Mass Spectrometric Quantifications Of Organic Acids And Acylcarnitines In Early Random Urine Specimens Of Newborns With Perinatal Complications: Feasibility Study For The Prediction Of The Neuro-developmental Outcome

P Mueller, E Robel-Tillig, D Hueckel, U Ceglarek, C Vogtmann

Citation

Abstract
Aim: The availability of indicators for neurodevelopmental outcome of newborns with perinatal complications is limited. This study advances previous biochemical findings in biological fluids during complicated adaptation of newborns with techniques of mass spectrometry.

Patients and Methods: Reference concentrations of organic acids, free carnitine and acylcarnitines were determined in urinary specimens of 71 newborns. The study cohort included 114 newborns with neonatal complications. For mass spectrometric investigations random urine samples were collected within 72 hours after birth. Data were correlated with the neurodevelopmental outcome at the end of the first year.

Results: A diagnostic sensitivity of 93 % and a diagnostic specificity of 99 % were obtained for the prediction of the neurodevelopment of newborns using the metabolic pattern screening.

Conclusion: Quantitative multiparametric profiling of metabolites in urine specimens of neonates are useful supplements of clinical findings for the prediction of the neurological outcome at the end of the first year of life.

INTRODUCTION
Recent advances in neonatal medicine have resulted in an increase of the survival rate of preterm infants. However, the rate of morbidity has increased, too. For evaluating of new therapeutic strategies in respect to their somatic and neurodevelopmental outcome it is of great importance to have measurable criteria, which describe a relevant posthypoxic metabolic pattern. The Apgar score is a useful tool to assess the state of newborns immediately after birth, but is not well suitable for prediction of the further psychomotoric development [1,3,4,7]. Even if other parameters were included such as lactic acid concentration in blood, pH-value or base excess, a weak validity in identifying infants at risk for morbidity and disability was obtained, because of the poor correlation to the Apgar scores [1]. Other scores did not enhance the results, too [1]. Nevertheless, the postasphyxial hypoxic-ischemic encephalopathy (HIE) with/without microcephaly is of essential prognostic importance [1].

Recently, several reports described the predictive values of several biochemical markers [9,10], of ultrasonographic [11] and neurophysiologic parameters [12] and of cerebral MRI findings [13]. All these publications relied on pathophysiologic changes as precursors of HIE after severe asphyxia. The risk of neurodevelopmental disturbances due to such crucial injuries is in the range of 40 % in extremely preterm infants. Even less severe hypoxic episodes may alter the metabolism of the immature brain and cause cognitive deficits later on [14]. Such metabolic changes are well reflected in urine so that the pattern of urinary metabolites may be an important diagnostic tool for the assessment of neurological outcome. Therefore, our study was aimed to analyze urine of hypoxic newborns to identify biomarkers of
metabolic disturbances and to correlate them with the neurological development. Analysis was performed using gas chromatography mass spectrometry (GC/MS) and electrospray ionisation tandem mass spectrometry (MS/MS). Both diagnostic tools are noninvasive, of high specificity and need small amounts of samples. Therefore, these methods are particularly suitable for metabolic investigations on intensively cared neonates.

PATIENTS AND METHODS
Subjects: The study cohort included 114 preterm and term infants aged 72 hours at maximum where one or more of the following criteria were fulfilled: umbilical artery pH-value ≤ 7.20, 5-minute-Apgar score ≤ 7, respiratory distress syndrome ≥ II., need for mechanical ventilation > 24 hours, therapy with surfactant or catecholamines, inappropriate weight for gestational age, and in cases of preterm infants with birth weights below 1500 g a critical risk index for babies ≥ 3 (CRIB) [11]. Infants with malformations, anuria, genetic, metabolic or renal-tubular diseases were excluded from the study. The comparison group (71 neonates) with a maximum age of 72 hours were grouped from nonselected patients without these criteria mentioned above for inquiring reference concentrations of organic acids and acylcarnitines in urine. Investigations were performed using GC/MS and MS/MS. Predictive values of quantified urinary metabolites for the neurological outcome were tested retrospectively. For that purpose neonatal data of intermediary metabolism were correlated with the results of a neurodevelopmental examination at the end of the first year of life. The clinical follow-up investigation consisted of a neurological assessment using standardized procedures in pediatric practice adapted on Baylay Scales of Infant Development with following modifications [10]: (I) spontaneous motoric activity, (II) active and passive muscle tone, (III) deep-tendon reflexes and postural reflex movements, (IV) social behavior, and (V) hearing and visual abilities. These five characteristics were proved and graded with 0 up to 2 points:

0 points = inconspicuous findings, i.e. neurologic and psychosocial development corresponds with corrected age,
1 point = mild disturbance, i.e. slight or moderate statomotoric and mental disabilities, slight or moderate disturbances of central nervous coordination, muscular hypotonia,
2 points = adverse disturbances, i.e. muscular hypertonicity or spasticity, severe disturbed central nervous coordination, blindness or deafness. In consequence, a score from 0 (minimum) up to 10 (maximum) was possible. This clinical score was used to classify the patients into three groups: favorable outcome (score 0), mild disturbed development (score 1 – 3, i.e. no parameter was graded with two points) and severe disturbed development (score 4 – 10, i.e. all patients with at least one parameter scaled with two points).

All neurological examinations were performed in the hospital out-patient clinic by an independent and qualified specialist during routine consultations of formerly premature infants to follow up their development. This pediatrician was not aware of the mass spectrometric results.

Gas chromatography mass spectrometry: All solvents used for analysis of organic acids after a previously described method were of high purity [12]. All reagents were purchased from SIGMA-ALDRICH (Munich, Germany) with exception of 4-nitrophenole-D₉ (CDN Isotopes, Quebec, Canada). Analysis was performed with a GC Hewlett Packard 5890 in combination with a MS Hewlett Packard 5972 on a narrow bore capillary (fused silica HP-5) with slight modifications as previously described [13]. Concentrations of the following organic acids were automatically generated with target ion orientation and control of corresponding qualifier ions and were subsequently adjusted to urinary creatinine: lactic, glycolic, 3-hydroxybutyric, 2-hydroxyisovaleric, malonic, 3-hydroxyisovaleric, methylmalonic, 4-hydroxybutyric, ethylmalonic, succinic, fumaric, glutaric, malic, glyoxylic, adipic, pyruvic, 2-hydroxyglutaric, 3-hydroxyglutaric, acetoacetic, pelimelic, 3-hydroxy-3-methylglutaric, 3-hydroxyphenylactic, 4-hydroxyphenylacetic, N-acetylaspartic, suberic, acconitic, homovanillic, azelaic, citric, homogentisic, 3-methoxy-4-hydroxymandelic, sebacic, 4-hydroxyphenyllactic, 2-oxoglutaric, indol-3-acetic, 5-hydroxyindolactic, 4-hydroxyphenylpyruvic acids, uracil, thymine, oxoproline, N-acetyltirosine and succinylacetone.

Concentrations of the following organic acids were automatically generated with target ion orientation and control of corresponding qualifier ions and were subsequently adjusted to urinary creatinine: lactic, glycolic, 3-hydroxybutyric, 2-hydroxyisovaleric, malonic, 3-hydroxyisovaleric, methylmalonic, 4-hydroxybutyric, ethylmalonic, succinic, fumaric, glutaric, malic, glyoxylic, adipic, pyruvic, 2-hydroxyglutaric, 3-hydroxyglutaric, acetoacetic, pelimelic, 3-hydroxy-3-methylglutaric, 3-hydroxyphenylactic, 4-hydroxyphenylacetic, N-acetylaspartic, suberic, acconitic, homovanillic, azelaic, citric, homogentisic, 3-methoxy-4-hydroxymandelic, sebacic, 4-hydroxyphenyllactic, 2-oxoglutaric, indol-3-acetic, 5-hydroxyindolactic, 4-hydroxyphenylpyruvic acids, uracil, thymine, oxoproline, N-acetyltirosine and succinylacetone.

Tandem mass spectrometry: The reference standard kit for acylcarnitines (AC) and free carnitine (NSK-B, Cambridge Isotope Laboratories, Inc., Andover, USA) was used as internal standard, containing [2H₉]-carnitine, [2H₆]-acylcarnitine, [2H₅]-propionylcarnitine, [2H₄]-butyrylcarnitine, [2H₃]-isovalerylcarntine, [2H₂]-octanoylcarnitine, [2H₁]-myristoylcarnitine, and [2H₁]-palmitoylcarnitine. Methanol, acetonitrile (gradient grade) and 98% formic acid were purchased from MERCK.
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(Darmstadt, Germany). All other commercially available chemicals were of highest purity. Sample preparation was performed as previously described \([19]\). An API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX) with TurboIonSprayTM interface in combination with a PE 200 Autosampler and a PE series 200 microgradient system was used. 25 µl of the sample were injected directly at a solvent flow rate of 60 µl/min resulting in a run-time of 2 min for each sample. For detection of acylcarnitines the precursor-ion scan of m/z 85 and scanning from m/z 200 - 510 in positive ion mode was used. Quantitative data analysis was performed with ChemoViewTM software (Applied Biosystems/MDS SCIEX) by comparing the signal intensities of the analyte and its corresponding internal standard or the standard next to the spectrum. Concentrations of free carnitine (C0) and AC's (C2 – C18) were related to the creatinine concentration of the urine sample.

Material / urine sampling: Random urine samples were collected during monitoring of the fluid balances of the patients within 72 hours after birth and stored at –20°C until analysis. An informed consent of the parents was obtained before.

Creatinine measurement: Creatinine concentration was determined by automatic analysis system BM/Hitachi 904 according to the method of Jaffé \([20]\).

Data analysis: Probability values were determined using Kruskal-Wallis-test and Mann-Whitney-U-test. All statistical tests were two-sided. Statistical significance was defined as p ≤ 0.05. Test validity was performed as previously described \([12]\). Kappa-index is defined as a degree of conformity between the assessments of two examinators, when both worked on the same subjects independent from the prevalence of the investigated test parameter. Values over 0.80 are defined as very good assessments. A value nearly zero means, that the observed assessment was really by chance \([22]\).

RESULTS

67 patients (58.8 %) of the treated neonates were scored zero and developed appropriate to biological age in the 4th trimenon (i.e. between the 9th and 12th month of life). 28 patients (24.5 %) were classified with mild developmental delay (score 1 – 3, median 1.0). Severe neurodevelopmental impairment (score 4 – 10, median 5.0) was diagnosed in 19 patients (16.7 %). Birth weight, gestational age, umbilical artery pH-value and base deficit were not significantly different between all groups incl. comparison group (Table 1). In order to preselect diagnostic relevant parameters the Kurskal-Wallis test was used to compare each of the organic acids and each of the acylcarnitines between patient groups and comparison group. Differences of high significance were shown for urinary concentrations of specific organic acids and most fractions of acylcarnitines which are summarized then as total AC's. Only those parameters with statistical significance are considered after that (Table 2). With exception of 4-hydroxyphenyllactic acid there were also significant differences in the concentration of organic acids between the groups with mild (score 1 – 3) and severe neurological symptoms (score 4 – 10). Individual data of urinary metabolite excretion are exemplary presented for methylmalonic acid (Figure 1). The median values are higher in patients with adverse outcomes compared to the group with favorable outcome. This observation is based mainly on the percentage of non-excretors of methylmalonic acid in urine. Patients with poor neurological outcome showed a higher total renal excretion of organic acids. However, no significant changes in the profile of organic acids could be observed. Interestingly, lower urinary acylcarnitine concentrations in infants with appropriate outcome and in infants with poor outcome related to the comparison group were found. Furthermore, no significant differences were detectable when urinary concentrations of organic acids and acylcarnitines of the groups “score 0” versus “score 1 – 3” were compared. Thus, for predicting an adverse statomotoric and psychosocial development threshold concentrations were defined as the 90th percentile from patients with “score 1 – 3” and mild neurological symptoms (Table 2).
**Figure 1**
Table 1: Characteristics of the comparison group and patients (n = 114) with the results of neurodevelopmental examinations. Plus A– minus values are means A± SD with exception of the score (median). Data of the comparison group were previously described [1].

<table>
<thead>
<tr>
<th></th>
<th>Comparison group</th>
<th>Normal development</th>
<th>Mild developmental impairment</th>
<th>Severe developmental impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (percent of patient cohort)</td>
<td>71</td>
<td>67 (58.3)</td>
<td>28 (24.5)</td>
<td>19 (16.7)</td>
</tr>
<tr>
<td>Score</td>
<td>4.07</td>
<td>0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Corrected age at examination (months)</td>
<td>9.79 ± 5.45</td>
<td>11.20 ± 7.19</td>
<td>9.82 ± 6.48</td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2065 ± 833</td>
<td>1851 ± 990</td>
<td>1412 ± 851</td>
<td>1618 ± 1213</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>32.8 ± 3.7</td>
<td>32.2 ± 5.8</td>
<td>29.9 ± 6.9</td>
<td>29.7 ± 6.6</td>
</tr>
<tr>
<td>Umbilical artery pH value</td>
<td>7.28 ± 0.06</td>
<td>7.13 ± 0.08</td>
<td>6.97 ± 0.13</td>
<td>6.90 ± 0.14</td>
</tr>
<tr>
<td>Base deficit (mmol/L)</td>
<td>-4.1 ± 3.0</td>
<td>-6.2 ± 4.1</td>
<td>-3.6 ± 4.7</td>
<td>-5.8 ± 7.0</td>
</tr>
</tbody>
</table>

Anot determined

**Figure 2**
Table 2: Creatinine-adjusted concentrations of metabolites in urine (always 90th percentile in mmol/mol creatinine) and statistics of comparison group and patients in relation to the neurological outcome during the 4th trimester. Shaded squares indicate significant differences (p < 0.05) between the groups. Data of the comparison group were previously described [1].

**Figure 3**
Figure 1: Urinary concentration of methylmalonic acid (indexed to creatinine) of healthy infants (comparison group) and asphyxiated infants according to their neurodevelopmental outcome at the 4th trimester of life. The solid lines represent median values.
Score 0 = normal neurologic development
Score 1 – 3 = mild impairment
Score 4 – 10 = severe impairment

The predictive values of various urinary metabolites were determined on the assumption that no perinatal induced adverse neurodevelopmental outcome (score < 4) is expected if the threshold concentration of organic acids and acylcarnitines in urine is not exceeded. Results of test validity are shown in Table 3. Analysis showed sufficient sensitivities and specificities and a positive prediction value > 90% for single parameters. In addition, a qualitative improvement of validity parameters were obtained for the investigation of combined organic acid profiles. However, no improvements of the parameters of test validity were obtained after additional considerations of the total urinary concentration of acylcarnitines.

**Figure 4**

Table 3: Validities of urinary metabolites for prediction of the neurological outcome of preterm and term newborns at the end of the first year of life

<table>
<thead>
<tr>
<th>Parameter or combination of parameters</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Prevalence (%)</th>
<th>Efficacy (%)</th>
<th>Kappa index</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1] Lactate acid</td>
<td>89.0</td>
<td>57.1</td>
<td>85.7</td>
<td>87.7</td>
<td>85.1</td>
<td>0.92</td>
</tr>
<tr>
<td>[2] 3-hydroxyisovaleric acid</td>
<td>83.6</td>
<td>63.2</td>
<td>82.6</td>
<td>83.1</td>
<td>87.7</td>
<td>0.88</td>
</tr>
<tr>
<td>[3] 3-hydroxyisovaleric acid</td>
<td>87.0</td>
<td>42.9</td>
<td>81.6</td>
<td>87.0</td>
<td>81.0</td>
<td>0.91</td>
</tr>
<tr>
<td>[4] Methylmalonic acid</td>
<td>95.1</td>
<td>30.9</td>
<td>90.6</td>
<td>80.6</td>
<td>78.0</td>
<td>0.79</td>
</tr>
<tr>
<td>[5] 4-hydroxyphenyl lactate</td>
<td>85.0</td>
<td>28.6</td>
<td>80.5</td>
<td>81.5</td>
<td>76.1</td>
<td>0.78</td>
</tr>
<tr>
<td>[6] 5-Oxoproline</td>
<td>87.5</td>
<td>66.7</td>
<td>96.8</td>
<td>92.0</td>
<td>85.0</td>
<td>0.92</td>
</tr>
<tr>
<td>[7] Acylcarnitines (total)</td>
<td>82.9</td>
<td>71.4</td>
<td>90.9</td>
<td>91.6</td>
<td>81.9</td>
<td>0.90</td>
</tr>
<tr>
<td>[1]+[2]</td>
<td>92.6</td>
<td>61.3</td>
<td>86.4</td>
<td>87.9</td>
<td>88.8</td>
<td>0.89</td>
</tr>
<tr>
<td>[1]+[3]</td>
<td>93.2</td>
<td>60.0</td>
<td>97.6</td>
<td>94.6</td>
<td>91.4</td>
<td>0.91</td>
</tr>
<tr>
<td>[1]+[2]+[4]</td>
<td>83.0</td>
<td>66.7</td>
<td>88.7</td>
<td>96.6</td>
<td>92.0</td>
<td>0.93</td>
</tr>
<tr>
<td>[1]+[2]+[3]+[4]+[5]</td>
<td>98.6</td>
<td>50.0</td>
<td>86.6</td>
<td>97.5</td>
<td>92.5</td>
<td>0.92</td>
</tr>
<tr>
<td>[1]+[2]+[3]+[4]+[5]+[6]</td>
<td>93.4</td>
<td>100.0</td>
<td>98.6</td>
<td>96.7</td>
<td>93.5</td>
<td>0.93</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Modern mass spectrometric technologies, commonly used in selective and neonatal screening for inborn errors in metabolism were applied in this study. Synopsis of combining results of GC/MS and MS/MS opened a new dimension for comprehensive interpretation of intermediary metabolism in neonates with perinatal complications on the basis of 65 quantitative parameters (42 organic acids, 22 acylcarnitines, free carnitine) and 15 ratios.

Hypoxic-ischemic encephalopathy (HIE) caused by severe asphyxia is of decisive prognostic importance for the later neurological development. It is essential to identify infants at risk for HIE soon after birth if successful neuroprotective therapy is considered. Lactic acid is widely used as an indicator of tissue hypoxia. Investigations of umbilical artery pH-values in own patients demonstrated a general tendency to higher degrees of adverse outcome according to dimensions of acidity (Table 1). Reliable prediction for the development of HIE was demonstrated with metabolite monitoring of the lactic acid/creatinine ratio in urine of asphyxiated newborns. Measurements obtained within the first 6 hours after birth had a sensitivity of 94% and specificity of 100% in cases with HIE where the lactic acid/creatinine ratio exceeded a limit of 0.64. But the usage of proton nuclear magnetic resonance spectroscopy in this study as a highly selective method is very expensive and rarely available. Different characteristics of investigated subjects in both studies may explain lower diagnostic sensitivity and specificity for lactic acid in own data with 89% and 57%, respectively. These deficits in prediction of the neurological development at the age of one year could be equalized with combined assessments of various informative intermediary metabolites in urine. Five parameters of ketone metabolism (3-hydroxybutyrate) and amino acid metabolism (5-oxoproline, 3-hydroxyisovaleric, 4-hydroxyphenylactic and methylmalonic acids) were statistically proved to be of predictive relevance in addition to lactic acid. Altered profiles of urinary organic acids during hypoxic episodes were studied previously despite limitations, caused by the nonspecific source of urinary metabolites. The investigated organic acids may not represent only brain pathology owing to limited transfer of some metabolites across the blood barrier. Nevertheless, moderate increased concentrations of methylmalonic acid in urine as shown in Figure 1 are common findings not only in critical ill neonates. Such transient accumulations are often caused by respiratory induced tissue hypoxias, which influence mitochondrial energy metabolism. It is hypothesized that additional elevated metabolites lead to cumulative effects with the result of disturbed cerebral energy production and subsequently neuronal lesions as our follow-up evaluation study demonstrates in preterm and term born neonates with perinatal complications. According to this presumption the total amounts of excreted organic acids were not significantly different between comparison group and patient groups with diverse outcomes. This points to
qualitative alterations of intermediary metabolism with the consequence of increased potential of brain damage in asphyxiated preterm and term neonates. Generally, a tendency was observed with higher urinary metabolite concentrations in the patient groups than in the comparison group, also depending from the degree of the neurological impaired outcome. It was an unexpected finding that the total amount of urinary acylcarnitines differed not significantly between the comparison group and the patient group with severe neurological defects (score 4 – 10, Table 2). One possible explanation could be that in the comparison group urinary concentrations of various unsaturated and hydroxylated long-chain acylcarnitines are higher in preterm neonates with lower capacity of renal re-absorption after enteral nutrition started. The fraction of short-chain acylcarnitines in urine of critically ill neonates especially of patients with HIE is mainly elevated due to disturbed mitochondrial energy metabolism, mimicking inherited metabolic disorders like ethylmalonic-adipic aciduria (EMA) or short-chain acyl-CoA dehydrogenase deficiency (SCAD) [7]. Nutrition effects could account for higher renal excretions of acylcarnitines within the comparison group than in patients with appropriate outcome (score 0) or mild neurological impairments (score 1 – 3) because in neonates with perinatal complications enteral nutrition starts usually later. Generally, it is recognized but not yet validated that the study of acylcarnitines in urine is more difficult than for organic acids because long-chain acylcarnitines are missed due to reduced water solubility which is causing a diagnostic gap for detecting carnitine-palmitoyltransferase deficiency or very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD).

From the analytical point of view investigations of urine samples are very effective methods because of higher concentrated metabolites compared to plasma and the excellent reflected metabolic conditions over the last hours before urine voiding and sampling. In the present study the concentrations of urinary metabolites were adjusted to the concentration of urinary creatinine. The reliability of the results is strongly dependent on the reliability of creatinine measurement. Internal and external laboratory quality controls showed that the results of creatinine determination obtained for a wide range of concentration are stable during a long period. The coefficient of variation was acceptable and ranged between 7.8 and 10.3 % (data not shown). Referring to creatinine may cause problems especially in premature infants because of the low muscular mass and tubular immaturity of the kidneys in those patients. However, recently published studies have demonstrated that urinary metabolites can be related to creatinine levels with high precision [6]. Collecting urines over a period of hours and the relation to creatinine, to the body weight or to the body area might be more reliable, but the probability of pre-analytical errors is increased due to bacterial contamination during collection time.

Our study presents first results of multiparametric investigations (metabolomics) in newborns with asphyxia and shows that mass spectrometry of urinary metabolites is useful for predicting the neurological and psychosocial outcome. The results suggest that concentrations of organic acids and acylcarnitines within 72 hours after birth are related to the occurrence and the severity of hypoxic-ischemic encephalopathy. Therefore, further studies are necessary using methods with short analysis time and high sample throughput to specify these preliminary findings.

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