Newborn Screening for Hepatorenal Tyrosinemia: Tandem Mass Spectrometric Quantification of Succinylacetone

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Background: False-positive and false-negative results occur in current newborn-screening programs for hepatorenal tyrosinemia, which measure tyrosine concentrations in blood spots, sometimes in combination with other metabolites, including succinylacetone. We present our experience with a newly described method for succinylacetone quantification in routine newborn screening.

Methods: Succinylacetone was extracted from blood spots that had already been extracted with absolute methanol for acylcarnitine and amino acid analysis. The solvent was acetonitrile–water (80:20 by volume) containing formic acid, hydrazine hydrate, and 100 nmol/L 5,7-dioxooctanoic acid as internal standard. Analysis was performed by tandem mass spectrometry in a separate run.

Results: Of 61,344 samples, 99.6% had succinylacetone concentrations ≤5 μmol/L. With a cutoff of 10 μmol/L, no false-positive results were obtained. In 2 patients, the succinylacetone concentrations in the dried blood spots from the 36th and the 56th hour of life were 152 and 271 μmol/L, respectively, and the tyrosine concentrations were 54 and 129 μmol/L. Hepatorenal tyrosinemia was subsequently confirmed in both patients. Retrospective analysis of the neonatal screening samples of 2 additional known patients revealed increased succinylacetone concentrations of 46 and 169 μmol/L, respectively.

Conclusions: Tandem mass spectrometric quantification directly from residual blood spots is a useful method for the early detection of hepatorenal tyrosinemia in newborn-screening programs.

Accumulation of succinylacetone (SA)⁵ is generally considered to be pathognomonic for hepatorenal tyrosinemia (HT; MIM 276700), a rare autosomal recessive metabolic disorder characterized by life-threatening progressive liver and kidney dysfunction and hepatocellular cancer. Very early diagnosis allows immediate introduction of specific treatment, with significant reduction of morbidity and mortality. Increased blood concentrations of tyrosine are neither specific nor sensitive enough to screen for HT in newborns (1, 2); it therefore seems promising to screen for SA rather than tyrosine in a routine newborn-screening program.

A simple method for quantifying SA in dried blood spots has recently been described by Allard et al. (3). The method is based on extraction of SA from blood spots with acetonitrile and water containing formic acid and hydrazine hydrate. Instead of deuterated SA, which is not yet available commercially, unlabeled 5,7-dioxooctanoic acid was used as an internal standard. Under the conditions given, hydrazine is thought to cleave covalently linked SA-protein adducts (4) and to simultaneously form a hydrazone derivative (Fig. 1), which is extracted (5). The analysis was performed by tandem mass spectrometry (MS/MS) with a total run time of 80 s per sample.

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⁵ Nonstandard abbreviations: SA, succinylacetone; HT, hepatorenal tyrosinemia (tyrosinemia type I); MS/MS, tandem mass spectrometry; and NTBC, 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (Nitisone).
Residual blood spots that had already been extracted for the analysis of acylcarnitines and amino acids were shown to be suitable for testing. It appeared, therefore, that inclusion of SA in our existing screening program could be achieved with little additional manual work. Here we report our experience using this test for newborn screening.

**Materials and Methods**

For this 16-week study, we used unselected samples from our routine screening program for inborn errors of metabolism and endocrinopathies, which we received from various parts of Germany. Blood collection on S&S 903 filter paper for metabolic screening in Germany is recommended at 36 to 72 h after birth. Sample shipping takes an average of 2.3 days (55.2 h). According to German regulations, reporting of results should be completed within 72 h after blood collection, leaving only 1 day to perform all laboratory work and data reporting. Routine neonatal metabolic screening for amino acids and acylcarnitines in our laboratory is therefore done on 4 different MS/MS instruments (MS/MS microTM and Quatro LCTM; Waters/Micromass Inc.), and 2 additional tandem mass spectrometers used for scientific purposes are available for backup. With laboratory work starting at 0700 in the morning, this instrumentation allows us to finish sample preparation and the complete analytical run for up to 1000 samples per day by late afternoon. Final results for more than 90% of newborns are available by the end of the first week of life.

For this study, SA was extracted from residual blood spots (3.2 mm) with 100 µL of a solution of acetonitrile and HPLC-grade water (80:20 by volume) containing, per liter, 1 mL of formic acid, 15 mmol of hydrazine hydrate (1 mL), and 100 nmol of 5,7-dioxooctanoic acid as described by Allard et al. (3). Stability of the extract was excellent with ≤10% degradation of the derivative over the course of 60 h. Analytical preparations require some precaution (use of a exhaust system and gloves) because hydrazine is a second-class carcinogen. The microtitration plates were agitated gently and incubated at 37 °C covered with aluminum foil. After 45 min, the extract was transferred to a second plate, which was covered with aluminum foil, for MS/MS analysis. The blood spots had earlier been extracted with 100 µL of absolute methanol for the measurement of amino acids and acylcarnitines. Using the residual spots not only saved sample material but also reduced sample preparation time considerably. Quantitative results did not differ in residual spots compared with direct preparation, but the analytical background was considerably less in residual spots, giving higher sensitivity. Quantification of SA was done in separate runs after the regular metabolic screening program on 2 of the routine instruments. This allowed us to use our laboratory’s analytical capacity during a time when it was otherwise not needed.

For calibrators and quality-control samples, EDTA whole blood was fortified with 2, 5, 10, 20, 50, and 100 µmol/L SA, and 25 µL of each calibrator or quality-control sample was spotted on S&S filter cards and subsequently dried at ambient temperature overnight. Samples were then stored at 4 °C. The donor blood had no detectable SA. We calculated the recovery from the calibrator blood spots by repeating the analysis 6 times and comparing the results with those of the respective aqueous solutions. The results are shown in Table 1.

Results for SA were generated in positive ion mode with cone energy set at 20 V, collision voltage at 10 eV, and dwell time at 9.1 s. The injection volume was 30 µL. The analysis was performed in multiple-reaction monitoring mode. Chemicals were of analytical grade: acetonitrile, methanol, and formic acid were from Merck, and SA, 5,7-dioxooctanoic acid (internal standard), and hydrazine hydrate were from Sigma-Aldrich. We measured 2 mass-specific transitions for SA (m/z 155.2→137.1 and 155.2→109.1) and 1 transition for 5,7-dioxooctanoic acid (m/z 169.3→151.2; Fig. 2).

We also measured the amino acids in multiple-reaction monitoring mode. Samples were prepared according to standard methods (6, 7). Briefly, blood spots 3.2 mm in diameter were extracted with 200 µL of methanol containing the deuterated internal standards as described previously (8). After the solvent was evaporated, the amino acids were butylated by incubation with 50 µL of butanol–HCl at 65 °C for 15 min, after which the sample was again dried. The residue was dissolved in acetonitrile–water (80:20 by volume).

During the 16-week study, we prospectively analyzed 61 344 unselected neonatal blood samples. Two affected children (patients 1 and 2) were identified. Two additional samples were analyzed retrospectively, these were

<table>
<thead>
<tr>
<th>Concentration, µmol/L</th>
<th>CV, % Intraassay</th>
<th>Interassay</th>
<th>Mean (SD) recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10</td>
<td>13</td>
<td>80 (15)</td>
</tr>
<tr>
<td>10</td>
<td>6.1</td>
<td>10</td>
<td>75 (9.5)</td>
</tr>
<tr>
<td>50</td>
<td>4.6</td>
<td>5.8</td>
<td>72 (5.6)</td>
</tr>
</tbody>
</table>
original filter-paper cards from patients known to have HT. The samples had been stored at ambient temperature for 7 and 11 months, respectively. We also examined 1 blood sample each from the parents of patient 3.

**Results**

**Analytical Characteristics**

The calibration curve was linear from 1 to 100 μmol/L (Fig. 3) with a correlation coefficient of 0.996 (0.999 for the range 1–50 μmol/L). The lower limit of quantification was 0.5 μmol/L (signal-to-noise ratio, 10:1). Precision increased with higher SA concentrations, and the recovery ranged from 72% to 80% (Table 1). To improve the assay specificity, we used a second mass fragmentation of SA ($m/z$ 155.2→109.1). This fragmentation showed similar precision but lower sensitivity for lower concentrations. The lower limit of detection was 0.1 μmol/L.

**Findings in Screening Samples**

SA concentrations were ≤5 μmol/L in 99.6% of 61,344 blood samples from neonates tested in our routine screening program. Apart from the samples from patients 1 and 2, all results were <10 μmol/L. SA was not significantly correlated with the tyrosine concentration, gestational age, or birth weight. The cutoff of 10 μmol/L produced no false positive results. To date, no false-negative results have been reported yet; that is, we are not aware of any patients, other than the 2 described below, who were born during the study period and subsequently had a diagnosis of HT.

**HT Cases**

The test results for 2 prospectively and 2 retrospectively diagnosed patients are presented in Table 2. For comparison, we calculated means and 99th percentiles for tyrosine, phenylalanine, and methionine for our screening population (Table 3).

SA concentrations in samples from HT patients were significantly higher than those in the reference population. In the samples from both parents of patient 3, who are obligate heterozygotes for a HT mutation, the SA concentrations were <1 μmol/L. In only 1 HT patient did the tyrosine concentration exceed the 99th percentile for the gestational age-matched group, in the other 3 HT patients the tyrosine concentrations were much lower, and methionine did not reach the 99th percentile in the blood spots of any of the patients.

**Patient 1**

Patient 1 is the second child of a nonconsanguineous couple from Albania. The first child and both parents are healthy. The patient was born spontaneously after an uneventful pregnancy of 39 weeks (Table 2). Mother and child were discharged in good condition at 36 h post partum, immediately after blood collection for newborn screening. The SA concentration in that sample was 152 μmol/L. The result was reported when the baby was 110 h old to the pediatrician, who immediately transferred the patient to a pediatric metabolic unit. At that time the boy did not show any clinical symptoms, and alkaline phosphatase, transaminases, coagulation factors, and ammonia were within reference values. However, large amounts of SA and phenolic acids were being excreted with the urine. Therapy with precursor substrate reduction and 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) was initiated on the 7th day of life. To date, his development has been normal (9 weeks of observation time).

Interestingly, the tyrosine concentration in the blood spot was only 54 μmol/L (1st percentile on day 2 of life, 31.5 μmol/L). Sample quality was visually unsuspicious, and other typical metabolites measured in the same run were in the upper ends of the reference intervals: carnit-
tyrosine, 47.8 μmol/L (mean for control group, 23.7 μmol/L); acetylcarnitine, 45.6 μmol/L (mean for control group, 23.7 μmol/L); palmitoylcarnitine, 4.9 μmol/L (mean for control group, 2.6 μmol/L). Because the tyrosine concentration was unusually low for a case of HT, amino acid analysis on fresh spots from the same test card was repeated 4 times; tyrosine results were in the range 52–55 μmol/L. In a repeat sample on the 7th day of life under NTBC treatment (1 mg/kg of body weight per day) the tyrosine concentration had increased to 280 μmol/L.

**Patient 2.** Patient 2 is the second son of second cousins of Kurdish origin. Their first son is healthy. The patient was born 2 weeks before term without complications. Body length and weight were appropriate for gestational age. The early neonatal period was uneventful. Blood for neonatal metabolic screening was obtained at an age of 56 h, and the child was discharged to home. Newborn screening results were reported on the 5th day of life. Liver function tests then were normal. A urine sample tested positive for SA and tyrosine metabolites at day 7, confirming the diagnosis of HT. When the baby was 11 days of age, laboratory tests revealed compromised liver function with abnormal global coagulation but normal ammonia, transaminases, and alkaline phosphatase. Occult blood was identified in his stool. Specific therapy with NTBC was started at the age of 17 days, when precursor substrate reduction was achieved by partial feeding with an amino acid–defined formula devoid of phenylalanine and tyrosine together with his mother’s milk. This treatment led to normalization of blood coagulation tests within 5 days. The patient was discharged in good general condition on day 22.

**Patient 3.** After an uneventful pregnancy, the boy was born at term to consanguineous, healthy parents of Turkish origin. The older brother suffers from HT and is treated with NTBC, whereas his sister does not show any clinical or biochemical signs of tyrosinemia. Because of the family history, the parents had been strongly advised to present future children soon after birth to the outpatient metabolic clinics or at least to send a urine sample for SA analysis. However, the parents failed to do so. The child was admitted to hospital with pulmonary infection at the age of 4 months. The parents complained that he was not thriving. Subsequently, blood and urine samples were analyzed for amino acids and SA, respectively. The blood concentrations of tyrosine, methionine, and several other amino acids were increased, and SA was detectable in urine at a concentration of 50 mmol/mol of creatinine. He had severe coagulopathy, which required treatment with fresh-frozen plasma. Clinically, there were no signs of hepatopathy. After the diagnosis of HT was made, NTBC treatment and protein restriction were started, leading to prompt recovery of liver function.

**Patient 4.** Patient 4 is the third child of second cousins of Turkish origin. The older brothers are healthy. Newborn metabolic screening was performed with normal results at day 2. Her development was unremarkable until the end of the 9th week, when she began bleeding from the mouth and nose during a respiratory infection with a fever of 39.5 °C. On her admission to a children’s hospital, laboratory tests revealed thrombocytopenia and profound synthetic liver failure with severe coagulopathy but transaminases and ammonia concentrations within reference values. The patient was treated symptomatically with repeated supplementation of fresh-frozen plasma. A repeat newborn screening at the age of 57 days revealed increased methionine (392 μmol/L) and tyrosine (375 μmol/L) concentrations and led to the suspicion of HT, which was then confirmed by increased SA concentrations in her urine. Starting at age 59 days, she received NTBC at 1 mg/kg of body weight per day. Supplementation with fresh-frozen plasma could be discontinued after 2 more days, and her liver function improved steadily. The patient was discharged home at the age of 76 days.

### Table 2. Characteristics of patients diagnosed with HT.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at blood collection, days</th>
<th>SA in blood, μmol/L</th>
<th>Tyrosine, μmol/L</th>
<th>Phenylalanine, μmol/L</th>
<th>Methionine, μmol/L</th>
<th>Birth weight, g</th>
<th>Gestational age, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>152</td>
<td>54</td>
<td>48</td>
<td>19</td>
<td>3870</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>271</td>
<td>129</td>
<td>38</td>
<td>20</td>
<td>2625</td>
<td>38</td>
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<tr>
<td>3</td>
<td>4</td>
<td>46</td>
<td>260</td>
<td>68</td>
<td>17</td>
<td>3950</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>169</td>
<td>170</td>
<td>93</td>
<td>23</td>
<td>3400</td>
<td>39</td>
</tr>
</tbody>
</table>

### Table 3. Reference values for amino acids from our screening population.

<table>
<thead>
<tr>
<th>Gestational age, weeks</th>
<th>No. of tests</th>
<th>Tyrosine, μmol/L Mean</th>
<th>99th percentile</th>
<th>Phenylalanine, μmol/L Mean</th>
<th>99th percentile</th>
<th>Methionine, μmol/L Mean</th>
<th>99th percentile</th>
<th>Phe/Tyr ratio Mean</th>
<th>99th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>25–31</td>
<td>505</td>
<td>126</td>
<td>494</td>
<td>93</td>
<td>171</td>
<td>0.98</td>
<td>2.45</td>
<td>24</td>
<td>56</td>
</tr>
<tr>
<td>32–37</td>
<td>5146</td>
<td>124</td>
<td>402</td>
<td>95</td>
<td>158</td>
<td>0.84</td>
<td>1.80</td>
<td>32</td>
<td>46</td>
</tr>
<tr>
<td>≥38</td>
<td>32 792</td>
<td>99</td>
<td>183</td>
<td>84</td>
<td>106</td>
<td>0.91</td>
<td>1.62</td>
<td>27</td>
<td>32</td>
</tr>
</tbody>
</table>
Discussion

HT constitutes an important health problem, particularly in regions with high incidence of the disease, as in parts of Canada (1, 9), India (10), and Tunisia (11). In most Caucasian populations, the incidence is lower, ~1 in 100,000 births, which equals the frequency of biotinidase deficiency. In central Europe, the incidence may be higher in immigrant populations because consanguinity is usually more frequent. The parents of the patients described here originate from Albania, Turkey, and Iraq.

The clinical phenotype of HT is variable (1, 12, 13). It is characterized by progressive liver failure and a high risk of hepatocellular carcinoma, renal tubular disease, and porphyria-like neurologic crises. Onset of the disease may be acute during infancy, with fulminant liver failure possible before the age of 6 months. A subacute form presents with failure to thrive, hepatomegaly, and rickets. Chronic forms have also been described. If left untreated, the disease leads to death within the first 2 years of life in 90% of affected children with the acute and subacute forms of the disease. In cases in which restriction of phenylalanine and tyrosine was the only available therapy, hepatocellular cancer developed and caused death in at least one-third of the patients (14). The authors of one study reported that 90% of the patients studied died within the first 12 years of life (15). Another study found that liver transplantation improved the clinical outcome significantly, but 17% of the patients in that study died during the first 2 years after transplantation (16). Therapeutic options have changed dramatically with the introduction of NTBC. Only 10% of patients do not respond to this drug, and adverse effects of long-term treatment are extremely rare. Since the first trial on patients in 1991, more than 300 cases have been enrolled in an international study (17).

Neonatal screening for HT has been performed in various screening programs for more than 3 decades (1). An American expert group (18) has recently reintroduced HT into the core panel of target diseases of screening programs, using quantification of tyrosine as the basic test. This was done for historical reasons as well as with respect to the considerable improvements in therapy in recent years. In screening programs relying on blood collection on the 2nd or 3rd day of life, as German programs do, the numbers of false positives as well as false negatives are unacceptably high when tyrosine is used as the key diagnostic indicator. In our routine program in which tyrosine concentrations were used to screen for tyrosinemia over several years, among the 1 million babies tested, we correctly suspected HT only twice, based on increased tyrosine concentrations. None of the patients described here were detected with tyrosine as the diagnostic marker (cutoff, 300 μmol/L). There are 5 more patients with HT known to us for whom routine neonatal screening results were normal based on tyrosine concentrations alone.

Before the description of the method used in this study, other methods for direct quantification of SA have been applied to neonatal screening, and no false-negative results were obtained (19–21). These methods, however, are quite time-consuming and are not suitable for mass screening. Indirect quantification of SA based on inhibition of δ-aminolevulinate dehydratase is also possible and has been used. Measurement of the activity of this enzyme, however, can give false-positive results because it is influenced by a variety of factors, such as the presence of EDTA, samples being exposed to high temperatures, lead poisoning, and hereditary deficiency of this enzyme (22). A rather small rate of false positives was observed for fumarylacetoacetase measurements by an ELISA (23).

In our study, quantification of SA in neonatal blood spots by the method of Allard et al. (3) indicated that this test is specific for the presymptomatic detection of HT, and it appears to be sensitive. The diagnostic sensitivity, however, has not been evaluated with follow-up of all 61,344 newborns for HT. Neither second-tier tests nor additional analytes are needed. Heterozygotes present with blood SA concentrations within reference values. The technology used allowed us to operate in a high-throughput setting. The ability to use residual blood spots allows for higher sensitivity as well as for short total analytical times. Adding the method to an existing MS/MS screening program requires little additional manual work; in our laboratory, performance of 800 tests per day added less than one-third of a full-time technician equivalent. In addition, the required reagents are inexpensive.

The use of MS/MS instruments in a separate run would require ~2.25 h for 100 samples, which may mean that additional instruments are necessary to implement the test on all samples. Under some conditions it might be more appropriate to use the method only for samples that are preselected according to tyrosine concentrations and/or additional criteria, such as gestational age or birth weight. Selecting a fraction of samples, however, makes it cumbersome to use residual blood spots. Measuring SA in all samples by MS/MS is not only faster but also safer than selecting samples, as even a low cutoff of 190 μmol/L tyrosine can lead to several false-negative results (1). The tyrosine concentration in patient 1 in our study was only 54 μmol/L, possibly because of the early blood collection. Selecting samples according to ethnicity might be useful in some populations to focus the analytical efforts on those babies with the highest risk of HT, but testing all samples avoids any bias that might be seen in such a procedure. Case-finding expenses in our laboratory have been in the range of the costs for other target diseases such as galactosemia and hypothyroidism.

In conclusion, we believe that use of the method of Allard et al. (3) for testing unselected residual blood spots in an established neonatal screening program fulfills most of the Wilson–Jungner criteria (24) for validity of a screening program. Presymptomatic diagnosis of HT at the age of
several days can prevent liver disease and possibly may lead to a lower rate of hepatocellular carcinoma in patients with HT.

References


