Article

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Mimicking Ketonuria in the Ketogenesis Defect 3-Hydroxy-3-Methylglutaryl-Coenzyme A Lyase Deficiency: An Artefact in the Analysis of Urinary Organic Acids

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Abstract

3-Hydroxy-3-methylglutaryl-coenzyme A lyase (HMGCL, *HMGCL*) deficiency is a rare inborn error of ketogenesis. Even if the ketogenic enzyme is fully disrupted, an elevated signal for the ketone body acetoacetic acid is a frequent observation in the analysis of urinary organic acids, at least if derivatization is performed by methylation. We provide an explanation for this phenomenon and trace it back to degradation of the derivatized 3-hydroxy-3-methylglutaric acid and high temperature of the injector of the gas chromatograph.

Keywords

ketogenesis, ketone body synthesis, leucine degradation, fatty acid metabolism, organic aciduria, enzyme activity, organic acid analysis

Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency (HMGCLD) is a rare inborn error of ketone body synthesis and leucine degradation, caused by mutations in the *3-hydroxy-3-methylglutaryl-coenzyme A lyase* gene. It has first been described by Faull et al[1] in 1976; recently, we have reported the largest series so far of patients with this disease.[2]

3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency patients present with a characteristic pattern of urinary organic acids that is dominated by leucine metabolites. Although HMGCLD impairs ketone body synthesis both from fatty acids and ketogenic amino acids, an elevated signal for the ketone body acetoacetic acid is a frequent observation in a laboratory where the derivatization in organic acid analysis is performed by methylation.[3,4] Although acetoacetate is normally interconverted enzymatically with 3-hydroxy-*n*-butyric acid, no alteration in 3-hydroxy-*n*-butyric acid levels is noted in such cases.

It has been speculated that the acetoacetate may be formed by bacterial degradation of 3-hydroxy-3-methylglutaric acid, but so far no evidence-based explanation of the origin of the acetoacetate has been given.[4] We have now tested the hypothesis that the formation of an acetoacetic acid signal is due to conversion of 3-hydroxy-3-methylglutaric acid during the analysis by gas chromatography-mass spectrometry (GC-MS) that is performed when patterns of urinary organic acids are obtained.

Methods

Most laboratories determine urinary organic acids by extraction from acidified urine followed by trimethylsilylation or methylation and subsequent GC-MS analysis.[5,6] Our methylation approach followed the description by Lehnert,[3] while the setup for trimethylsilylation using silylation agent (BSTFA + TMCS, 99:1, 33148 Supelco; Sigma-Aldrich,

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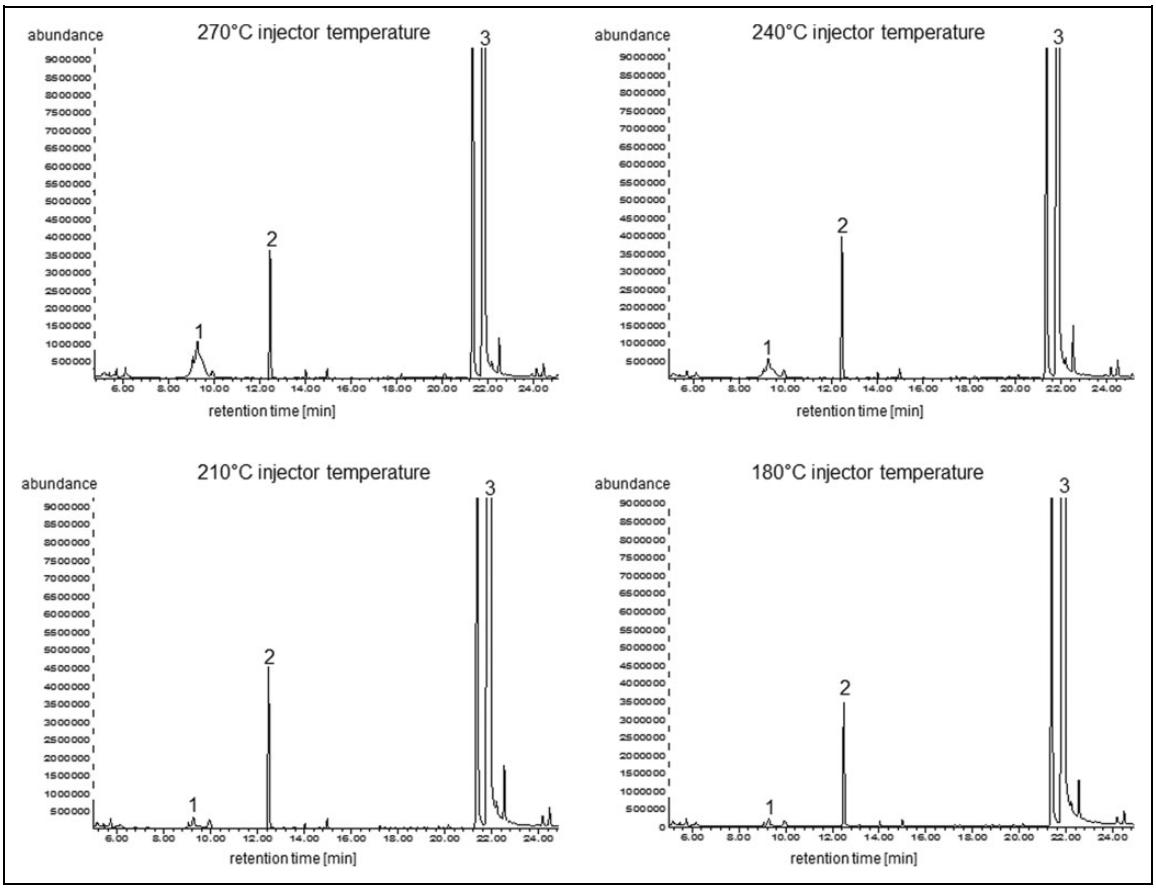

Figure 1. Impact of the injector temperature in GC-MS organic acid analysis (methylation) of 1 mM 3-hydroxy-3-methylglutaric acid. Signal 1 (acetoacetate methylester) increases with the injector temperature. Signal 2: Isopropylmalonic acid dimethylester (internal standard). Signal 3: 3-hydroxy-3-methyl glutaric acid dimethylester.

Taufkirchen, Germany) resembled that of Guneral and Bachmann,[7] although simple ethylacetate extraction was applied instead of solid-phase extraction. Internal standards were 0.04 mmol/L isopropylmalonic acid (for methylation) and 0.44 mmol/L tricarballylic acid (for trimethylsilylation), respectively.

For both types of derivatization, a 7890A gas chromatograph (Agilent) was used which was coupled to a 5975C mass-sensitive detector (Agilent, Waldbronn, Germany). Split injection was performed at a ratio of 1:5. New liners used were of the Ultra Inert Liner type (Agilent 5190-3168). For our experiments, different injector temperatures between 180°C and 270°C were applied.

For analysis after methylation, an FFAP column (25 m × 0.25 mm, 0.2 µm film; CP7717 Agilent) was used and a temperature program was started with 3 minutes constant temperature at 50°C, followed by rapid heating to 70°C. For separation, a gradient of 4°C/min has been used. The GC was then baked out at 260°C for 15 minutes.

For analysis after trimethylsilylation, a CP-Sil 5 CB column (25 m × 0.25 mm, 0.12 µm film; CP7710 Agilent) was used. The temperature program for separation started at initial 70°C and then heated with 4°C/min up to 200°C, followed by a gradient of 8°C/min up to 270°C and then baking out at the same temperature.

In order to avoid memory effects due to incomplete vaporization at low temperatures, we always inserted a blank run with the commonly used injector temperature (270°C) and injection of methanol or the silylation agent between 2 regular analyses.

In order to create stable, reproducible standard conditions and to avoid rapid contamination of the injector of the GC-MS system, aqueous solution of 1 mM 3-hydroxy-3-methylglutaric acid (Sigma H4392; Sigma-Aldrich, Taufkirchen, Germany) was studied instead of patient urine.

Results

Analysis of the methylated sample with the commonly used injector temperature of 270°C yielded a major signal of 3-hydroxy-3-methylglutaric acid dimethylester that was accompanied by a signal of acetoacetate methyl ester as it is known from urine of patients with HMGCLD (Figure 1). However, when the injector temperature was decreased, the signal of the acetoacetate methyl ester became smaller and almost disappeared at 180°C. This finding was reproducible in all 3 experiments performed.

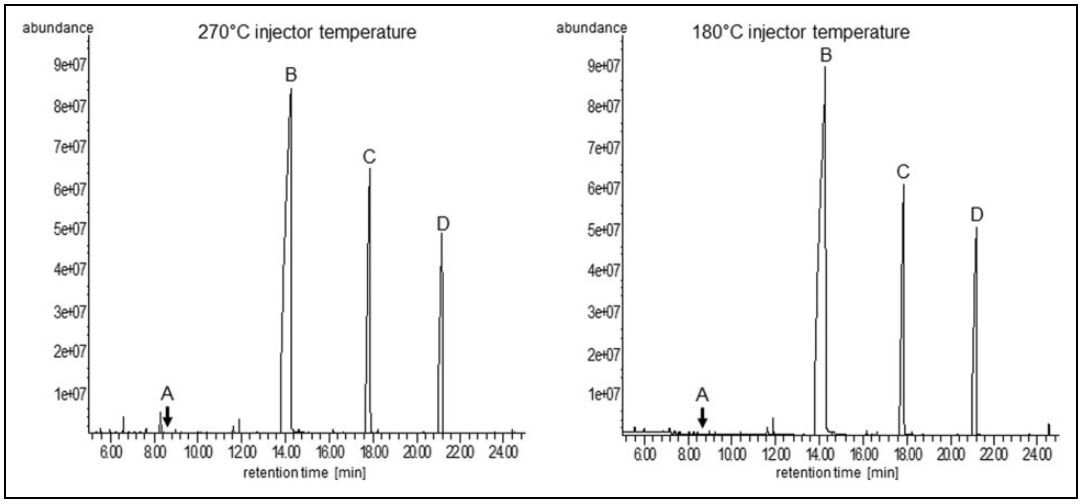

Figure 2. No effect of the injector temperature in GC-MS organic acid analysis (trimethylsilylation) of 1 mM 3-hydroxy-3-methylglutaric acid was noted. No signal was detected at the retention time of derivatized acetoacetate (signal A). Signal B: 3-methylglutaconic acid-bis(trimethylsilyl) ester. Signal C: 3-methyl-3-glutaric [(trimethylsilyl)oxy]-bis(trimethylsilyl) ester. Signal D: tricarballylic acid, tris(trimethylsilyl) ester (internal standard).

However, when a new Ultra Inert liner (Agilent 5190-3168) was inserted into the injector, initially, only a trace amounts of the acetoacetate methyl ester were detected. After just 10 regular injections with methylated urine samples, major formation of acetoacetate methyl ester was observed again. This suggests that the liner is soon activated and then catalyzes the degradation of dimethylated 3-hydroxy-3-methylglutaric acid at high temperatures.

In contrast, no derivatized acetoacetic acid was observed if trimethylsilylation was applied (Figure 2). However, a major signal of 3-methylglutaconic acid-bis(trimethylsilyl) ester was noted in addition to 3-hydroxy-3-methylglutaric acid ([trimethylsilyl]oxy)-bis(trimethylsilyl) ester and even exceeded the peak height and area of the latter. This is well-explained by dehydratation of 3-hydroxy-3-methylglutaric acid in analogy to that of 2-hydroxyglutaric acid.[8] In agreement with this and because the 3-hydroxy-group should have been protected from participation in the dehydration by trimethylsilylation, it is concluded that the dehydration occurs not in the injector, but already when the sample is derivatized. The extent of this conversion revealed no direct dependency on the injector temperature (270°C vs 180°C) or the age of the liner.

Discussion

Ketogenesis, the -mainly hepatic- formation of the ketone bodies acetoacetic acid and 3-hydroxy-n-butyric acid is the main path to the synthesis of ketone bodies[4]. Extrahepatic pseudoketogenesis based on reversal of ketone metabolism has especially been presented as an analytical problem for tracer studies.[9,10] Now, we present the formation of methylated acetoacetic acid as an artifact in the analysis of urinary organic acids. Its formation in the injector of the GC instrument can explain the unexpected signal of a ketone body in the organic acid patterns of patients with the ketogenesis defect HMGCLD. As a decrease of the injector temperature deteriorates the resolution of the chromatogram, one cannot simply reduce the injector temperature. However, now, after the mechanism that underlies the signal of methylated acetoacetic acid has been elucidated, it should not result in irritation any more when metabolite profiles are evaluated.

The production of 3-methylglutaconic acid during the sample preparation by trimethylsilylation might provoke questions regarding the biological origin of this metabolite in HMGCLD in general. However, the formation of this leucine metabolite has not only been observed by GC-MS (also after methylation), and is plausible in view of the pathway, but has also been observed by NMR spectroscopy where dehydratation of 3-hydroxy-3-methylglutaric acid can be excluded.[11,12] Thus, the production of some 3-methylglutaconic acid derivative during the sample preparation is not in contrast to its accumulation in vivo in HMGCLD.

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Declaration of Conflicting Interests

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Correction Notice

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