analysis (Fig. 1B, lower panel, line 3) confirmed that the result obtained in reaction 3 was a false negative.

In the experiments of this study, the RICO was added to the reverse transcription mixture. It is possible to co-extract the RICO with the sample RNA to control for the extraction procedure. In this case, however, careful validation is necessary: because of the small size of the RICO, its extraction efficiency might be low or variable depending on the method used. We have noticed these effects when using Qiagen columns for RNA extraction.

In summary, we have developed a simple, inexpensive method that allows laboratories to produce their own RNA controls. Sufficient RICO for millions of RT-PCR assays can be produced from a synthetic oligonucleotide in <5 h with little hands-on time. The RICO technique could be applied to many real-time amplification protocols that use a probe format and an instrument that can perform a melting-point analysis after amplification. Because the method does not require a second, control-specific probe, it conserves detection channels on the real-time amplification instrument and reduces reagent costs. Because of some difficulty in titrating the amount of RICO needed for reliable detection of partial inhibition of the reverse transcription step, however, a separate probe should still be used to detect the RICO during amplification in quantitative RT-PCR assays.

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References


Identification of 19 New Metabolites Induced by Abnormal Amino Acid Conjugation in Isovaleric Acidemia, Du Toit Loots,1,2 Erardus Erasmus,2 and Lodewyk J. Mienie2 (1 Department of Nutrition, School of Physiology, Nutrition and Consumers Science, and 2 Department of Biochemistry, School of Chemistry and Biochemistry, North-West University, Potchefstroom, South Africa; * address correspondence to this author at: North-West University, Private Bag X6001, Potchefstroom, 2531, South Africa; fax 27-(0)18-299-2464, e-mail vgedtl@puk.ac.za)

Isovaleric acidemia (IVA) is an autosomal recessive genetic disorder of the enzyme isovaleryl-CoA dehydrogenase, which is involved in leucine metabolism (1). Clinical symptoms include poor feeding, tachypnea, vomiting (2), listlessness, lethargy, coma (2,3), and dehydration (4). Individuals homozygous for this defect are characterized primarily by the excretion of isovalerylglycine (2). The metabolic profile may be further complicated by intermediate metabolites such as 3- and 4-hydroxyisovaleric acid (5), methylsuccinic acid (6), methylfumaric acid (7), isovalerylglucuronic acid (8), isovalerylglutamic acid (9), N-isovalerylalanine and N-isovalerylarscine (10), isovalerylcarnitine (11), 3-hydroxyisohexanoic acid (12), and alloisoleucine (4). Diagnosis of IVA is based on clinical symptoms and the presence of isovalerylglycine and 3-hydroxyisovaleric acid (13), with some of the above-mentioned metabolites also occurring to a greater or lesser extent (4). Despite the large number of already identified excreted metabolites, their occurrence still does not entirely explain all of the clinical symptoms experienced by these patients. The statement made in 1982 by Duran et al. (14) that “[t]he continuing search for ‘new’ metabolites may eventually lead to a better understanding of the relationship between the clinical conditions and their biochemical abnormalities” is the motivation for our search for previously unidentified metabolites in IVA. In this study, we analyzed the urine of IVA patients for the presence of induced amino acid metabolites not previously identified in IVA.

Most of the amino acid conjugates occurring in the urine of patients with IVA have been detected via gas chromatography–mass spectrometry (GC-MS). Because this technique is not suitable for detection of acetyl and isovaleryl conjugates of tryptophan, lysine, histidine, and asparagine because of their polar characteristics, we identified and quantified these metabolites by electrospray tandem mass spectrometry (ESI-MS-MS). Mass spectra (GC-MS) and daughter-ion profiles (ESI-MS-MS) were obtained by analyses of the chemically synthesized calibrators. These spectra were then used to identify the metabolites in the urine of these patients.

Six IVA patients were investigated for the presence of new urinary metabolites. The concentrations of the diagnostic metabolites found in urine samples from these patients are summarized in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/.

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N-Acetyl-amino acid conjugates to be used as calibrators were prepared with the appropriate organic acid chlorides or anhydrides as reported by Hagenfeldt and Naglo (15) and Liebich and Först (16), respectively. N-Isovaleryl-amino acid conjugates were synthesized by adaptation of the same method.

Because of the complexity of the profiles and the coelution of organic acids in metabolic disorders, fractionation of the organic acid extract by thin-layer chromatography before GC-MS analysis was necessary. Silica gel 60 F254 thin-layer chromatography plates (Merck) and a mobile phase consisting of 20 mL of tert-amyl alcohol (Merck Chemicals), 70 mL of chloroform (SARchem), 10 mL of formic acid (Merck), and 5 mL of hexane (BDH) were used for fractionation of the organic acids. A volume of urine equivalent to 26.5 μmol of creatinine was extracted by the above-mentioned standard organic acid extraction procedure. After the organic acid solvent was evaporated, the organic acid extract was resuspended in a small volume of methanol for easy application on the thin-layer chromatography plates. The development time of the thin-layer plates was ~2 h. The plates were allowed to dry, and the bands were visualized under ultraviolet light and marked with a pencil. The choice of fractions varied depending on the complexity of the visible bands, but the number of bands usually ranged from 6 to 11 per plate. The silica gel containing each band was carefully scraped off the plate; the material was then powdered and placed in a column. The organic acids were eluted from the silica gel with methyl acetate and methanol. The eluate was subsequently dried under nitrogen and derivatized with bis(trimethylsilyl)trifluoroacetamide (Sigma) and trimethylchlorosilane (Sigma). GC-MS analysis was conducted on an Agilent 6890 gas chromatograph equipped with an SE 30 capillary column [25 m × 0.33 mm (i.d)] and ported to an Agilent 5973 mass spectrometer. Extraction and derivatization of urinary isovaleryl- and acetyl-amino acid conjugates were performed by the standard organic acid solvent extraction method as described previously (17).

ESI-MS-MS identification of the amino acid conjugates was done by use of a characteristic fragment in the daughter-ion profile and identification of the mother ion of mass characteristic of the pseudomolecular ion (M⁺) of the conjugate of interest, with the characteristic daughter-ion and pseudomolecular ion masses for each compound shown in Table 1. A stock solution of stable-isotope–labeled amino acids was formulated in which the concentrations of the amino acids in the final analysis mixture were 2594.7 μmol/L glycine-d2, 1175.2 μmol/L ring-d5-phenylalanine, 394.2 μmol/L methyl-d4-methionine, 1198.5 μmol/L d5-valine, and 1275.1 μmol/L d15-isoleucine (all synthesized by Dr. Herman J. ten Brink of the Academic Hospital VU, The Netherlands). Samples were prepared by adding 400 μL of the labeled amino acid stock solution to 10 μL of urine or synthesized sample. These were then dried at 70 °C under reduced pressure. Butanolic HCl (3 mol/L) was then added, and the mixture was butylated for 10 min at 70 °C and dried at 70 °C under reduced pressure. After the dried samples were resuspended in 100 μL of 50:50 acetonitrile–water containing 10 mL/L formic acid, they were centrifuged at 16 000g for 10 min to remove any materials blocking the capillary tube. The samples were introduced into the electrospray source at a rate of 10 μL/min. ESI-MS-MS analyses and conditions were carried out as described previously (18).

Formerly unidentified isovaleryl conjugates of the following amino acids were detected and identified in the urine of IVA patients: α-amino butyric acid, aspartic acid, serine, phenylalanine, threonine, valine, leucine, and tyrosine by GC-MS; and N-isovalerylasparagine, -histidine, -lysine, and -tryptophan by ESI-MS-MS. Acetylated conjugates of the following amino acids were also detected: threonine, valine, glycine, β-alanine, α-alanine, and tyrosine by GC-MS and tryptophan by ESI-MS-MS. Both the concentrations and the characteristic mass spectra of all of the above-mentioned conjugates are given in the online Data Supplement (Supplemental Data Table 2 and Supplemental Data Figs. 1–19, respectively). As expected, the concentrations of the acetylated conjugates were higher in the 2 patients with ketosis at the time of urine collection because of the increased acetyl-CoA production that occurs during ketotic episodes.

These results indicate that as a detoxification mechanism the body uses a variety of amino acids to conjugate accumulating isovaleric and acetic acid. The 19 isovaleryl- and acetyl-amino acid conjugate metabolites identified in this study give a more complete biochemical profile of IVA patients, which may lead to better understanding and treatment of this disease. The new mass spectra and the methods used to obtain them also may serve as a useful resource for investigation of other inherited metabolic disorders.

Table 1. Amino acid conjugates and their characteristic ESI-MS-MS daughter-ion fragments and M⁺ masses.

<table>
<thead>
<tr>
<th>Amino acid conjugate</th>
<th>Characteristic daughter ion, m/z</th>
<th>M⁺, amu⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isovalerylasparagine</td>
<td>144</td>
<td>273</td>
</tr>
<tr>
<td>Isovaleryhistidine</td>
<td>212</td>
<td>296</td>
</tr>
<tr>
<td>Isovalerylysine</td>
<td>129</td>
<td>287</td>
</tr>
<tr>
<td>Isovalerytryptophan</td>
<td>243</td>
<td>345</td>
</tr>
<tr>
<td>Acetyltryptophan</td>
<td>201</td>
<td>303</td>
</tr>
</tbody>
</table>

a ESI-MS-MS daughter-ion fragments and M⁺ masses used to identify isovaleryl- and acetyl-amino acid conjugates in the urine of IVA patients.

b amu, atomic mass units.

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Relationship of Plasma Homocysteine with the Severity of Chronic Heart Failure, Markus Herrmann, Ingrid Kindermann, Stephanie Müller, Thomas Georg, Michael Kindermann, Michael Böhm, and Wolfgang Herrmann

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Chronic heart failure (CHF) is a major public health problem causing considerable morbidity and mortality (1–3). Prevention of CHF by identifying risk factors is therefore a major issue. Previous studies found that hypertension, smoking, diabetes mellitus, obesity, and advancing age are the most important risk factors for CHF (4). Recently, plasma homocysteine (Hcy) has been suggested as a newly recognized risk factor (5, 6). However, there are no data regarding the association between Hcy and various objective as well as subjective measures of CHF. The demonstration of such relationships would help to clarify the role of hyperhomocysteinemia in CHF. We hypothesized that plasma Hcy is associated with clinical and echocardiographic signs of CHF as well as with N-terminal pro-brain natriuretic peptide (NT-proBNP), suggesting a relationship between Hcy and the severity of CHF. Accordingly, we investigated the relationships of plasma Hcy with serum NT-proBNP and clinical and echocardiographic indices of CHF in patients and in controls.

For this study, 95 patients with systolic CHF and 12 healthy persons without cardiac diseases were interviewed and examined by the same 2 experienced cardiologists, who were blinded to the study. All participants had a medical history, physical examination, venous blood sampling, 6-min walking test (6-MWT), electrocardiography, and echocardiography. Eighty-two patients underwent a cardiac catheterization according to the American Heart Association guidelines (7). Additionally, 37 patients performed a symptom-limited bicycle exercise test (Ergoline cardio-systems) with gas-exchange analysis (MedGraphics CPX/D spiroergometry system; Medical Graphics Corporation) to determine maximum oxygen uptake ($V_{O2max}$). Informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Board.

Nonfasting venous blood samples (plasma and serum) were drawn during the office visits and centrifuged within 45 min. Total Hcy was measured in EDTA-plasma by an HPLC application (Immundiagnostik) according to the method of Araki and Sato (8). Inter- and intraassay CVs were <5.1%. Serum NT-proBNP and cardiac troponin T were measured with commercial chemiluminescence assays on an Elecsys 2010 analyzer (Roche Diagnostics). Intra- and interassay CVs were <2.7% and <3.2%, respectively, at concentrations ≥175 ng/L.

Anthropometric data are provided as the mean (SD) and were compared by a Student t-test. Because Hcy and NT-proBNP were not normally distributed, we performed a logarithmic transformation before further data exploration. We then performed a Pearson correlation analysis. Hcy and NT-proBNP are influenced by renal function and age; we therefore also calculated a partial correlation controlling for age and creatinine. Calculations were done with the software package SPSS 11.0 (SPSS Inc.).

Most of the patients investigated were classified as New York Heart Association (NYHA) classes II ($n$ = 27) and III ($n$ = 39; Table 1). The mean age increased ($P$ = 0.03) with increasing NYHA class I controls, 44 (10) years; NYHA class I, 51 (16) years; NYHA class II, 53 (11) years; NYHA class III, 55 (12) years; NYHA class IV, 61 (15) years. Weight and height did not differ among the NYHA classes. As expected, physical performance decreased with increasing NYHA classes. The 6-MWT decreased from 1530 (70) m in controls to 26 (82) m in NYHA class IV patients ($P < 0.001$). Additionally, $V_{O2max}$ decreased from 25 (13) mL·min$^{-1}$·(kg body weight)$^{-1}$ in NYHA class I patients to 16 (4) mL·min$^{-1}$·(kg body weight)$^{-1}$ in NYHA class IV patients ($P < 0.001$).