Introduction of a Simple Second Tier Screening Test for C5 Isobars in Dried Blood Spots: Reducing the False Positive Rate for Isovaleric Acidaemia in Expanded Newborn Screening

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Abstract In 2015 the English Newborn Screening programme expanded to include Isovaleric Acidaemia (IVA). Screening is performed by flow injection analysis tandem mass spectrometry of isovalerylcarnitine. Isovalerylcarnitine is isobaric with pivaloylcarnitine which can be present in blood due to the use of pivalic ester pro-drugs or pivalic acid derivatives used as emollients in some nipple creams; the potential for false positives (FP) is well documented. A pilot study in England screened 438,164 babies, 18 had presumptive positive results but only 4 were confirmed as true positives (TP). We developed a simple test to separate the isobaric compounds and investigate these samples further.

We studied newborn screening blood spots from 122 randomised controls and 34 infants with an initial raised C5 result. Dried blood spots were eluted with 30% acetonitrile (150 µL) and injected into a Waters Acquity UPLC coupled to a Waters Premier XE tandem mass spectrometer operating in positive ion mode. Isocratic separation of isovalerylcarnitine, pivaloylcarnitine, valerylcarnitine and 2-methylbutyrylcarnitine was achieved within 8 min. Assay performance characteristics were acceptable and non-parametric reference ranges ($n = 122$) were determined for each analyte.

If this method had been used as a second tier test for the 34 presumptive positive samples, the number of FP's would have reduced from 24 to 8 and the positive predictive value of the screening test would have increased from 29 to 56%. Introduction of this test into the screening protocol has the potential to significantly reduce FP results for IVA and prevent unnecessary anxiety.

Introduction

Isovaleric acidaemia (IVA) is an autosomal recessive disorder of leucine metabolism, with an estimated prevalence of 1 in 123,457 in western populations (Moorthie et al. 2013). The phenotypic abnormalities seen in IVA are due to the accumulation of isovaleric acid, which is toxic to the central nervous system and it is the excess 3-hydroxyisovaleric acid which is responsible for the unusual “sweaty foot” odour associated with these patients during an acute episode.

IVA can present as one of three forms, acute neonatal, chronic intermittent (Tanaka 1990; Vockley et al. 1992) or the more recently recognised milder phenotype (Ensenauer et al. 2004). The biochemical diagnosis of IVA is based upon an abnormal acylcarnitine profile, with a prominent increase in isovalerylcarnitine, and a urine organic acid profile with increased excretion of 3-hydroxyisovaleric acid and isovalerylglycine. IsovalerylCoA dehydrogenase (IVD) activity can also be measured in fibroblasts and lymphocytes if required. More than 25 mutations in the IVD gene have been described, some of which result in complete absence of the enzyme. Recent evidence indicates that the 932C > T (A282V) IVD mutation is associated with a

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milder phenotype whereas heterogeneous IVD gene mutations tend to correlate with more severe disease (Ensenauer et al. 2004).

In recent years, IVA has been added to newborn screening programs in many centres across Europe, America and Australasia. IVA is a good candidate for newborn screening; there is a simple and acceptable screening test available and ready access to confirmatory testing. The characteristic presentation of acute IVA is in the first 2 weeks of life and if left untreated, IVA is associated with approximately 50% mortality rate and there is a likelihood of severe neurological symptoms and learning disability.

Newborn screening for IVA is based on flow injection analysis tandem mass spectrometry of isovalerylcarnitine. Isovalerylcarnitine is isobaric with pivaloylcarnitine which can be present in blood due to maternal use of pivalic ester pro-drugs or pivalic acid derivatives used as emollients in some nipple creams; the potential for FP results is well documented. Abdenur et al. (1998) first reported a FP newborn screening result on a baby whose mother was taking pivaloyl containing antibiotics. Further investigation of a urine sample by Gas Chromatography Mass Spectrometry revealed the presence of methylpivaloate and the pivaloylcarnitine was shown to decrease once the antibiotics were withdrawn. Common examples of such drugs include Pivmecillinam, the pivaloyloxymethyl ester of Mecillinam and Pivampicillin, the pivaloylmethyl ester of Ampicillin. Boemer et al. (2014) reported 50 FP IVA results detected in Belgium during an 18-month period which were subsequently investigated and found to be due to the use of a nipple fissure ungent containing pivallic acid derivatives. This particular nipple cream was routinely supplied to breastfeeding mothers in a specific hospital and correlated with an increase in the number of positive results from 0.2 to 1.4% over an 18-month period. The pivalate derivative acts as an emollient and is absorbed from the nipple cream by the baby during breastfeeding. In the UK, many nipple fissure ungents are based on lanolin and do not appear to contain pivalic acid derivatives, however, at least one product has been identified as containing pivaloyl derivatives (described as “neopentanoate” or “isodecylneopentanoate”). This was “Mustela Nursing Comfort Balm” and was being used by at least one of the mothers of the FP IVA babies described here. Boemer et al. (2014) successfully developed an LCMSMS method to separate the isovalerylcarnitine from its isobars. However, this method was based on derivatisation of the blood spot extract prior to analysis making it less suitable to rapid turnaround. The method described here is simple and does not require derivatisation of the blood spot prior to analysis making it suitable for inclusion as a second tier screening test. In the event of a screen positive sample being detected, isobar

analysis could be rapidly performed ensuring that there was minimal delay in clinical referral of a TP sample.

Materials and Methods

Isovaleryl-L-carnitine and Tridecafluoroheptanoic acid were purchased from Sigma Aldrich. Pivaloyl-L-carnitine hydrochloride, valeryl-L-carnitine hydrochloride and 2-methylbutyryl-L-carnitine hydrochloride were purchased from VU University Medical centre, Amsterdam. [$^2\text{H}_9$]-isovalerylcarnitine was purchased from CDN Isotopes. A stock standard containing 10 mM isovalerylcarnitine, pivaloylcarnitine, valerylcarnitine and 2-methylbutyrylcarnitine in 100% methanol was prepared. This was used to spike whole blood to a concentration of 2.0 $\mu\text{mol/L}$. 40 μL of blood was spotted out on to filter paper (903, Whatman) and allowed to dry at ambient temperature. Stock internal standard solution containing 20 $\mu\text{mol/L}$ of [$^2\text{H}_9$]-isovalerylcarnitine was prepared in 30% acetonitrile and diluted 1 in 10 to prepare a working internal standard solution.

We tested 122 newborn screening spots from healthy controls and 33 newborn screening samples with C5 results above the analytical cut-off value. During the pilot study the cut-off value was 1.0 $\mu\text{mol/L}$ and this was increased to 2.0 $\mu\text{mol/L}$ following review of the evidence. Confirmatory investigations were performed on all presumptive positive samples and included urinary organic acid analysis, derivatised blood spot acylcarnitines and DNA analysis for the benign mutation. All blood spot samples were collected between days 5 and 8 of age. Blood taken from a heel prick is spotted on to filter paper and transported to the screening laboratory via courier or first class post. Blood spot samples were stored at room temperature until analysis.

A 3.2 mm filter paper disc containing approximately 3.1 μL of whole blood was punched from each dried blood spot into a 96-well polypropylene plate. Samples were extracted for 20 min with 150 μL of 30% acetonitrile containing 2.0 $\mu\text{mol/L}$ [$^2\text{H}_9$]-isovalerylcarnitine internal standard.

The hardware configuration includes a Waters Premier XE triple quadrupole mass spectrometer equipped with ESI source operating in positive ion mode at a capillary voltage of 3,500. The source temperature was 120 $^{\circ}\text{C}$, desolvation gas temperature 350 $^{\circ}\text{C}$ and gas flow 800 L/h. Optimised cone voltage was 35 and collision energy 21. All data was acquired in multiple reaction monitoring mode (MRM) with a dwell time of 20 ms per channel. The transitions monitored were 246 > 85 and 255 > 85.

Chromatography was performed on a Waters Acquity UPLC. Isovalerylcarnitine, pivaloylcarnitine, valerylcarnitine and 2-methylbutyrylcarnitine were separated isocrati-

Fig. 1 Isobaric species as a percentage of total C5 in 33 presumptive positive blood spot samples. Samples 1–10 are TP's, samples 11–17 are FP's and samples 18–33 are FP due to pivaloylcarnitine (one FP sample was not available for analysis)

cally by injecting sample extract (10 μ L) onto an Acquity BEC C18 column (1.7 μ m, 2.1 $\times$ 100 mm) fitted with a Vanguard BEH C18 pre-column (1.8 μ m, 2.1 $\times$ 5.0 mm). The column assembly was maintained at 40 $^{\circ}$ C. Mobile phase was composed of 0.1% tridecafluoroheptanoic acid (74%) and 0.1% tridecafluoroheptanoic acid in acetontirile (26%) and the flow rate was 450 μ L/min. The total run time was 8.0 min and inject-to-inject time was 9.0 min. Isovalerylcarnitine, pivaloylcarnitine, valerylcarnitine and 2-methylbutyrylcarnitine eluted with retention times of 4.0, 4.2, 4.6 and 5.0 min, respectively.

Figure 1 shows the 2 μ mol/L blood spot standard and two patients with presumptive positive results for IVA, a FP due to the presence of pivaloylcarnitine and a TP with increased isovalerylcarnitine.

Results

Ion suppression experiments demonstrated that in blood spots the main region of ion suppression occurred between 6.0 and 6.2 min, with no significant ion suppression occurring near the compounds of interest. The assay was linear to at least 10 μ mol/L isovalerylcarnitine, intra- and interassay precision were <6% for all four analytes and carry-over was <0.1%. The limit of detection (mean plus 3SD of blank) and the limit of quantification (mean plus 10SD of blank) were 0.06 and 0.2 μ mol/L, respectively. Reference ranges (non-parametric, $n = 122$) were determined for each analyte and as % of total C5 they were 44–80%, 19–54%, <3% and <2% for isovalerylcarnitine, 2-methylbutyrylcarnitine, valerylcarnitine and pivaloylcarnitine, respectively.

To assess whether second tier testing would be effective, we analysed the C5 isobars in 33 of the 34 presumptive positive samples identified by the English Newborn Blood Spot Screening (NBSS) program between 2012 and 2016 (one FP sample was not available for analysis). Of these samples, 18 were identified during the pilot study when the cut-off value was 1.0 μ mol/L and 16 were identified post pilot when the cut-off value was 2.0 μ mol/L. Confirmatory investigations were performed on all samples and included urinary organic acid analysis, derivatised blood spot acylcarnitines and DNA analysis for the 932C > T (A282V) mutation. Table 1 summarises the outcome data for the 33 presumptive positive samples identified by the English NBSS programme.

Of the 18 presumptive positive samples identified during the pilot using a cut-off value of 1.0 μ mol/L, 4 were confirmed as TP's and 14 as FP's. Analysis of C5 isobars on these samples demonstrated that 6/14 FP's contained predominantly pivaloylcarnitine (mean 94%, median 96%, range 86–98%). The inclusion of C5 isobar analysis as a second tier test would have increased the PPV from 22.2 to 33.3%. In the post pilot study, using a cut-off value of 2.0 μ mol/L, 6 were confirmed as TP's and 10 as FP's. Analysis of C5 isobars on these samples demonstrated that 10/10 contained predominantly pivaloylcarnitine (mean 97%, median 98%, range 90–100%). The inclusion of C5 isobar analysis as a second tier test would have increased the PPV from 38 to 100%. Figure 1 shows the isobaric species as a percentage of total C5 in 33 of the presumptive positive blood spot samples (one sample was not available for analysis) and demonstrates the ability of the test to discriminate between the TP cases of IVA, and the FP's due to pivaloylcarnitine. Samples 1–10 are TP's, samples 11–17 are FP's and samples 18–33 are FP's due to pivaloylcarnitine.

Table 1 Summary of results from 34 newborn blood spot screening samples with presumptive positive result for Isovaleric Acidaemia

Case	Initial C5 on screen $\mu\text{mol/L}$	% of total C5 isobars				Mutation analysis	Diagnostic C5	Urine organic acids	Additional information	Outcome
		Pivaloyl	Methylbutyryl	Isovaleryl	Valeryl					
1	1.4	98	4	3	0	c.941C>T not detected	NAD	Not measured		FP
2	1.3	0	6	94	0	c.941C>T not detected	0.15 (<0.3)	Trace IVG		FP
3	1.0	0	7	93	0	c.941C>T not detected	0.24 (<0.3)	Trace IVG		FP
4	2.0	0	47	49	3	Not tested	1.42 (<1.2)	NAD		FP
5	1.5	94	2	4	0	c.941C>T not detected	1.54 (<1.2)	NAD		FP
6	1.3	94	3	3	0	c.941C>T not detected	1.54 (<1.2)	NAD		FP
7	1.1	86	3	11	0	c.941C>T not detected	2.3 (<1.2)	NAD		FP
8	1.1	0	13	87	0	c.941C>T not detected	0.45 (<0.5)	1.2 (<2.6)		FP
9	1.0	0	7	93	0	c.941C>T not detected	0.46 (<0.5)	1.8 (<2.6)		FP
10	1.3	0	8	92	0	c.941C>T not detected	0.49 (<0.5)	0.7 (<2.6)		FP
11	1.2	0	11	89	0	c.941C>T not detected	0.17 (<0.5)	0.5 (<2.6)	Breastfed	FP
12	1.9	97	2	1	0	c.941C>T not detected	0.79 (<0.6)	0.4 (<2.6)	Maternal pivampicillin	FP
13	3.6	97	2	1	0	c.941C>T not detected	0.96	0.3 (<2.6)	Maternal pivampicillin	FP
14	1.3					c.941C>T not detected	Not detected	1.1 (<2.6)	No sample for analysis of isobars. SBCAD deficiency	FP
15	11.9	0	0	100	0	c.941C>T not detected	8.4 (<0.3)	Increased IVG		TP
16	1.1	0	2	98	0	c.941C>T not detected	1.6 (<0.5)	54.6 (<2.6)	No antibiotics. Breastfed + formula	TP
17	1.4	0	3	97	0	c.941C>T not detected	2.0 (<0.5)	4.9 (<2.6)		TP
18	6.7	0	12	88	0	Heterozygous for c.941C>T	7.4 (<0.3)	82.3 (<2.6)		TP
19	64.0	0	0	100	0	Homozygous for c.280G>A	13.3 (<0.5)	Not analysed	Sibling of known patient. Diagnosed pre NBS	TP
20	70.0	0	0	100	0	Homozygous for c.367G>A	8.9 (<0.5)	Increased IVG	Sibling of known patient. Diagnosed pre NBS	TP
21	2.9	0	2	98	0	homozygous	5.9 (<0.5)	Increased IVG		TP
22	28.4	0	0	100	0	Homozygous for c.941C>T	13.9 (<0.5)	Increased IVG	Sibling of known patient	TP
23	3.9	0	1	99	0	Heterozygous for c.941C>T	2.6 (<0.5)	Increased IVG	Breastfed	TP
24	1.1	0	2	98	0	c.941C>T not detected	3.7 (<0.5)	Increased IVG	Sibling of known patient	TP
25	2.1	90	6	4	0	c.941C>T not detected	0.6 (<0.5)	NAD		FP
26	1.9	97	2	1	0	Not done	1.5 (<0.5)	NAD	Maternal antibiotics	FP
27	2.3	98	0	2	0	c.941C>T not detected	0.3 (<0.5)	NAD		FP
28	2.3	98	0	2	0	c.941C>T not detected	0.13 (<0.5)	NAD	Breastfed. Mum using Mustela cream	FP
29	3.0	98	0	2	0	c.941C>T not detected	1.4 (<0.3)	NAD		FP
30	4.1	98	1	1	0	c.941C>T not detected	NAD	NAD	Lanolin nipple cream	FP
31	4.4	100	0	0	0	c.941C>T not detected	0.84 (<0.5)	NAD	Maternal antibiotics	FP
32	5.4	100	0	0	0	c.941C>T not detected	1.11 (<0.5)	NAD		FP
33	5.8	100	0	0	0	c.941C>T not detected	0.56 (<0.5)	NAD		FP
34	2.1	2	91	0	9	c.941C>T not detected	1.08 (<0.3)	NAD	Maternal pivmecillinam	FP

Key: Bold = samples collected during pilot

Discussion

The impact of FP screening results has been considered previously. One recent study conducted by health economists reported that whilst patients would wish to avoid the possibility of a FP result if they could, it was not considered to be a major factor. Conversely a study that looked at the outcome of actual FP cases determined an increased parental stress index score, a significant and lasting increase in parental anxiety and an increase in hospital stays of the screen positive children (21% versus 10%) (Bonham 2013). It is evident that screening has the potential to do harm as well as good and so it is important to develop strategies to minimise FP results as well as FN's.

Although the mean concentration of isovalerylcarnitine in the 10 TP samples was 19.1 $\mu\text{mol/L}$, the median concentration was only 5.3 $\mu\text{mol/L}$ and the range 1.1–70 $\mu\text{mol/L}$. In comparison, the FP results due to pivaloylcarnitine had a mean initial C5 result of 2.4 $\mu\text{mol/L}$, median of 2.1 $\mu\text{mol/L}$ and range 1.4–4.4 $\mu\text{mol/L}$. Data from 173 screening laboratories registered with the CLIR Region 4 website (<https://www.clir-r4s.org/>) indicates that the 99th centile of C5 in the normal population is 0.38 $\mu\text{mol/L}$, whilst the 5th centile of the IVA disease range is 0.48 $\mu\text{mol/L}$. Based on this data CLIR suggests a target range of 0.38–0.48 $\mu\text{mol/L}$ for the C5 cut-off value which is approximately 4.5 times lower than that used in England.

The incidence of FP results due to pivaloylcarnitine is likely to be directly influenced by the prescribing patterns of common pivaloyl containing drugs, such as Pivmecillinam, which is used to treat bacterial infections such as lower tract urinary infections. In July 2012, GP practices in England wrote 2,408 prescriptions for Pivmecillinam whereas in July 2016 this had increased almost fivefold to 11,045. If use of this drug continues to grow at the current rate, the frequency with which FP results due to pivaloylcarnitine occur is also likely to increase. Recent guidance published by Public Health England (2016) recommending pivmecillinam as an alternative therapy when widespread bacterial resistance to ampicillin, amoxicillin and trimethoprim is reported, supports the likelihood of this. Prescribing patterns also vary geographically, with many CCGs in the south of England currently not prescribing the drug at all, compared with relatively frequent use by Airedale, Wharfedale and Craven, Greater Preston and Chorley and South Ribble CCGs (107, 88, 88 and 87 per 1,000 items of Penicillin) (Powell-Smith and Goldacre 2015). This could lead to small clusters of FP results in certain regions and indeed the 8 FP results identified post pilot have originated from the following screening laboratories: Yorkshire ($n = 1$), Sheffield ($n = 4$), Manchester ($n = 2$), West Midlands ($n = 1$), Bristol ($n = 1$) and South East Thames

($n = 1$). Whilst the latter case had no history of antibiotic use (mum was found to be using Mustela nursing balm which contains pivalic acid), at least five of the other cases were confirmed as being linked to maternal antibiotic use. Evidence indicates that the use of pivmecillinam is likely to increase throughout the rest of Europe too: it is now recommended as the treatment for uncomplicated UTIs by the Infectious Diseases Society of America, the European Society for Clinical Microbiology and Infectious Diseases and the European Association of Urology (Dewar et al. 2014).

It can be difficult to draw firm conclusions when only limited numbers of true cases are available. The data collected on samples post pilot, with a cut-off value of 2.0 $\mu\text{mol/L}$, indicate that incorporation of C5 isobars as second tier test would increase the PPV to 100%. However, it is important to remember that during this time approximately 1 million babies have been screened and 16 screen positive samples identified, 10 of which were FP due to pivaloylcarnitine.

The methodology described here can reliably identify FP IVA screening results due to the presence of pivaloylcarnitine. It is a simple, rapid and robust method that could easily be implemented by newborn screening laboratories. In our opinion, the inclusion of this method as a second tier screening test for IVA will significantly reduce the number of FP results and prevent unnecessary stress and anxiety to parents. In addition, the cost of implementing this second tier test is marginal in comparison to the expense associated with an unnecessary clinical referral.

Synopsis

Introduction of C5 isobars as a second tier screening test for Isovaleric Acidaemia has the potential to significantly reduce the FP rate.

General Rules

The name of the corresponding author: Rachel Carling.

A competing interest statement: No competing interests.

Details of funding N/A.

Conflict of Interest

Rachel Carling, Deborah Burden, Ian Hutton, Rachel Randle, Kate John and Jim Bonham declare no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the

Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Details of the contributions of individual authors:

Rachel Carling performed the data analysis, researched the literature and wrote the first draft of the manuscript. All authors contributed to the data interpretation, review and approval of the manuscript.

References

- Abdenur JE, Chamoles NA, Guinle AE et al (1998) False positive result due to pivaloylcarnitine in a newborn screening programme. *J Inherit Metab Dis* 21:624–630
- Boemer F, Schoos R, de Halleux V et al (2014) Surprising causes of C5-carnitine false positive results in newborn screening. *Mol Gen Metab* 111:52–54
- Bonham JR (2013) Impact of new screening technologies: should we screen and does phenotype influence this decision? *J Inherit Metab Dis* 36:681–686
- Dewar S, Reed L, Koerner R (2014) Emerging clinical role of pivmecillinam in the treatment of urinary tract infection in the context of multidrug-resistant bacteria. *J Antimicrob Chemother* 69(2):303–308
- Ensenauer R, Vockley J, Willard JM et al (2004) A common mutation is associated with a mild, potentially asymptomatic phenotype in patients with isovaleric acidemia diagnosed by newborn screening. *Am J Hum Genet* 75:1136–1142
- Moorthie S, Cameron L, Sagoo G, Burton H (2013) Birth prevalence of five inherited metabolic diseases. A systematic review. PHG Foundation
- Powell-Smith A, Goldacre B (2015) [OpenPrescribing.Net](https://openprescribing.net)
- Public Health England (2016) Management of infection guidance for primary care for consultation and local adaptation
- Tanaka K (1990) Isovaleric acidemia: personal history, clinical survey and study of molecular basis. *Prog Clin Biol Res* 321:273–290
- Vockley J, Parimoo B, Nagao M, Tanaka K (1992) Identification of the molecular defects responsible for the various genotypes of isovaleric acidemia. *Prog Clin Biol Res* 375:533–540