JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2006; **41**: 263–265
Published online 13 January 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.964

JMS

JMS Letters

Received 13 July 2005; Accepted 11 October 2005

Dear Sir,

Falsely elevated C4-carnitine as expression of glutamate formiminotransferase deficiency in tandem mass spectrometry newborn screening

After the first report by Millington *et al.*,[1] the development of electrospray tandem mass spectrometry (MS/MS) has enabled in recent years the introduction of expanded newborn screening programs in many countries.[2,3] This has led to an increase in the capacity to detect rare metabolic disorders during the neonatal period.

The increase of the only C4-acylcarnitine, as detected by tandem mass spectrometry in newborn screening, is reported to be related to short chain acyl-CoA dehydrogenase (SCAD) deficiency (butyrylcarnitine) or isobutyryl-CoA dehydrogenase (IBD) deficiency (isobutyrylcarnitine).[4]

Expanded Newborn Screening Pilot Program using MS/MS is being conducted in three provinces of Tuscany since January 2001, and, being officially mandated by a legislative action, screens all babies born in Tuscany since November 2004 (approximately 30 000/year) for selected acylcarnitines and amino acids. We report on a newborn in which the screening showed an isolated apparent elevation of C4-acylcarnitine usually associated with SCAD or IBD deficiency. A careful evaluation of the results and the subsequent biochemical investigations allowed us to demonstrate that this alteration was due to accumulation of formiminoglutamate (FIGLU).

Acylcarnitines and amino acids in all dried blood spots from newborn were analysed as butyl esters with the same methodology described by la Marca *et al*.[5]

An Applied Biosystems-Sciex (Toronto, Canada) API 4000 triple-quadrupole mass spectrometer equipped with a Turbo V-Spray source was employed.

MS and MS/MS spectra were collected in continuous flow mode by connecting the infusion pump directly to the Turbo V-Spray source. A standard solution of 1 ng/µl of each amino acid and acylcarnitine in water : acetonitrile (30 : 70) containing 0.05% formic acid was infused at 5 µl/min. Measurements on samples were performed by using a Series 1100 Agilent Technologies (Waldbronn, Germany) CapPump coupled to an Agilent Micro ALS autosampler, both fully controlled from the API 4000 data system. Experimental flow rate was 70 µl/min using water : acetonitrile (30 : 70) containing 0.05% formic acid. The eluent from the column was directed to the Turbo V-Spray probe. The acquired data were processed using the Analyst 1.4.1 proprietary software including the 'Explore' option (for spectral interpretation) and the ChemoView software (for quantitative information generation).

The child was born from consanguineous Pakistani parents (first cousins) after five uneventful pregnancy. The family history was unremarkable: two brothers and two sisters were normal. The delivery was made by caesarean section because of foetal distress at the 36th week of gestation. At birth, the APGAR score was 9^{I} and 10^{V}, weight 2700 g, length 49 cm, and head circumference 33 cm. Since the first days of life, the infant presented poor sucking and brought up milk. Expanded newborn screening for metabolic diseases by tandem mass spectrometry showed levels of C4-acylcarnitine mildly increased (using a cutoff value of the mean + 2SDs). The physical examination at 20 days of life showed normal cardiac, respiratory and abdominal findings and no dysmorphic features. Increased tendon reflex and mild axial hypotonia with hypertonia of legs were present. EEG showed diffuse paroxystic activity. Abdominal and cerebral ultrasound investigations as well as brain auditory–evoked potentials (BAEPs) and visual-evoked potentials (VEPs) were normal. Fundus oculi, ECG and cardiac ultrasound were normal. Laboratory investigations revealed normocytic anaemia (Hb 10,9, MCV 80,1 fl). Plasma and urine amino acid and urinary organic acid were normal. Folate plasma level was normal (13 ng/ml; n.v. 3–17). At the age of 5 months, the child showed a mild persistent axial hypotonia and a mild hypertonia of legs. EEG showed interhemispheric asymmetry and excess of slow waves during sleep. Development Quotient (DQ) score (Brunet-Lezine psychometric scale-early childhood) was 0.85. An informed consent for these studies was obtained from the parents.

Apparent C4-acylcarnitine in dried blood spot collected on the third day of life for newborn screening was 0.68 µM (normal 0.01–0.63). This abnormal value was confirmed twice on the same neonatal spot before the first recall. At the recall, apparent C4-acylcarnitine was 0.8 µM. Urinary organic acid analysis did not show ethylmalonic acid, methylsuccinic acid, butyrylglycine or isobutyrylglycine as expected metabolites of SCAD or IBD deficiency. These negative findings pushed us for a careful re-evaluation of results provided by the MS/MS analysis. In acylcarnitine profile, close to the increased signal of 288.2 m/z ion (C4-acylcarnitine), a very high signal of 287.2 m/z ion was present, which is usually absent in any other sample (Fig. 1). This ion has been reported by Pitt *et al*.[6] in a study of urinary metabolites of inborn errors of metabolism as the butyl-ester of FIGLU acid for which a transition in MRM 287.2 → 157 m/z is assigned. The product ion scan spectrum collected on a FIGLU chemical standard in our laboratory has confirmed that the 157.1 m/z ion was the most prominent, but a less intense fragment at 85.0 m/z was also present. Since the latter is a well-known signature fragment for acylcarnitines, it enabled us to conclude that in acylcarnitine profile the specific signal at 287.2 m/z should be assigned to FIGLU. Moreover, since the precursor ion scan of 85.0 m/z ion on the FIGLU chemical standard showed that the 287.2 m/z quasi-molecular ion was accompanied by the 288.2 m/z ion (16.8% of 287.2 ion, sensitivity-wise), the latter rationalised as the first ^{13}C isotopomer.

FIGLU levels subsequently measured with MRM scan function for the m/z 287.2 → 157.1 transition (specifically suggested for FIGLU quantitation) on patient's blood spot ranged from 5.6–7.1 µM (normal value 0.01–1.52 from 1000 healthy newborns). Plasma and urine FIGLU levels are reported in Table 1.

The high levels of FIGLU were also confirmed by the levels found in urine. High FIGLU concentration could be due to glutamate formiminotransferase deficiency, an autosomal recessive disorder of folate metabolism. The enzyme involved in this metabolic defect is a bifunctional protein that contains the activities of glutamate formiminotransferase and formimino-H_4folate cyclodeaminase. This enzyme permits the transfer of formimino group, coming from the catabolism of histidine, to tetrahydrofolate followed by the formation of 5,10-methenyl-H_4folate and with the release of ammonia group.[7] Patients affected by glutamate formiminotransferase deficiency show two distinct phenotypes, severe and mild. Clinical manifestations of the severe phenotype include mental retardation, failure to thrive, vomiting,[8] cortical atrophy, megaloblastic anaemia and hypersegmentation of neutrophils, while biochemical findings show elevated levels of FIGLU in the urine and in plasma especially after histidine loading, normal or high serum folate. Hyperhistidinemia can be also present. In the mild phenotype, mental retardation is not present, but sometimes mild developmental delay, no haematological abnormalities, massive excretion of FIGLU in the urine without histidine loading and normal serum folate are manifest.[8–11] Megaloblastic anaemia is not always a constant feature.[6] Some subjects are asymptomatic and it is not clear if the above reported symptoms are due to bias of ascertainment.[12]

In our patient, the main biochemical findings have been the high levels of FIGLU in blood or plasma and the massive excretion in urine without histidine loading. Serum folate and histidine levels were normal; megaloblastic anaemia was absent. The clinical findings at the age of 5 months showed only mild psychomotor development delay (DQ 0.85) and few EEG alterations. For the described patient, biochemical and clinical data allowed us to suspect the mild form of glutamate formiminotransferase deficiency.

The expanded newborn screening by tandem mass spectrometry in Tuscany has brought about the identification of a defect not included in our program of newborn screening. This defect could have poor clinical significance but can be an

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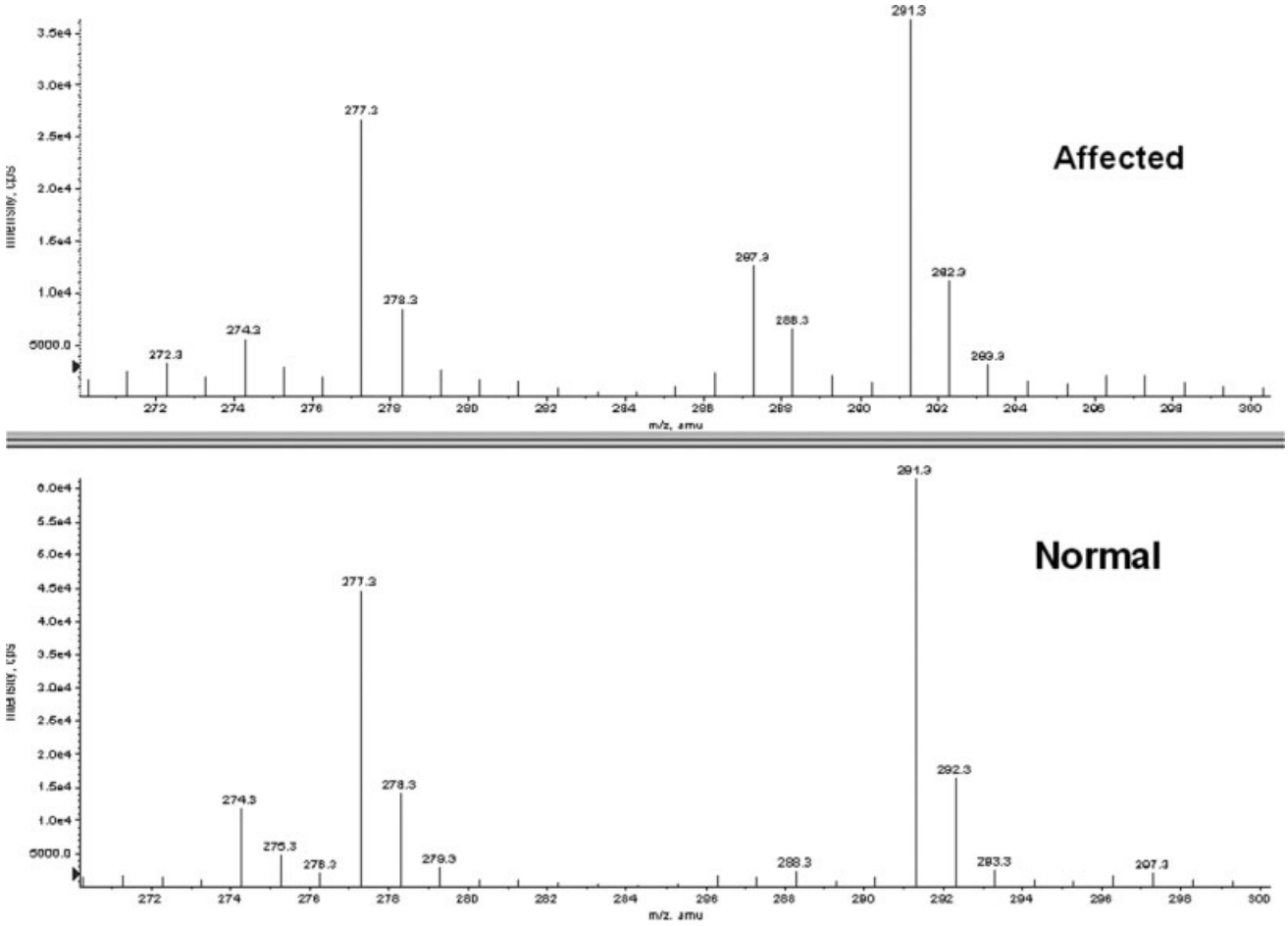

Figure 1. Acylcarnitine profile of affected newborn *vs* control. In the affected patient, besides the expected 288.2 *m/z* ion (assigned to the butylated C4-acylcarnitine), there was a very intense signal at mass 287.2 *m/z*, which is usually not expected and not related in any way to the above-mentioned C4-acylcarnitine butyl-ester.

Table 1. FIGLU and C4-acylcarnitine levels on patient's blood, plasma and urine

Age at sampling	C4-acylcarnitine in dried blood spot (μm/l)	C4-acylcarnitine in plasma (μm/l)	FIGLU in dried blood spot (μm/l)	FIGLU in plasma (μm/l)	FIGLU in urine (μm/mol creat)
3 days	0.68	–	3.66	–	–
11 days	0.8	–	5.66	–	–
17 days	0.97	–	7.1	–	–
19 days	–	2.00	–	14.75	399
26 days	–	1.78	–	6.95	–
30 days	–	1.4	–	9.01	301
2 months	–	1.1	–	13.5	–
3 months	–	0.88	–	3.73	–
5 months	–	1.23	–	3.4	193.5
Normal values	0.01–0.63	0.12–0.44	0.01–1.52	0.21–1.57	0.9–9.3

example of misinterpretation in newborn screening by tandem mass spectrometry. A false interpretation of metabolic alterations with unknown or poor clinical significance could create stress in parents and an increase of professional work in care.[13]

In conclusion, before investigating for SCAD or IBD deficiency, it is important to exclude from the acylcarnitine profile the presence of signal of 287.2 m/z ion near the 288.2 m/z ion (C4-acylcarnitine), whenever the latter shows an abnormal intensity.

Yours,

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