Combined Newborn Screening for Succinylacetone, Amino Acids, and Acylcarnitines in Dried Blood Spots

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BACKGROUND: Tyrosinemia type I (TYR 1) is a disorder causing early death if left untreated. Newborn screening (NBS) for this condition is problematic because determination of the diagnostic marker, succinylacetone (SUAC), requires a separate first-tier or only partially effective second-tier analysis based on tyrosine concentration. To overcome these problems, we developed a new assay that simultaneously determines acylcarnitines (AC), amino acids (AA), and SUAC in dried blood spots (DBS) by flow injection tandem mass spectrometry (MS/MS).

METHODS: We extracted 3/16-inch DBS punches with 300 μL methanol containing AA and AC stable isotope-labeled internal standards. This extract was derivatized with butanol-HCl. In parallel, we extracted SUAC from the residual filter paper with 100 μL of a 15 mmol/L hydrazine solution containing the internal standard $^{13}C_5$-SUAC. We combined the derivatized aliquots in acetonitrile for MS/MS analysis of AC and AA with additional SRM experiments for SUAC (m/z 155–137) and $^{13}C_5$-SUAC (m/z 160–142). Analysis time was 1.2 min.

RESULTS: SUAC was increased in retrospectively analyzed NBS samples of 11 TYR 1 patients (length of storage, 52 months to 1 week; SUAC range, 13–81 μmol/L), with Tyr concentrations ranging from 65 to 293 μmol/L in the original NBS analysis. The mean concentration of SUAC in 13 521 control DBS was 1.25 μmol/L.

CONCLUSION: The inclusion of SUAC analysis into routine analysis of AC and AA allows for rapid and cost-effective screening for TYR 1 with no tangible risk of false-negative results.

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Tyrosinemia type I (TYR 1;[3] OMIM 276700) is caused by autosomal recessive fumarylacetoacetate hydrolase (EC 3.7.1.2) deficiency, which causes development of severe liver disease in infancy, hypophosphatemic rickets, and neurologic crises. If left untreated, most patients die of liver failure in the first years of life (1). Over the last decade, treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC; Nitisinone) became available. It is particularly effective when initiated early in life, providing a major incentive to identify affected patients by newborn screening (2, 3). Measurement of tyrosine in newborn screening blood spots is routinely performed by tandem mass spectrometry (MS/MS) and other methods. However, hypertyrosinemia is neither a sensitive nor a specific marker for TYR 1 and most often is associated with common and benign transient tyrosinemia of the newborn. In addition, the concentration of tyrosine in affected newborns overlaps significantly with the range observed in unaffected individuals (1). Succinylacetone (SUAC) is a specific marker for TYR 1, but has been adopted as primary marker only in the newborn screening program of Quebec, where this condition has an incidence of approximately 1 in 20 000 live births (4). A method for determination of SUAC in dried blood spots (DBS) by flow injection MS/MS was previously developed by Allard et al. (5). This approach makes use of leftover blood spots already extracted with methanol for acylcarnitine and amino acid analysis. Because methanol does not allow extraction of SUAC, it is achieved by an additional extraction procedure that involves the incubation of the residual DBS at 37 °C with a hydrazine-containing acidic solution. The extract is then analyzed by MS/MS. This method requires dedicated MS/MS equipment, which hinders adoption of this method into routine screening (6). Several other programs, for example in Minnesota,

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[3] Nonstandard abbreviations: TYR 1, tyrosinemia type I; SUAC, succinylacetone; DBS, dried blood spots; AA, amino acid; AC, acylcarnitine; SRM, selected reaction monitoring.

have implemented a second-tier approach where SUAC is determined only when the concentration of tyrosine in the primary screening is above a conservatively chosen cutoff value (7, 8). However, whereas this approach effectively eliminates false-positive results for TYR 1, approximately 25% of TYR 1 cases could be missed with this strategy because the concentration of tyrosine in newborn screening samples is still below the cutoff value.

To remedy this situation, we developed and validated a method for the simultaneous determination of SUAC, amino acids (AA), and acylcarnitines (AC) by flow injection tandem mass spectrometry (FIA-MS/MS), which requires minor increases in reagent costs and labor but no additional equipment.

Materials and Methods

MATERIALS AND METHODS
We purchased succinylacetone, hydrazine monohydrate (98%), glycine, alanine, valine, leucine, methionine, phenylalanine, tyrosine, aspartic acid, glutamic acid, ornithine, citrulline, arginine, and L-carnitine from Sigma-Aldrich; isotopically labeled succinylacetone, amino acid, carnitine, and acylcarnitine internal standards from Cambridge Isotope Laboratories; acetyl-L-carnitine-HCl, propionyl-L-carnitine-HCl, butyryl-L-carnitine-HCl, valeryl-L-carnitine-HCl, octanoyl-L-carnitine-HCl, tetradecanoyl-L-carnitine-HCl, and hexadecanoyl-L-carnitine from Herman J. ten Brink, Kinische Genetica, VU Medical Center, Amsterdam; and 3 mol/L HCl in n-butanol from Regis Technologies. All other chemical and solvents were of the highest purity available from commercial sources and used without further purification.

PREPARATION OF CALIBRATORS AND CONTROLS
We prepared DBS for SUAC calibration, recovery, stability, and imprecision studies as follows: aliquots of pooled whole blood were spiked with SUAC to achieve final concentrations of 0, 5, 10, 20, 50, and 100 μmol/L, then spotted on filter paper (Whatman ProteinSaver 903) and dried overnight at room temperature. The spotted cards were transferred to a zip-lock bag with desiccator and stored at −20 °C.

SAMPLES
For the validation of this method, we analyzed 13 521 leftover newborn screening blood spots that were initially submitted to Mayo Clinic's supplemental newborn screening program and were not suggestive of TYR 1, based on a previously published 2-tier screening approach (8). In addition, blood spots from the original newborn screening samples of 11 confirmed TYR 1 patients were made available by their respective physicians and screening laboratories with informed consent.
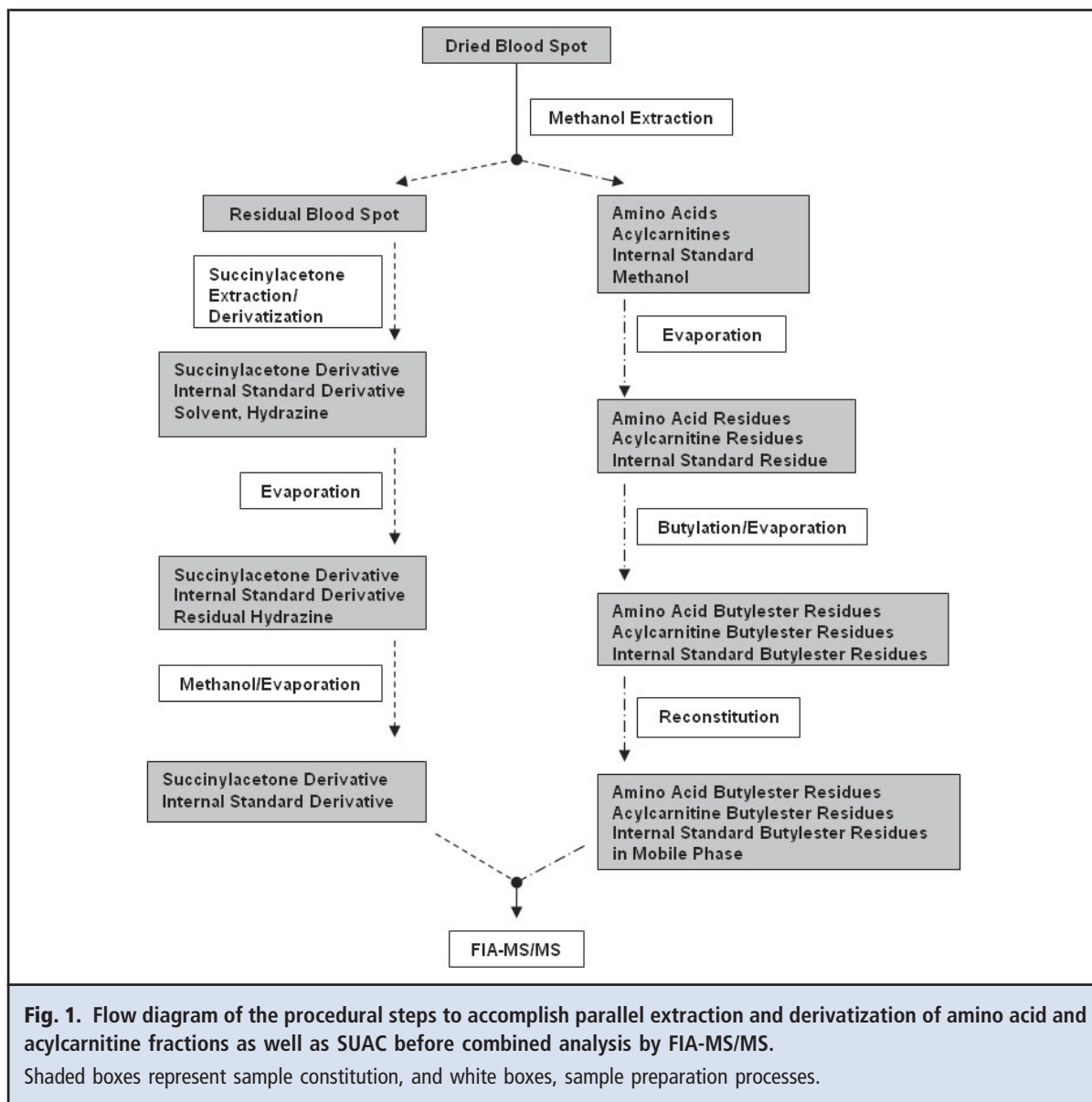
METHODS
Sample preparation entails a parallel work-up of eluates from the same DBS that contain either amino acids and acylcarnitines or SUAC. The analytes are subsequently recombined for ESI-MS/MS analysis as outlined in Fig. 1.

We punched single 3/16-inch discs (Wallac DBS Puncher; PerkinElmer Life and Analytical Sciences) from controls and samples into wells of a flat-bottom 96-well plate (Fisher Chemical Co.). We added methanol solution containing the amino acid, carnitine, and acylcarnitine internal standards (300 μL) to each well. The plate was covered and the discs were eluted by mixing using an orbital rotator for 30 min at 120 rpm. We transferred the methanol eluates to another round-bottom 96-well plate, leaving the residual filter paper discs for subsequent elution of SUAC for 30 min at 65 °C by addition of 100 μL acetonitrile/water/formic acid solution (80:20:0.1, vol:vol:vol), which also contains 0.1% hydrazine monohydrate (15 mmol/L) and the internal standard $^{13}C_5$-SUAC (0.25 μmol/L). During the extraction of the residual filter paper discs, the plate containing the methanol eluates was evaporated under heated (40 °C) nitrogen to dryness (approximately 8–12 min). We added 3 mol/L HCl in n-butanol (50 μL) to the dried residues, which were then covered and incubated for 15 min at 65 °C. After incubation, excess reagent was evaporated to dryness (approximately 5–7 min) under heated (40 °C) nitrogen, and the residue, containing butyl esters of the AA and AC, was reconstituted in 100 μL of mobile phase (acetonitrile:water:formic acid; 50:50:0.02, vol:vol:vol).

After extraction of SUAC from the leftover dried filter paper spots, the eluates were transferred to another round-bottom 96-well plate and dried under heated (40 °C) nitrogen for approximately 7 min. We removed any residual hydrazine reagent by addition of 100 μL methanol to each well, mixing, and evaporation under heated nitrogen. We transferred the mobile phase in the plate containing butylated amino acids and acylcarnitines to corresponding wells in the plate containing the SUAC-hydrazone/IS residues. This plate was covered, gently mixed by agitation, and ready for analysis by FIA-MS/MS.

MS/MS PROCEDURE
We used a triple-quadrupole MS/MS system (Applied Biosystems/MDS Sciex API 3000) operated in positive ion mode (source voltage, 5500 V). Mass calibration and resolution of both resolving quadrupoles were automatically optimized by the use of a poly(propylene)glycol solution introduced by an infusion pump.

Fig. 1. Flow diagram of the procedural steps to accomplish parallel extraction and derivatization of amino acid and acylcarnitine fractions as well as SUAC before combined analysis by FIA-MS/MS.

Shaded boxes represent sample constitution, and white boxes, sample preparation processes.

We performed method optimization for the detection of SUAC by selected reaction monitoring (SRM) by infusing a 10 μmol/L solution of SUAC and its internal standard as hydrazones at 0.6 mL/h. The instrument was optimized automatically by an internal algorithm to monitor the transitions m/z 155.0 to 137.0 and m/z 160.0 to 142.0 for SUAC and the internal standard, respectively. These SRM experiments (100-ms dwell, each experiment) were then added to precursor, neutral loss, and SRM scans for acylcarnitine and amino acid analysis in blood spots as described (9–12). Sample was introduced into the atmospheric pressure ionization source by a Leap Technologies HTC PAL Autosampler and a Perkin-Elmer Micro LC pump.

Autosampler injections of 10 μL per sample were made into the mobile-phase (acetonitrile:water:formic acid; 50:50:0.02, vol:vol:vol) flow of 0.025 mL/min. Analysis time was 1.2 min/injection.

SAFETY PRECAUTIONS

Hydrazine is a known toxic and carcinogenic chemical. To prevent exposure to this agent and its hazardous fumes, samples are prepared by use of a robotic pipetter (Freedom Evo; Tecan Systems Inc.) equipped with Plexiglas shielding and a custom-made fume evacuation system (see Supplemental Fig. in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/

Table 1. Average SUAC stability in extracts of control blood spots with either 6.4 μmol/L (control 1, n = 2) or 64 μmol/L (control 2, n = 2) SUAC added, as well as random newborn screening samples (n = 23).

	SUAC day 1, μmol/L	SUAC day 2, μmol/L	% Difference
Newborn screening DBS	1.39	1.48	6.08
Control 1	5.81	5.97	2.71
Control 2	66.4	65.2	−1.82

issue4). Hydrazine containers are not opened outside of this system, and its effectiveness and safety were tested and documented by Mayo Clinic's Section of Occupational Safety and Security.

Results

LINEARITY AND IMPRECISION

DBS calibrators of SUAC at 5 different concentrations (0, 5, 20, 50, and 100 μmol/L) showed detectable and reproducible signals with a linear response (R^2 = 0.9918, n = 6).

We determined intraassay imprecision by performing 5 replicate analyses of samples with 5 different SUAC concentrations, 1.1, 7.6, 26.5, 57.0, and 115.8 μmol/L. The mean CVs were 7.7%, 8.7%, 7.8%, 3.1%, and 12.3% respectively. We determined interassay imprecision by analysis of DBS with 2 different concentrations of SUAC, 6.0 and 60.7 μmol/L. Mean CVs of 192 analyses determined over 2 months were 16.7% and 15.8%, respectively.

STABILITY

We assessed the stability of extracted and prepared specimens by analysis of 23 newborn screening samples and 4 controls enriched with SUAC at 6.4 μmol/L (n = 2) and 64 μmol/L (n = 2) before and after 24 h under ambient conditions. Prepared specimens yielded their expected concentrations within 6.2% (Table 1).

METHOD COMPARISON FOR AA AND AC

We conducted a comparison using 290 newborn screening samples that were analyzed with and without the SUAC modification. Bland-Altman and x-y plots were generated for those analytes where corresponding isotopically labeled internal standards are available: alanine, valine, leucine/isoleucine, methionine, phenylalanine, tyrosine, asparagine, glutamic acid, ornithine, citrulline, arginine, glycine, free carnitine, and C2, C3, C4, C5, C6, C8, C14, C16 acylcarnitines. In addition,

we compared >10 000 newborn screening results obtained before and after the inclusion of SUAC analysis to our newborn screening method (Fig. 2). AC and AA concentrations in both of these studies compared well, with no clinically significant differences (Fig. 2). However, we noted that the modified screening method yielded a higher concentration of C12 acylcarnitine (Fig. 2B). Whereas isolated increases of C12 acylcarnitine are not indicative of a particular disorder, we studied the cause of this observation. The molecular ion of the butylester derivatives of C12 and C16 acylcarnitine species are detected by a precursor ion scan of m/z 85 at m/z 400.5 and 456.5, respectively. The same analysis of nonderivatized C16 acylcarnitine yields a signal at m/z 400.5. We therefore concluded that the higher abundance at m/z 400.5 observed in the modified method originated from nonderivatized C16 acylcarnitine extracted from the residual DBS along with SUAC. This was proven by reconstitution of the derivatized SUAC extract in mobile phase without the addition of the extracted and derivatized amino acids and acylcarnitines in DBS spiked with either AA (n = 4) or AC (n = 4). The contribution of nonderivatized acylcarnitines, when using the modified method, is negligible for most acylcarnitine species (Fig. 2B) and most likely notable only for C16 acylcarnitine secondary to the polarity and higher concentration of this acylcarnitine species in newborn DBS.

SUAC METHOD COMPARISON

We measured SUAC retrospectively in newborn screening samples of 11 patients with TYR 1 using our previously described LC-MS/MS method (8) and the modified newborn screening method. An x-y method plot yielded a slope, y-intercept, and coefficient of linear regression (R^2) of 1.7, −6.0, and 0.95, respectively. The difference between these methods is likely related to different extraction, sample preparation, and analytical procedures.

SUAC REFERENCE RANGE

The SUAC distribution for 13 521 random newborn screening samples is shown in Fig. 3. The mean, median, and 99.5th percentile of SUAC values were 1.25 μmol/L, 1.21 μmol/L, and 2.65 μmol/L, respectively. The lowest SUAC values determined in leftover newborn screening samples of 11 TYR 1 patients was 13 μmol/L, a value observed in 2 cases whose samples had been in storage at room temperature for 36 and 53 months, respectively (Table 2). As part of this retrospective study, we also compared the tyrosine concentrations determined during the initial newborn screening analysis to those measured in the retrospective analysis (Table 2). As we have shown previously, tyrosine is typically not stable over time. Reanalyzing 3
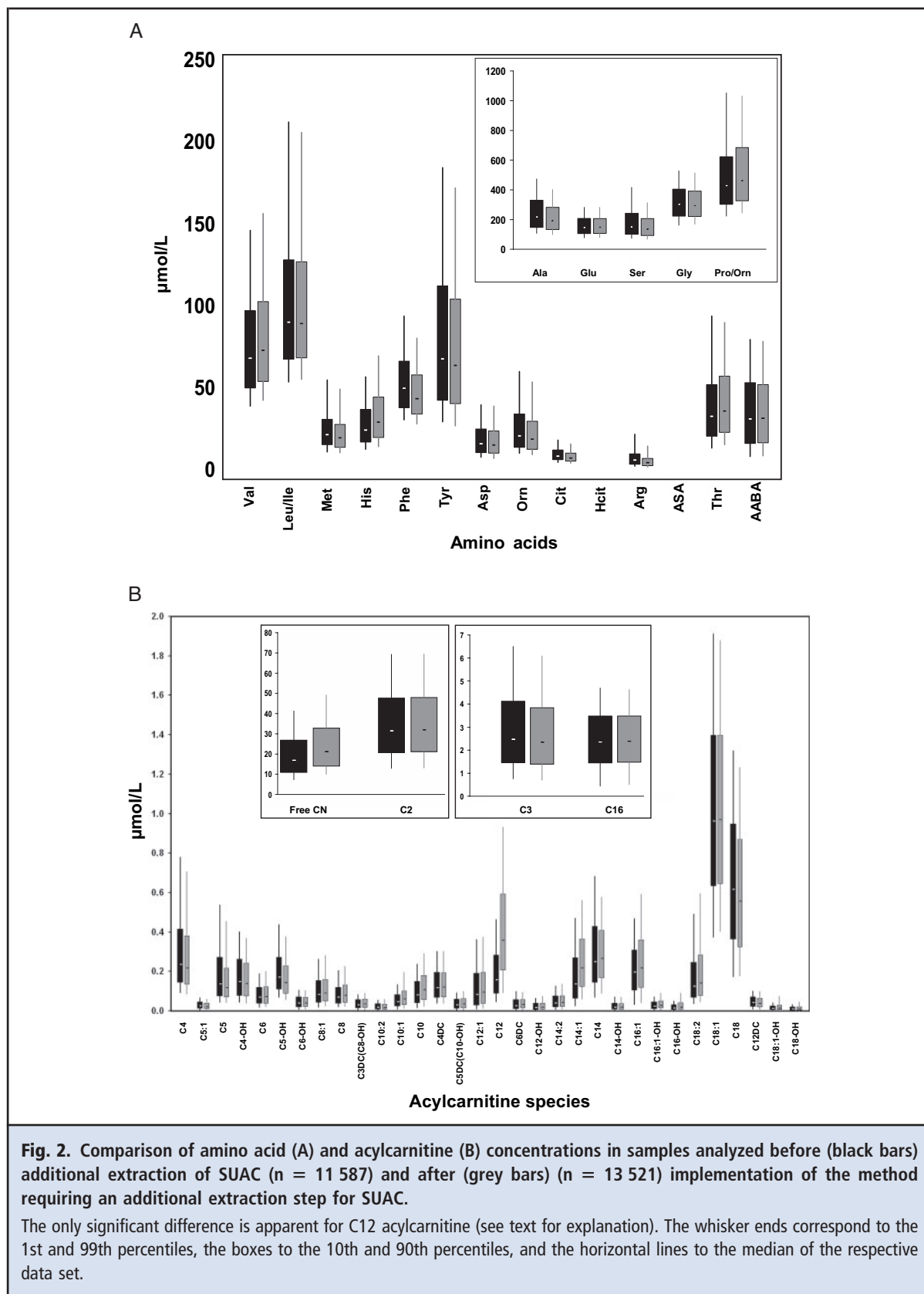
Fig. 2. Comparison of amino acid (A) and acylcarnitine (B) concentrations in samples analyzed before (black bars) additional extraction of SUAC (n = 11 587) and after (grey bars) (n = 13 521) implementation of the method requiring an additional extraction step for SUAC.

The only significant difference is apparent for C12 acylcarnitine (see text for explanation). The whisker ends correspond to the 1st and 99th percentiles, the boxes to the 10th and 90th percentiles, and the horizontal lines to the median of the respective data set.
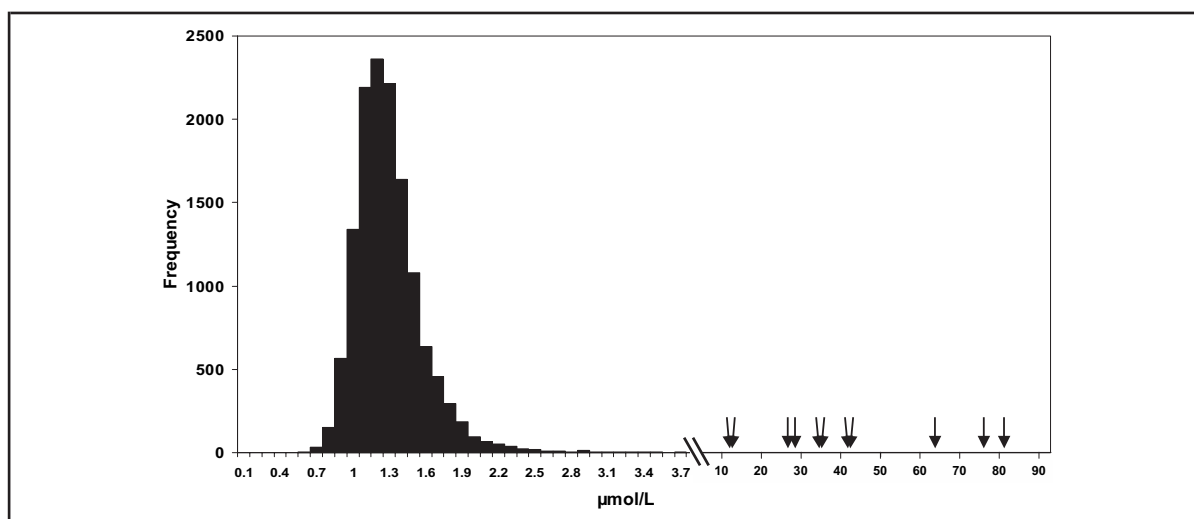
Fig. 3. Reference interval for SUAC as determined from 13 521 random newborn screening samples (mean 1.25 μmol/L, median 1.21 μmol/L; 99.5th percentile 2.52 μmol/L).

To demonstrate discrimination of healthy controls from newborns with TYR 1, SUAC values for 11 TYR 1 patients (Table 2) are indicated by arrows.

leftover DBS of patients with TYR 1 also revealed a decrease of the measurable SUAC concentration by 35%, 21%, and 9% after storage at room temperature for 6 months, 6 months, and 1 month, respectively. Based on these observations, we set the cutoff for SUAC

at 5.0 μmol/L, which allowed clear discrimination of the control population from TYR 1 patients.

Discussion

Newborn screening for TYR 1 is highly desirable because of the availability of effective treatment for this otherwise life-threatening condition. It was therefore included in the American College of Medical Genetics' core panel of conditions that every newborn should be screened for *(13)*. However, tyrosine is a poor marker for TYR 1, and not every laboratory has the ability to provide testing for SUAC either as a primary screen or as a 2-tiered approach. Between January 2005 and April 2007, we had applied a 2-tier screening strategy *(8)*. The cutoff for tyrosine was set at 150 μmol/L, and any newborn screening sample with tyrosine concentrations above this threshold was submitted for second-tier testing of another DBS punch from the same screening card for determination of SUAC by a liquid-chromatography MS/MS method. Of the 217 942 samples submitted to our newborn screening program during this time, 6479 (2.8%) were tested for SUAC following this strategy. In addition, our laboratory receives newborn screening DBS for second-tier testing of samples with increased tyrosine concentrations from other screening laboratories (Kentucky, Michigan, Ohio, and South Carolina). None of these revealed abnormal SUAC concentrations, and we are not aware that any patient tested by our laboratory has been di-

Table 2. Retrospectively determined SUAC and tyrosine concentrations in the original newborn screening samples of 11 patients with TYR 1, original tyrosine concentrations as determined by the respective screening laboratory, and storage time from birth to retrospective analysis.

Patient	Retrospective analysis, μmol/L		Original NBS result for tyrosine, μmol/L (state)	Storage time
	SUAC	Tyrosine		
1	13	13	250 (MI)	52 months
2	13	41	>200 (NC)	36 months
3	35	70	226 (CA)	35 months
4	77	114	293 (CA)	31 months
5	81	87	101 (IA)	12 months
6	42	95	213 (NC)	10 months
7	42	136	203 (CA)	7 months
8	35	68	113 (CA)	4 months
9	63	68	90 (OH)	1 week
10	29	176	141 (WA)	1 week
11	28	35	65 (IA)	1 week

agnosed with TYR 1 (*14*). However, review of the original newborn screening results of 57 patients with TYR 1 revealed that 16 (28%) had tyrosine concentrations <150 μmol/L (see Region 4 Collaborative Project web site, www.region4genetics.org). In light of these observations, newborn screening for TYR 1 must include testing for SUAC of every sample to achieve adequate diagnostic sensitivity (*15*). However, a first-tier assay for SUAC that requires a separate assay and additional equipment—in particular when MS/MS technology is used for testing (*6*)—is not affordable by most laboratories and has led some programs to abandon screening for TYR 1 (*16*). Incorporation of SUAC determination in the primary screening requires a small incremental effort and a minor cost increase for reagents ($0.13 per sample). The amount of blood spot used and equipment required remain the same, with the exception of a 1-time investment of approximately $600 to outfit the liquid handler with a custom-made venting system to prevent exposure of our personnel to the toxic fumes of hydrazine (see Supplemental Fig. in the online Data Supplement).

Sample preparation for this method includes a double extraction of the DBS sample, which raises concern as it may increase the recovery of analytes and therefore require determination of new cutoff concentrations. Comparing the results of >10 000 samples analyzed before and after implementation of the new method revealed mostly negligible increases in recovery of amino acids and acylcarnitines by the additional extraction. The only notable exception is the calculated concentration of C12 acylcarnitine, which is higher because it now represents a mixture of the butylester of C12 acylcarnitine with contribution of nonderivatized C16 acylcarnitine, the most abundant long-chain acylcarnitine species in newborn blood spots. This effect can be dealt with either by increasing the cutoff for what is considered C12 acylcarnitine or, as we have done, by avoiding setting an active cutoff value altogether, because this analyte by itself is not associated with a particular disorder. Laboratories that do not derivatize their samples would likely need to adjust their cutoff values for C16 acylcarnitine following similar validation studies as described here. Most importantly, the interpretation of newborn screening results is not affected by the new method which we implemented in May 2007. Since then, >40 000 samples were analyzed with no negative impact on newborn screening performance metrics, as the false-positive rate (0.08%), positive predictive value (42%), and detection rate (1:1688 live births) remained stable (*17*). So far, no newborn screening sample revealed an increased SUAC concentration. However, we assume that diagnostic sensitivity is 100% because no patient was diagnosed clinically with TYR 1 in Minnesota since June 2004.

In conclusion, we have developed and validated a new assay that successfully combines existing methods for newborn screening for disorders of fatty acid, organic acid, and amino acid metabolism including TYR 1. This method is sensitive, rapid, and cost-effective, with no increased risk of either false-positive or false-negative results and could be implemented by all laboratories already using FIA-MS/MS for newborn screening to the benefit of the population they serve, including patients with TYR 1.

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