Original Article

Integrated Multianalyte Second-Tier Testing for Newborn Screening for MSUD, IVA, and GAMT Deficiencies

Journal of Inborn Errors of Metabolism & Screening
2016, Volume 4: 1–7

DOI: 10.1177/2326409816666296
iem.sagepub.com
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Graham B. Sinclair, PhD, FCCMG[1,2,3,4], Manuel Ester[1], Gabriella Horvath, MD, PhD, FRCPC, FCCMG[4,5,6], Clara D. van Karnebeek, MD, PhD, FRCPC, FCCMG[2,3,4,5,6], Sylvia Stockler-Ipsirogu, MD, FRCPC[2,3,4,5], and Hilary Vallance, MD, FCCMG[1,2,3,4]

Abstract

Advances in mass spectrometry have allowed for expansion of newborn screening test panels over the last decade but with increased numbers of disorders have come increased concerns with false-positive rates. The introduction of second-tier testing has improved the specificity of screening for a number of disorders without any corresponding sacrifice in sensitivity. Such testing does, however, put pressure on scarce laboratory resources including instrument and personnel time and even the bloodspot sample itself. The British Columbia Newborn Screening Program has developed an integrated second-tier screening approach to improve test performance without the requirement to resample and reprocess the original bloodspot specimen. By utilizing the residual extract from the first-tier assay and introducing a chromatography step as the second tier, we have been able to reduce false-positive rates due to interfering isobaric compounds for 3 different disorders (maple syrup urine disease, isovaleric aciduria, and guanidinoacetate methyltransferase) in a single multianalyte assay.

Keywords

newborn screening, guanidinoacetate, second tier, isovaleric aciduria, MSUD, GAMT deficiency

Introduction

Population-wide newborn screening is widely accepted as a highly effective public health intervention and has seen a significant expansion in the number of disorders covered in the last decade, driven primarily by advances in mass spectrometry. With these advances, however, have come increasing concerns regarding false-positive rates and the impact that reduced overall test performance has on the screening system as a whole.[1] Second-tier testing, through the use of a lower throughput but higher specificity test on a subset of high-risk infants identified on the initial screen, provides a technological approach to drastically improve test performance for a number of disorders.[2-4] Although such approaches can greatly reduce the number of infants who have to be recalled for follow-up testing, they do place significant pressure on a number of limited laboratory resources including the remaining dried bloodspot specimen itself along with personnel and instrument time. Given recent focus on the timeliness of newborn screening, minimizing the delays associated with second-tier testing is becoming increasingly important.[5]

[1] Department of Pathology and Laboratory Medicine, British Columbia Children's Hospital, Vancouver, BC, Canada
[2] Treatable Intellectual Disability Endeavour, British Columbia Children's Hospital, Vancouver, BC, Canada
[3] Child and Family Research Institute, British Columbia Children's Hospital, Vancouver, BC, Canada
[4] University of British Columbia, British Columbia Children's Hospital, Vancouver, BC, Canada
[5] Department of Pediatrics, British Columbia Children's Hospital, Vancouver, BC, Canada
[6] Centre for Molecular Medicine and Therapeutics, British Columbia Children's Hospital, Vancouver, BC, Canada

Received March 23, 2016, and in revised form May 17, 2016. Accepted for publication May 30, 2016.

Corresponding Author:
Graham B. Sinclair, Department of Pathology and Laboratory Medicine, British Columbia Children's Hospital, Room 2F24, 4500 Oak Street, Vancouver, BC V6H 3N1, Canada.
Email: gsinclair@cw.bc.ca

A number of the second-tier assays that are in routine use involve the reanalysis of a compound initially tested as part of the standard flow-injection tandem mass spectrometry assay (FIA–MS/MS) for acylcarnitines and amino acids. These assays are generally designed to introduce a chromatographic step prior to the MS/MS analysis (liquid chromatography [LC]–MS/MS) effectively separating the compound of interest from an interfering isobar. Examples of such isobaric interferences include branched-chain amino acid separation in maple syrup urine disease (MSUD), pivalic acid contamination in isovaleric aciduria (IVA), and an unidentified interferent in first-tier screening for guanidinoacetate methyltransferase (GAMT) deficiency.[3,6,7] Accordingly, these assays require the resampling of the original bloodspot specimen that can become an issue if the specimen is of poor or limited quantity, as is often the case for premature infants. Subsequent reextraction and derivatization of the samples require further technologist time and reagent costs that must be borne by the laboratory. Finally, given standard laboratory workflows, these second-tier tests can easily add 24 or more hours to the screening process.

The British Columbia Newborn Screening Program has utilized a laboratory developed assay for acylcarnitines and amino acids since 2001 that includes a 7-point external calibration curve, run daily, to control for day-to-day variability in extraction efficiency and minor lot-to-lot variability of internal standard concentration.[8] During our evaluation of a second-tier assay to improve test performance for MSUD screening, we realized that the residual derivatized extract from the first-tier test could act as the starting material for the second-tier assay, eliminating the need to resample the original specimen or even reextract and process the specimen. We report here the successful implementation of such integrated second-tier testing for MSUD, IVA, and GAMT deficiency screening in a single combined assay.

Materials and Methods

First-Tier Assay

A full panel of acylcarnitines and amino acids were analyzed by FIA–MS/MS, modified from our previous publication.[9] Three millimeter disks were punched from dried bloodspots using standard Whatman 903 filter paper (Western Business Forms, Friant, California). Analytes were extracted from the punches by shaking incubation at 60°C for 60 minutes in 200 µL of 80:20 methanol:ddH_2O containing 1.5 µmol/L hydrazine and isotope-labeled internal standard mixes NSK A and NSK B from Cambridge Isotopes supplemented with $^{13}C_5$-succinylacetone, $^{13}C_6$-isoleucine, d_{10}-alloisoleucine, and $^{13}C_2$-guanidinoacetate (Cambridge Isotopes, Andover, Massachusetts). 150 µL of the extract was transferred to a clean plate and dried under heated airflow at 55°C for 15 minutes. Then, 50 µL butanolic HCl (Sigma-Aldrich, Oakville, Ontario, Canada) was added to butylate the extract at 60°C for 15 minutes. Finally, the butylated product was again evaporated to dryness (55°C airflow for 15 minutes) and resuspended in 200 µl of 80:20 acetonitrile:ddH_2O with 0.1% formic acid. The final derivatized extract was analyzed in multiple reaction monitoring (MRM) acquisition on a Waters TQD mass spectrometer (Waters Canada, Mississauga, Ontario, Canada) with a 10-µL injection and flow rate 1.2 mL/min for 0.4 minutes, 0.6 mL/min for 0.8 minutes, and 1.2 mL/min for 0.6 minutes for a total 1.2-minute injection-to-injection time.

External dried bloodspot calibrators were created by preparing 10 mmol/L aqueous stocks of all saturated even-chain acylcarnitines (C2-C18) plus C3 and C5 species (H. Ten Brink, Amsterdam, the Netherlands). The acylcarnitines were combined in a master stock in methanol ranging from 100 µmol/L for C2-carnitine down to 10 µmol/L for C18-carnitine depending on the normal target range. Amino acids were prepared at 10 mmol/L in 0.1 N NaOH (Phe, Tyr, Ala, Cit, Met, Arg, Leu, Ile, Allo-ile, guanidinoacetate [GAA], and succinylacetone [SUAC]) and also diluted to a 100 µmol/L working stock in methanol for most amino acids (10 µmol/L for GAA and SUAC). Adult donor whole blood was spun (4000*g*, 10 minutes) to pellet the cells and sufficient serum was removed to achieve a 55% hematocrit. A 7-point calibration curve was created by diluting the master acylcarnitine and amino acid stocks into the 55% hematocrit whole blood with thorough mixing (30 minutes at room temperature) prior to spotting 100 µL per spot on Whatman 903 filter paper. Calibrators were dried and stored with desiccant in foil bags at −80°C for up to 1 year.

Calibrator Validation

Calibrators were punched and analyzed in duplicate over 6 analytical runs with calculation of slope and *x*-intercept relative to the nominal spiked values. Endogenous levels were calculated as the average *y*-intercept of these initial curves and the spiked values plus the calculated endogenous levels utilized for daily calibrations using a custom modification to our Specimen Gate Laboratory Software (Perkin Elmer, Waltham, Massachusetts).

Second-Tier Assay

For second-tier analysis, the residual extract from the first-tier assay was evaporated to dryness (55°C airflow for 15 minutes) and the wells were resuspended in 100 µL of 90:10 ddH_2O:-methanol with 0.1% formic acid. This sample was then chromatographically separated with a 10 µL injection onto a BEH 50 × 1.5 mm, 3.6 µmol/L ultra performance LC (UPLC) column (Waters Canada) at a flow rate of 0.6 mL/min, buffer A = 100% ddH_2O, 0.1% formic acid and buffer B = 100% methanol, 0.1% formic acid. The column was held at 90% buffer A for 1.5 minutes from injection, followed by a shallow gradient to 85% buffer A over 2 minutes. This was followed by a quick ramp to 50% buffer A over 1 minute and a final gradient back to starting conditions over 30 seconds with a 1-minute equilibration prior to the next injection. Total cycle time was 5 minutes from injection to injection utilizing an

Acquity UPLC system (Waters Canada). Data analysis was completed by MRM analysis on a Waters TQD mass spectrometer utilizing the TargetLynx software (Waters Canada). Transitions monitored include the following compounds and their corresponding isotope-labeled internal standards—Val (174.2 > 72.2), d_8-Val (182.2 > 80.2), Leu/Ile/Allo-ile (188.2 > 86.2), d_3-Leu (191.2 > 89.2), $^{13}C_6$-Ile (194.2 > 91.2), d_{10}-Allo-ile (198.2 > 96.2), GAA (174.2 > 101.2), $^{13}C_2$-GAA (176.2 > 103.2), isovalerylcarnitine (302.3 > 85.2), and d_9-isovalerylcarnitine (311.3 > 85.2). Pivaloylcarnitine (302.3 > 85.2) was also monitored with a separate injection of prederivatized pivaloylcarnitine as a retention time check and confirmation of baseline separation from isovalerylcarnitine.

Interference Testing

Common preanalytical interferences were tested by spiking whole blood with intralipid solution (0-1000 mg/dL), bilirubin (0-340 μmol/L), and collecting blood in ethylenediaminetetraacetic acid, Na Heparin, Li Heparin, and Na Citrate anticoagulant tubes prior to spotting on the filter paper.

Newborn Screening Workflow

The second-tier assay was run on an as-needed basis whenever a specimen flagged on the first-tier assay for MSUD, GAMT, or IVA (Figure 1). The first-tier assay was run overnight with data analysis completed by laboratory staff the following morning. If any samples flagged positive for the aforementioned assays based on established algorithms, the initial plate containing the calibration curves and initial quality control (QC), along with any plate with a flagged specimen were evaporated to dryness and resuspended in the chromatographic loading buffer. All calibrators, flagged patient samples and bracketing QC samples were then reinjected through the column. Results were generally available before noon on the same day. For MSUD and IVA assays, newborn screening reports were generated based on the results of the second-tier assay. For GAMT deficiency, a third-tier GAMT gene sequencing assay was conducted on all samples with an elevated GAA on the second-tier and reported in accordance with the sequencing results.[10]

Method Validation

Test performance for each analyte in the second-tier assay was validated using a combination of spiked bloodspot controls and deidentified residual newborn screening bloodspot samples. Limits of quantitation were set by the analysis of dried bloodspot samples spiked with decreasing concentrations of each analyte, with the quantitation limit defined as a signal-to-noise ratio >15 and an intraday coefficient of variation <15%. Given the high endogenous concentrations in bloodspot for the branched-chain amino acids, quantitation limits were set at the mean plus 3 standard deviation value for a blank specimen and verified by analysis of a nonmatrix spiked sample. Assay precision was established by the analysis of spiked samples at low (normal range) and high (disease range) levels with 5 replicates measured per day over 5 analytical runs. Linearity was assessed using spiked samples across the analytical range including a set of standards provided by the US Center for Disease Control (CDC; provided by Victor DeJesus). Accuracy was assessed relative to the nominal value of in-house spiked samples and those provided by the CDC. Analyses were also completed on organized external proficiency testing samples for the branched-chain amino acids (CDC Newborn Screening Quality Assurance Program).

Ethics

Deidentified residual newborn bloodspot samples were included in the development of MSUD and IVA second-tier assays as part of standard quality improvement / quality assurance processes in keeping with the British Columbia Newborn Screening Program specimen storage and use policy. Specimens included in the GAMT method development and pilot study were approved by the research ethics board of the University of British Columbia C&W who granted a waiver of individual consent for this deidentified study (UBC C&W #: H12-00026).

Results

Test Performance

Baseline chromatographic separation was achieved with a 5-minute run time for the branched-chain amino acids, pivaloylcarnitine, isovalerylcarnitine, and GAA relative to its unidentified interferant (Figure 2). Calibration curves also proved linear across the analytical range with r^2 values >.99 for all analytes (Table 1). Both intraday and interday precision were acceptable, with maximal interday coefficients of variation less than 8% for all analytes at both low and high QC levels (Table 1). Given the newborn screening application and a focus on pathological elevations, limits of quantitation were sufficient for all analytes with accuracy well within or even below the normal population range. No analytical interferences were detected from lipid or bilirubin spiking across the ranges tested (data not shown). Similarly, no significant impact on calculated concentrations was noted for any of the anticoagulants tested.

Screening Results

Newborn screening for MSUD was initiated in 2009 in British Columbia, 1 year before the second-tier assay was available. In order to minimize the false-positive rate in this intervening period, screening cutoffs were established as Xle >560 μmol/L or (Xle >450 μmol/L and Val >250 μmol/L and [Val/Phe >3.5 or Xle/Phe >6]) with a theoretical sensitivity of 79% based on Region 4 Collaborative Data.[11] In 1 calendar year (44 727 infants), there were 2 false positives and 0 MSUD cases detected. False positives were defined as those with abnormal

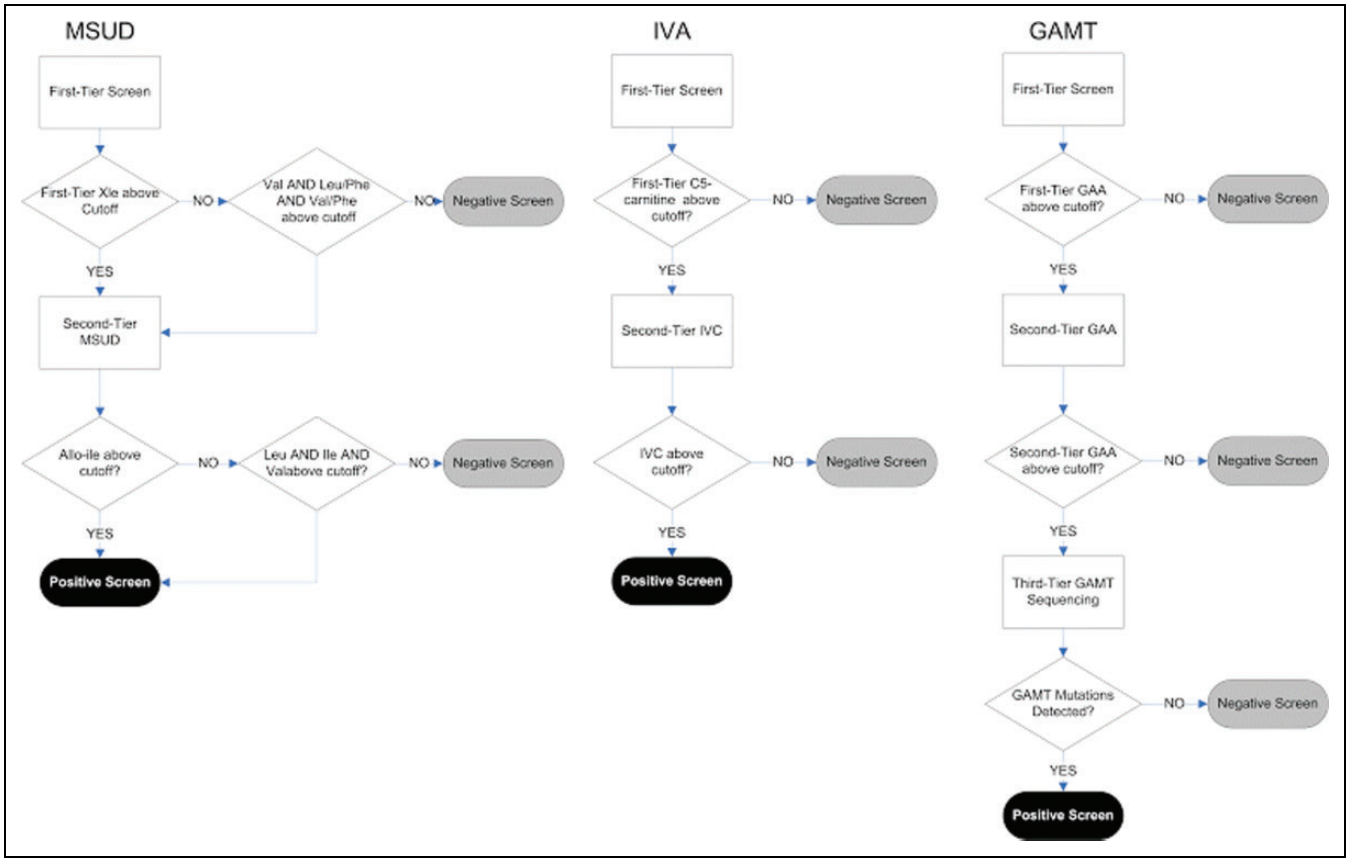

Figure 1. Screening algorithms integrating second-tier testing for MSUD, IVA, and GAMT deficiency. Allo-ile indicates alloisoleucine; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; Ile, isoleucine; IVA, isovaleric aciduria; IVC, isovalerylcarnitine; MSUD, maple syrup urine disease; Val, valine; Xle, combined signal of leucine, isoleucine, alloisoleucine, and hydroxyproline.

initial screen results for whom the balance of the data on follow-up testing ruled out the primary disorder in question.

Once the second-tier testing was live, the first-tier screening cutoffs were reduced in value to Xle >350 µmol/L or (Xle >250 µmol/L and Val >220 µmol/L and [Val/Phe >3 or Xle/Phe >3.5]) to improve the theoretical sensitivity to 93%. Second-tier screening results were flagged against Allo-ile (>2 µmol/L) or marked elevations in 2 of 3 other branched-chain species as a fail-safe (Leu >200 µmol/L, Ile >100 µmol/L, Val >350 µmol/L). In the subsequent 5 years of screening (223 954 infants), there have been 7 false positives and 3 true positive MSUD cases detected, for a positive predictive value of 30%. Allo-ile was normal in all false positives (flagged due to the fail-safe algorithm), whereas all true positives had clear Allo-ile elevations (38, 102, and 22 µmol/L). In that time period, 529 specimens were reflexed to second-tier MSUD testing. Accordingly, without second-tier testing, the positive predictive value would have been only 0.57% if the same theoretical sensitivity was maintained. There have been no identified false-negative screens in this time period.

The GAMT screening was introduced as a pilot project in British Columbia in 2012 and the results of that pilot have recently been reported.[10] Over a 3-year pilot period (135 372 infants), 259 samples were reflexed to the second-tier testing representing 0.19% of all infants screened. No true-positive nor false-positive cases were reported. Although the theoretical sensitivity of this assay is 100%, this is based on a very small number of known cases whose newborn bloodspots were tested retrospectively, so the true performance of this assay remains to be determined. Second-tier testing did, however, eliminate all potential false-positive screen results over the study period.

Isovaleric aciduria screening has utilized C5-carnitine as the single screening marker since its introduction in 2009. The established cutoff of 0.8 µmol/L has a theoretical sensitivity of 97% based on Region 4 Collaborative data.[11] In the first 5 years of screening (224 526 infants), we reported 18 false-positive screens, largely from premature infants, likely as a result of interference from pivalic acid-containing antibiotics. In late 2014, a marked C5-carnitine elevation (3.1 µmol/L) was noted in an infant and immediate metabolic referral was initiated. Subsequent bloodspot testing revealed a rapid normalization of C5-carnitine levels and no isovalerylglycine was noted in urine organic acid analysis for infant and mother. Based on the recent publication by Boemer et al of a string of false-positive IVA screens in Belgium due to contamination with pivaloylcarnitine from a nursing balm, we decided to evaluate our existing MSUD method as a potential second-tier assay.[7] This assay clearly separated the isovalerylcarnitine peak from pivaloylcarnitine (Figure 1), and this application for the assay

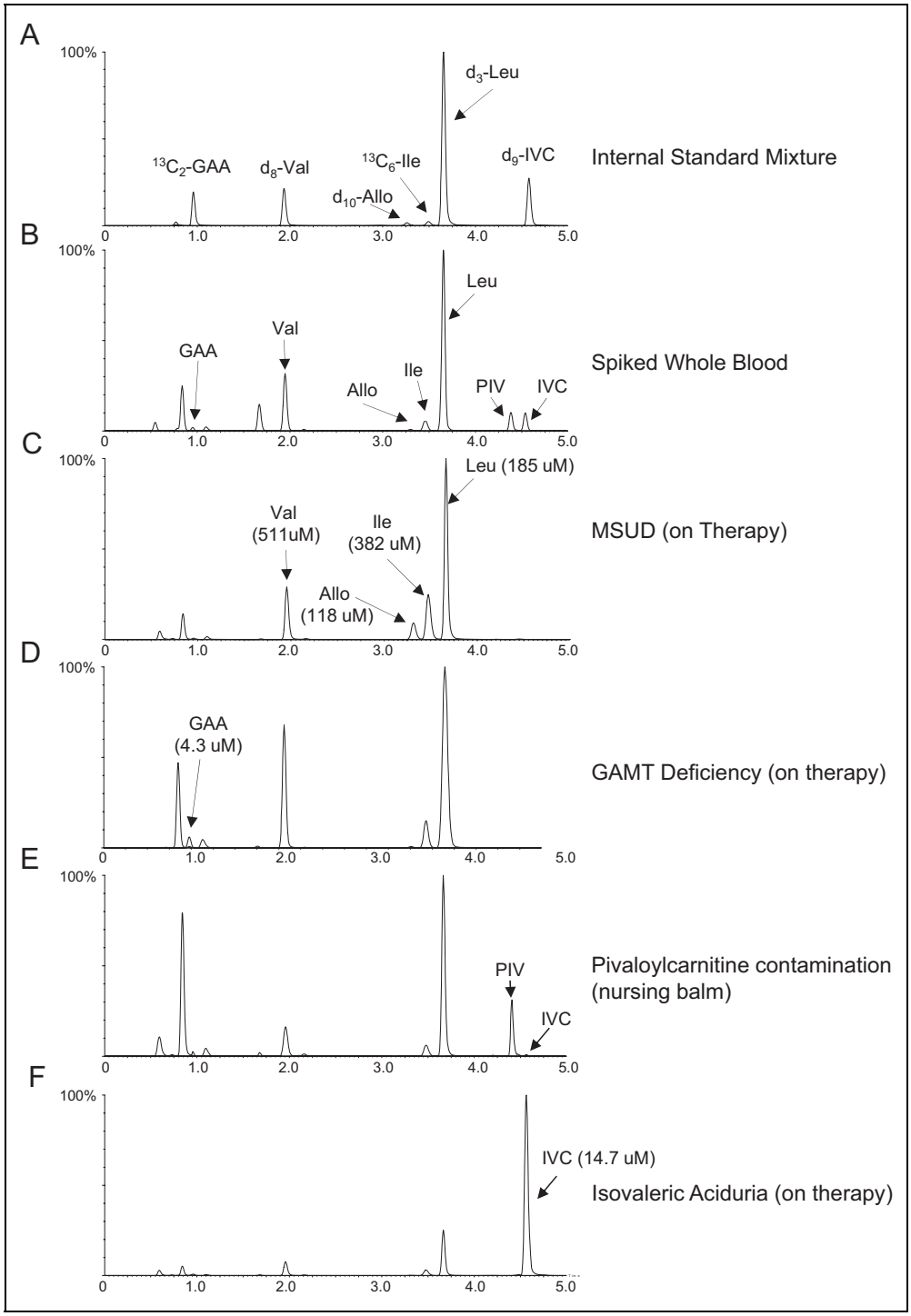

Figure 2. Chromatograms from the second-tier UPLC–MS/MS separation of isobaric compounds utilizing the residual extract from the first-tier flow-injection assay. A, Isotope-labeled internal standard mixture ($^{13}C_2$-GAA, guanidinoacetate standard; d8-Val, valine standard; d10-Allo, alloisoleucine standard; 13C6-Ile, isoleucine standard; d3-Leu, leucine standard; d9-IVC, isovalerylcarnitine standard). B, Dried bloodspot sample from a control donor spiked with nonlabeled standards. C, A bloodspot sample from a patient with MSUD on a leucine restricted diet. D, A bloodspot sample from a patient with GAMT deficiency on therapy. E, Bloodspot from an IVA false-positive screen due to pivaloylcarnitine contamination from a nursing balm. F, A bloodspot sample from a patient with IVA. GAMT indicates guanidinoacetate methyltransferase; IVA, isovaleric aciduria; IVC, isovalerylcarnitine; MS/MS, tandem mass spectrometry; MSUD, maple syrup urine disease; PIV, pivaloylcarnitine; UPLC, ultra performance liquid chromatography.

Table 1. Interassay Precision and Limits of Quantitation.

Analyte	Low QC CV% (Concentration), μmol/L	High QC CV% (Concentration), μmol/L	LOQ, μmol/L	Calibration Curve r^2
Alloisoleucine	6.1 (11.2)	6.8 (68.7)	1.0	.990
Isoleucine	5.5 (132)	6.8 (738)	5.0	.996
Leucine	5.5 (200)	5.2 (791)	5.0	.996
Valine	5.3 (408)	4.4 (1678)	20.0	.995
C5-carnitine	4.6 (1.7)	5.8 (11.5)	0.07	.991
Guanidinoacetate	7.5 (6.7)	7.2 (38.5)	0.3	.991

Abbreviations: CV, coefficient of variation; LOQ, limits of quantitation; QC, quality control.

was subsequently formally validated. Chromatographic separation of 2-methylbutyrylglycine, another potentially interfering isobar, was not assessed. The initial specimen from the infant in question was confirmed to contain a large pivaloylcarnitine peak with normal isovalerylcarnitine levels and the mother was confirmed to have been using the same nursing balm product reported in the Belgian publication.[7] Subsequent to the introduction of this second-tier IVA assay, we have had no true- or false-positive screen results but this represents a very limited time frame.

Discussion

A variety of multitiered testing approaches have been implemented to improve the positive predictive value of newborn screening assays including IRT/DNA protocols for cystic fibrosis, steroid profiling for congenital adrenal hyperplasia, and methylcitrate/methylmalonate assays for the differentiation of C3-carnitine-related disorders. All of these approaches, although effective in improving screening performance, do require resampling of the original newborn bloodspot card and associated sample processing steps. This can become a major issue as bloodspot material, technologist time, and available reporting time are all limited commodities. However, for those screening tests confounded by an isobaric interferant on FIA–MS/MS analysis, adding a chromatographic step to the existing bloodspot extract as an integrated second-tier assay avoids most of these limitations.

The introduction of integrated second-tier testing for MSUD in our laboratory allowed us to reduce the analytical values for our first-tier screening cutoffs to improve sensitivity, without affecting the false-positive rates of the assays. This is particularly important for MSUD given reports of false-negative screens due to very mild branched-chain elevations in biochemically attenuated cases.[12,13] Of equal importance in MSUD screening is the timeliness of reporting given the short latency period for severely affected cases.[14] The presence of alloisoleucine as a highly sensitive and specific marker for MSUD in the second-tier assay allows for an aggressive response to a positive screen result, including immediate referral to a tertiary-care center given the high positive predictive value of the result. Similarly, the integration of the second-tier testing into the first-tier workflow significantly reduces the time to reporting.

The interference of pivaloylcarnitine from pivalic acid containing antibiotic, and more recently, nursing balms containing isodecyl neopentanoate can significantly impact the positive predictive value of screening for IVA, particularly given the rarity of the disorder. The availability of second-tier testing can significantly reduce the false-positive rate for infants in the neonatal intensive care unit (NICU), a population already the recipient of a large percentage of abnormal screen results. Similarly, the confirmation of contamination by nursing balms can avoid the high cost of urgent metabolic referral given the marked false elevations that can be seen. Similarly, newborn screening for GAMT deficiency has been trialed in a number of jurisdictions but early attempts had high false-positive rates. The introduction of second-tier GAA testing has led to dramatic improvements in test performance leading to its recommendation and/or implementation in a number of jurisdictions.

The integration of second-tier testing utilizing residual extracts from the first-tier assay is not limited to the compounds described in this article, as the approach has the potential to be used in other assays where isobaric interference leads to a high false-positive rate by FIA–MS/MS. The presence of an external calibration curve in our initial analysis allowed for a fully quantitative assay without the need to prepare separate calibrators; however, laboratories without external calibration may also be able to implement this approach utilizing analyte—internal standard ratios and empirically determined response factors. A recent description of the analysis of lysophosphatidylcholines for X-linked adrenoleukodystrophy screening follows this basic approach.[15] Given increasing demands for screening expansion, the ability to increase the efficiency of second-tier testing with respect to sample consumption, instrument time, personnel costs, and time to reporting all support the utilization of such an integrated approach.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by a grant from the Rare Diseases Foundation, Motherhood Centred, and BC Children's Hospital Foundation [1st

collaborative Area of Innovation, Treatable Intellectual Disability Endeavour in British Columbia, TIDEBC].

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