan.

Although our pilot study is still experimental, modifications shown in this study now enable us to screen more newborns than before, and we will test

our methods to increase the disorders that can be screened for and to further improve sensitivity and selectivity in ESI–MS–MS screening.

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