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SHORT COMMUNICATION

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3-METHYL-3-BUTENOIC ACID: AN ARTEFACT IN THE URINARY METABOLIC PATTERN OF PATIENTS WITH 3-HYDROXY-3-METHYLGLUTARYL-CoA LYASE DEFICIENCY

C. JAKOBS [a,*], M. BOJASCH [a], M. DURAN [b], D. KETTING [b], S.K. WADMAN [b] and D. LEUPOLD [c]

[a] *Department of Pediatrics, Free University of Berlin, Kaiserin Auguste Victoria Haus, Heubnerweg 6, D-1000 Berlin 19, F.R.G.,* [b] *University Children's Hospital 'Het Wilhelmina Kinderziekenhuis', Nieuwe Gracht 137, 3512 LK Utrecht (The Netherlands) and*
[c] *Department of Pediatrics, University of Ulm, Prittwitzstrasse 43, D-7900 Ulm (F.R.G.)*

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Introduction

At present six patients with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, an inborn error of leucine metabolism have been reported [1—4 and C. Bachmann, personal communication]. Characteristic urinary organic acids in this disease are: 3-hydroxy-3-methylglutaric acid, 3-methylglutaconic acid, 3-methylglutaric acid and 3-hydroxyisovaleric acid. Endogenous accumulation of 3-methylcrotonyl-CoA leads to the formation of 3-methylcrotonylglycine [5]. However, urinary 3-methylcrotonic acid may also be formed artificially by decarboxylation of 3-methylglutaconic acid [6]. During the investigation of the decarboxylation of 3-methylglutaconic acid another compound was detected, probably an isomer of 3-methylcrotonic acid.

In this article the identification of this compound as 3-methyl-3-butenoic acid is described. Evidence is presented that 3-methyl-3-butenoic acid is not an endogenous metabolite.

Materials

3-Methylglutaconic acid, 3-methylglutaric acid and 3-hydroxyisovaleric acid were synthesized as described previously [6]. 3-Methyl-3-butenoic acid was prepared by the method of Eggerer and Lynen [7].

3-Methylcrotonic acid, *n*-valeric acid, and 3-hydroxy-3-methylglutaric acid were obtained from Sigma Chemie, Munich, F.R.G. and the TMS-reagent

* To whom correspondence should be addressed.

MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) was from Macherey-Nagel, Düren, F.R.G., *N*-methyl-*N*-nitroso-*N'*-nitroguanidine was obtained from Fluka AG (Buchs, Switzerland).

Methods

Analysis of free volatile fatty acids and their TMS-derivatives

The preparation of the samples from three patients for the analysis of free volatile fatty acids was performed as described previously [8]. However, in addition, TMS-derivatives from the same sample were made. For this purpose the dry ether layer at the end of the procedure was divided into two equal portions. An aliquot of the first portion was directly injected in the gas chromatographic system. To the second portion 50 μl pyridine was added to prevent loss of volatile acids by the next evaporation step of the ether phase. Finally 50 μl MSTFA were added and after 60 min at room temperature an aliquot was injected into the gas chromatographic system for the determination of the TSM-derivatives.

Volatile fatty acids were analyzed on a glass column (6 ft × 2 mm I.D.) packed with 5% DEGS-PS on Supelcoport, 100—120 mesh, (Supelco Inc., Bellafonte, PA, U.S.A.). The injection temperature was 180°C, detector temperature was 200°C and the column temperature was 100°C isothermal. Nitrogen-flow was 30 ml/min, hydrogen-flow 40 ml/min and air-flow 400 ml/min.

For the analyses of the TMS-derivatives a glass column packed with 3% OV-17 on Varaport, 100—120 mesh (Varian, Darmstadt, F.R.G.) (12 ft × 2 mm I.D.) was used. The injection temperature was 250°C, detector temperature was 250°C and the column temperature was programmed from 70°C (5 min isothermal) with a rate of 4°C/min. Nitrogen-flow was 35 ml/min, hydrogen-flow was 35 ml/min and the air-flow was 300 ml/min.

For combined gas chromatography-mass spectrometry we used a double focusing mass spectrometer (Type 311 A, Varian-MAT, Bremen, F.R.G.), which was normally operated with an ionisation energy of 70 eV. The gas chromatograph was equipped with the above-mentioned packed columns. Helium was the carrier gas.

Analysis of methyl esters

For the analysis of 3-methylcrotonylglycine the urine was extracted with ethyl acetate. The dry extract was methylated with diazomethane, which was generated from *N*-methyl-*N*-nitroso-*N'*-nitroguanidine. The methyl esters were separated on a column filled with 3.8% SE-30 on Chromosorb W AW-DMCS 80—100 mesh. The column temperature was programmed from 100°C to 220°C at a rate of 4°C/min. Identification of 3-methylcrotonylglycine was done by gas chromatography-mass spectrometry and selected-ion monitoring (monitoring of fragments m/z = 55, 82 and 83). Mass spectrometric conditions were as described previously [6].

Results and discussion

A typical gas chromatogram of urinary free volatile fatty acids in patients with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency is shown in Fig. 1A. The

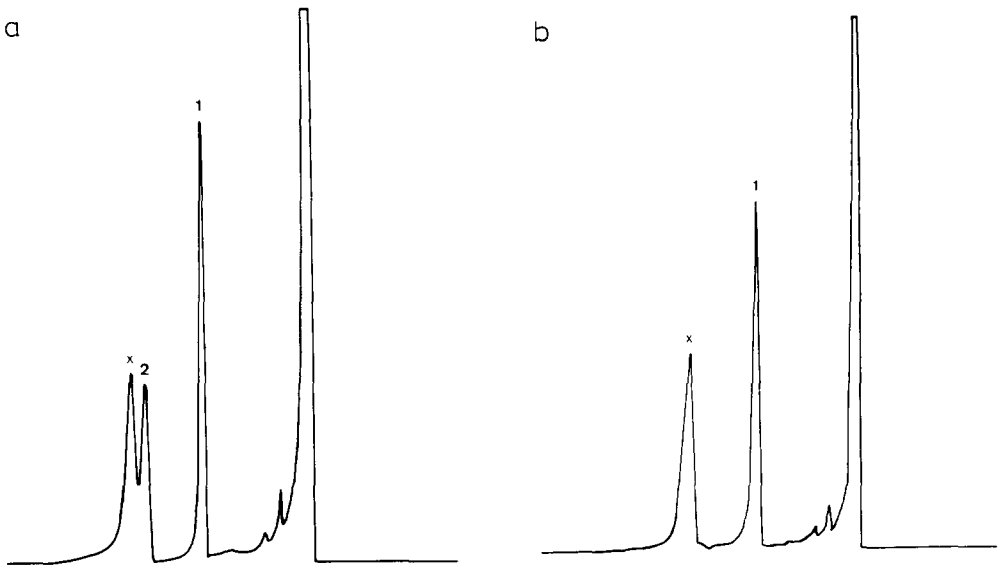

Fig. 1. Gas chromatograms of free volatile fatty acids. (a) injection of urine sample of a patient with 3-hydroxy-3-methyl-glutaryl-CoA lyase deficiency; (b) injection of authentic 3-methyl-3-butenoic acid. Column: 5% DEGS-PS (6 ft X 2 mm I.D.). Peaks: 1 = *n*-valeric acid (internal standard), 2 = 3-methylcrotonic acid, X = 3-methyl-3-butenoic acid.

first peak of the doublet had the same retention time and mass spectrum as 3-methylcrotonic acid (3-methyl-2-butenoic acid). The mass spectrum of the second peak (X) showed some similarities to that of 3-methylcrotonic acid; the compound was thought to be a structural isomer. Subsequently, 3-methyl-3-butenoic acid was synthesized; it appeared to have the same mass spectrum as the unknown compound (Fig. 2). Furthermore, the retention time of X was shown to be identical to that of 3-methyl-3-butenoic acid (Fig. 1B).

In order to establish the origin of 3-methyl-3-butenoic acid a sample of 3-methylglutaconic acid was carried through the whole procedure. The same peak combination as in the patients' urine was observed, proving the artificial formation of 3-methylcrotonic acid and 3-methyl-3-butenoic acid. In order to locate the site of decarboxylation of 3-methylglutaconic acid, the volatile fatty acid extract was trimethylsilylated and analyzed on an OV-17 column. No peak with the retention time and mass spectrum identical to that of silylated 3-methylcrotonic acid and/or 3-methyl-3-butenoic acid emerged after injection of the TMS-derivative of authentic 3-methylglutaconic acid. Moreover, by this method no free 3-methylcrotonic acid and 3-methyl-3-butenoic acid could be detected in the urines of the patients.

Analysis of trimethylsilyl organic acids in the urines of the patients after extraction with either ethyl acetate or diethyl ether failed to detect 3-methylcrotonylglycine. However, following methylation of the organic acid extract a positive identification of this conjugate could be made by means of mass fragmentography. The mass spectrum of methylated 3-methylcrotonylglycine showed intense peaks at m/z = 55, 82 and 83 [5]. These fragments were moni-

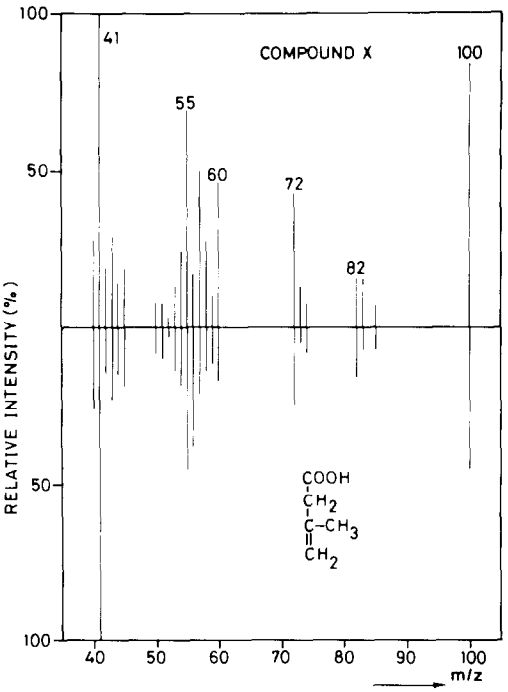

Fig. 2. Mass spectra of compound X in the urines of the patients and of authentic 3-methyl-3-butenoic acid.

tored in urine extracts from three patients. No interfering substances appeared to be present. Like Wysocki et al. [5] we have demonstrated that 3-methylcrotonyl-glycine occurs in 3-hydroxy-3-methylglutaryl-COA lyase deficiency. This implies that some accumulation of 3-methylcrotonyl-CoA must take place in this condition. Hence, urinary 3-methylcrotonic acid is not necessarily formed by decomposition only. On the other hand 3-methyl-3-butenoic acid is not likely to be formed endogenously. Using the mass fragmentography technique we were unable to find indications of the presence of 3-methyl-3-butenoylglycine.

It has been demonstrated that the sample preparation in itself does not lead to artefact formation. Thus, the injection port of the gas chromatograph with its high temperature is to be considered the most likely site for this process to take place.

Unfortunately, the formation of artefacts is a frequently occurring pitfall in the estimation of metabolic profiles, which should always be taken into account. Tanaka [9] mentioned the finding of isovaleric acid in blood and urine of a patient with maple syrup urine disease resulting from decarboxylation of 2-ketoisocaproic acid in his analytical procedure.

The spontaneous decarboxylation of 2-methylacetoacetic acid has been reported by Gompertz et al. [10]. Duran et al. [11] described the artificial formation of propionic acid from methylmalonic acid, which they presumed to take place in the injection port of the gas chromatograph. Frenkel and Kitchens

[12] made use of this phenomenon for the determination of propionic acid in the presence of methylmalonic acid. They lowered the injection port temperature to 130°C, at which temperature spontaneous decarboxylation does not take place.

The artefact described in this study is a further link in a chain which is, in spite of the examples given above, certainly not complete and will most probably grow in the future. Still, identification of all unknown metabolites in patients with metabolic disorders will remain necessary in order to find clues to the pathogenic mechanisms in these diseases.

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