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N-METHYLATION AND N,N-DIMETHYLATION OF AMINO ACIDS

AN ARTIFACT PRODUCTION IN THE ANALYSIS OF ORGANIC ACIDS USING DIAZOMETHANE AS DERIVATIZING AGENT

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SUMMARY

In the fractions of the methyl esters of urinary organic acids seventeen N-methylated or N,N-dimethylated amino acid methyl esters are identified by gas chromatography—mass spectrometry. It is shown for twelve amino acids that their amino group reacts with diazomethane to form these derivatives. Using deuterated reagents, in particular deuterated diazomethane, in the sample preparation procedure during the organic acid analysis, it is shown that the N-methylated and N,N-dimethylated amino acids are artifacts from diazomethane and are not biochemical N-methylation products.

INTRODUCTION

Diazomethane is frequently used as derivatizing agent in the gas chromatographic (GC) and gas chromatographic—mass spectrometric (GC—MS) analysis of organic acids. Its general applicability to various classes of the acids has been demonstrated by several authors [1—6]. Methylation with diazomethane is easy to perform, the resulting derivatives are stable and their MS fragmentation is usually informative and interpretable. Since diazomethane reacts with acidic hydrogen, carboxylic groups and phenolic —OH groups are methylated. In addition, other kinds of reactions can occur with diazomethane. It may react with carbonyl compounds and olefinic double bonds. Methylation of alcoholic hydroxyl groups, such as in hydroxydicarboxylic acids, with diazomethane has also been observed. This has to be taken into account when the substance is used as derivatizing agent.

The reaction of diazomethane with the amino group of amino acids is not necessarily expected, because the hydrogens at the nitrogen atom are not acidic. However, during our analyses of organic acids a series of substances was

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found in the acid fractions, whose mass spectra led to the class of N-methyl- and N,N-dimethyl-substituted amino acids. Some examples of this group of components have been described previously [4, 7]. This paper deals with a systematic study of the formation of N-methylated and N,N-dimethylated amino acids with diazomethane.

EXPERIMENTAL

Materials

The following chemicals were used: alanine, valine, leucine, isoleucine, threonine, methionine, cysteine, tyrosine, tryptophan, aspartic acid and glutamic acid from E. Merck (Darmstadt, F.R.G.), phenylalanine, Diazald® (N-methyl-N-nitroso-*p*-toluenesulphonamide), carbitol-d_1 [2-(2-ethoxyethoxy)-ethanol-d_1], a 30% solution of sodium deuterooxide in ^2H_2O, methanol-d_1, and ^2H_2O from EGA-Chemie (Steinheim, F.R.G.), and a 95% solution of formic acid-d_2 from A. Hempel (Düsseldorf, F.R.G.).

Samples

Urine samples were collected (24 h) from healthy individuals and analysed directly after the collection period.

Analysis of organic acids in urine

The sample preparation and the GC and GC—MS investigations were performed according to our previously described procedure [8]. The reference compounds were analysed according to the same GC and GC—MS conditions as the urinary components.

Synthesis of the methyl esters of N,N-dimethylalanine and N,N-dimethylphenylalanine

The selective methylation of the amino groups of alanine and phenylalanine was carried out as described by Bowman and Stroud [9]. The obtained N,N-dimethylamino acids were transformed into their methyl esters by the following procedure: 1 mg of each of the amino acid derivatives was reacted in srew-capped vials (Macherey-Nagel, Düren, F.R.G.) with 1 ml of a solution of 10% acetyl chloride in methanol for 10 min at 110°C.

Reaction of amino acids with diazomethane

One milligram of each amino acid was separately mixed with 5 ml of methanol, and the mixture was warmed up to facilitate dissolving of the amino acid. An ethereal solution of diazomethane prepared from N-nitroso-N-methylurea, was added until the N_2- development stopped and the yellow colour of the reaction mixture persisted. The reaction mixture was allowed to stand overnight (12 h) before it was concentrated under a stream of nitrogen.

Reaction of the urinary organic acids with deuterated diazomethane

The regular sample preparation procedure was used for the urinary acids except that the following changes were made: after the application of two equal portions of urine to the two anion-exchange columns, each column was

washed with 100 ml of isopropanol—^2H_2O (2:1) and subsequently with 100 ml of methanol-d_1. The acidic components were eluted with 200 ml of a solution of 4% formic acid-d_2 in methanol-d_1. The methylation was performed with deuterated diazomethane, which was prepared by the procedure of Campbell [10].

RESULTS AND DISCUSSION

Occurrence of N-methylated and N,N-dimethylated amino acids in the analysis of organic acids

In the GC—MS analyses of the organic acids a series of mass spectra was obseved in the fractions 2b and 3a—3d which suggested the presence of N-methylated and N,N-dimethylated amino acids. The substances occurred in low amounts, but were regularly found. Figs. 1—5 show the organic acid fractions in which the discussed components are labelled according to their GC—MS identifications. Derivatives of twelve amino acids are detected, the N-N-

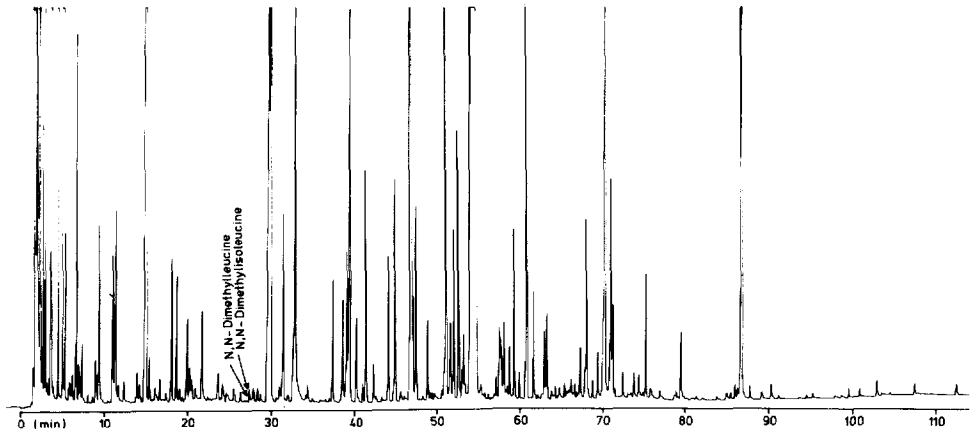

Fig. 1. Gas chromatogram of fraction 2b of the methyl esters of the organic acids in urine of a healthy individual.

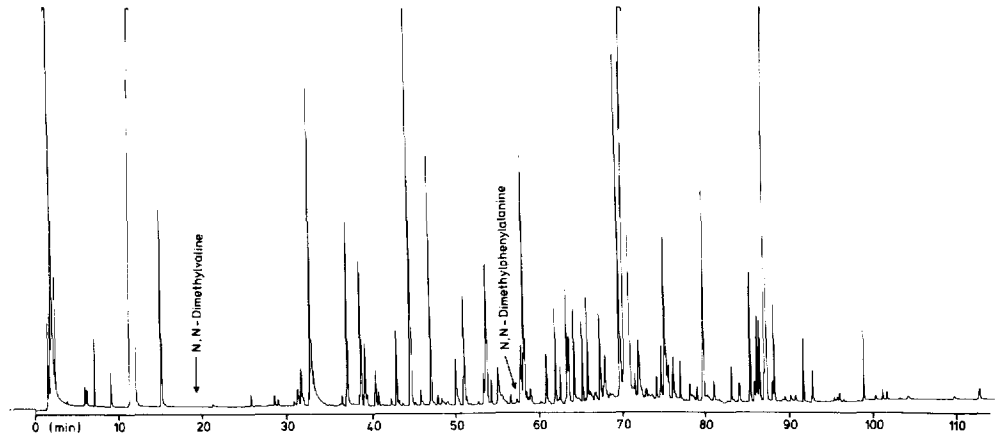

Fig. 2. Gas chromatogram of fraction 3a.

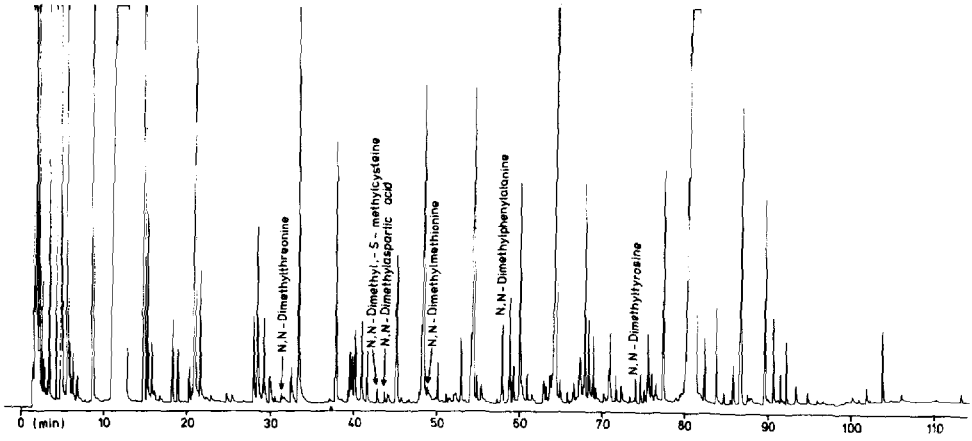

Fig. 3. Gas chromatogram of fraction 3b.

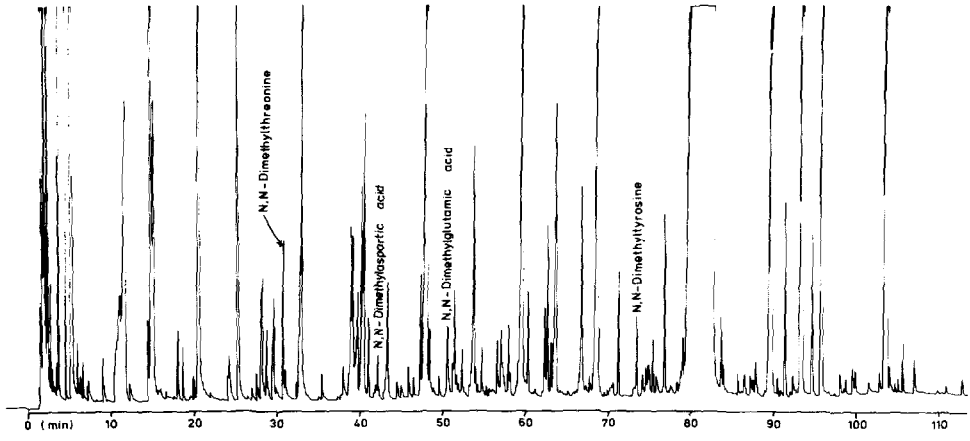

Fig. 4. Gas chromatogram of fraction 3c.

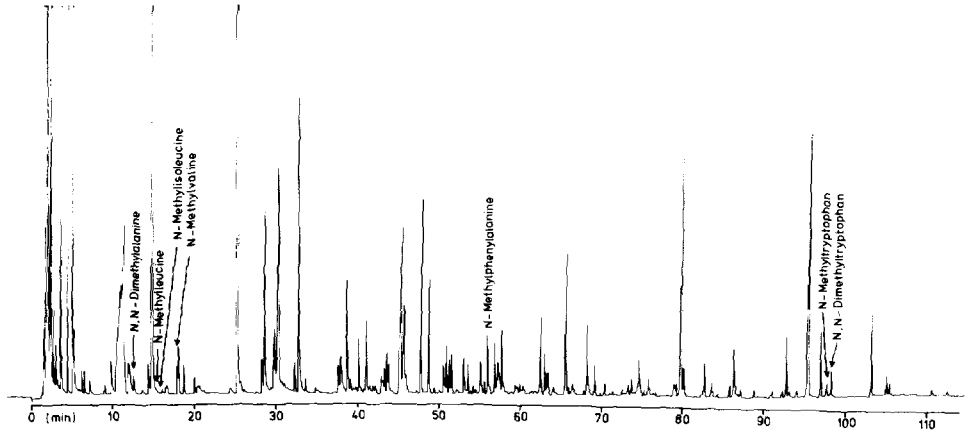

Fig. 5. Gas chromatogram of fraction 3d.

dimethylated products being found in all of the cases, the monomethylated substances only from five amino acids. Altogether seventeen peaks in the chromatograms of the organic acids correspond to derivatives of amino acids.

Synthesis of the methyl esters of N,N-dimethylated alanine and phenylalanine

To prove the assumption that the seventeen observed components are indeed methyl derivatives of amino acids, alanine and phenylalanine, as examples, were transformed into N,N-dimethylated amino acid methyl esters without using diazomethane. The reaction of the amino group with formaldehyde, hydrogen and palladium on activated charcoal [9] selectively forms the dimethylated product. To avoid diazomethane in the entire synthesis, the carboxyl group was methylated with methanol—acetyl chloride. The GC behaviour and the MS fragmentation of the synthetic substances were identical with those of the derivatives in the organic acid profiles.

Reaction of amino acids with diazomethane

The reaction of amino acids with diazomethane was assumed to be the source of the proposed amino acid derivatives in the acid profiles. To test their reaction behaviour, reference substances of the twelve amino acids expected from the mass spectra to occur in the organic acid fractions were reacted with diazomethane.

As demonstrated for leucine in Fig. 6, the amino acid is partially transformed into the N-methylated and the N,N-dimethylated substances. The sequence of the retention times of amino acid (A), N-methylated product (N) and N,N-dimethylated product (NN) varies for the different amino acids. For alanine, cysteine and aspartic acid the order A—N—NN is observed, for valine, leucine, isoleucine and phenylalanine the sequence is N—A—NN, and for

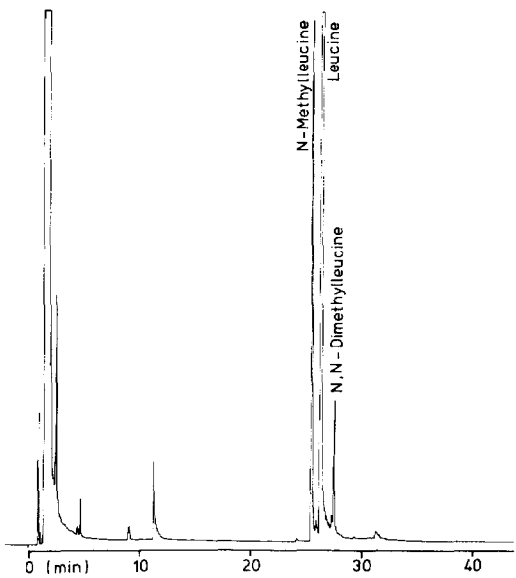

Fig. 6. Gas chromatogram of the products of the reaction between leucine and diazomethane.

threonine, methionine, tyrosine and tryptophan N—NN—A. In the experiment with the reference amino acids the amount of the N-methylated amino acid formed is larger than that of the N,N-dimethylated derivative. Other ratios are observed in the organic acid profiles. The N-methylated derivatives, which are the more polar substances, are found in lesser amounts than the N,N-dimethylated products or are not detected at all. Presumably they are not effectively extracted from the silica gel after the TLC pre-fractionation.

Mass spectrometric fragmentation

The mass spectrometric fragmentations of the N-methylated and N,N-dimethylated amino acid methyl esters are characterized by the loss of the carbomethoxy group and by the ion *m/e* 102 from the monomethylated substances and the ion *m/e* 116 from the dimethylated compounds (Fig. 7). These fragments usually occur with high abundance or as base peaks. The molecular peaks are of low intensity, in some cases not distinguishable. In Table I the fragments and their intensities of 23 reaction products of amino acids with diazomethane are summarized. The seventeen substances found in the fractions of the organic acids had the same mass spectra and showed the same

Fig. 7. Mass spectrometric fragmentation of N-methylated and N,N-dimethylated amino acid methyl esters.

TABLE I

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF N-METHYLATED AND N,N-DIMETHYLATED AMINO ACIDS

Substance	MW**	Fragments (intensities)
N-Methylalanine	117	117(9), 102(6), 70(2), 59(22), 58(100), 56(50), 42(38)
N,N-Dimethylalanine*	131	131(16), 116(5), 73(23), 72(100), 70(22), 56(31), 42(45)
N-Methylvaline*	145	102(67), 87(10), 86(100), 71(16), 70(10), 56(13), 42(28)
N,N-Dimethylvaline*	159	159(1), 116(50), 100(100), 85(18), 84(15), 70(10), 56(19), 42(38)
N-Methylleucine*	159	102(22), 101(9), 100(100), 84(2), 70(2), 58(41), 42(20)
N,N-Dimethylleucine*	173	173(3), 116(16), 115(13), 114(100), 98(2), 58(10), 42(7)
N-Methylisoleucine*	159	102(76), 100(90), 84(3), 70(16), 69(18), 42(100)
N,N-Dimethylisoleucine*	173	173(1), 116(80), 115(12), 114(100), 85(15), 70(7)
N-Methylthreonine	147	147(3), 132(6), 103(69), 102(100), 97(8), 88(97), 70(50), 42(88)
N,N-Dimethylthreonine*	161	146(4), 117(32),116(100), 102(50), 84(15), 58(57), 56(17)
N-Methyl-S-methylcysteine	163	163(2), 104(76), 102(100), 89(9), 70(14), 57(42), 56(19), 42(70)
N,N-Dimethyl-S-methylcysteine*	177	177(2), 118(35), 116(100), 103(3), 98(1), 84(10), 71(57), 70(19), 56(49)
N-Methylmethionine	177	177(3), 130(5), 129(10), 118(78), 102(27), 70(86), 61(100)
N,N-Dimethylmethionine*	191	191(9), 133(15), 132(100), 116(22), 84(90), 70(21), 61(81)
N-Methylphenylalanine*	193	191(1), 134(37), 102(100), 91(10), 77(8), 65(6), 63(2)
N,N-Dimethylphenylalanine*	207	148(32), 116(100), 91(11), 84(4), 77(9), 65(4), 63(2), 56(11)
N-Methyltyrosine	223	223(0.5), 164(15), 122(25), 121(44), 102(100), 91(7), 77(9), 65(6)
N,N-Dimethyltyrosine*	237	178(15), 163(4), 121(9), 117(10), 116(100), 91(5), 77(4), 65(3)
N-Methyltryptophan*	232	232(2), 173(4), 130(100), 116(1), 102(16), 77(9), 63(1)
N,N-Dimethyltryptophan*	246	246(4), 216(1), 187(14), 131(8), 130(30), 116(100), 101(1), 77(5), 63(1)
N-Methylaspartic acid	175	143(2), 117(9), 116(100), 102(40), 85(5), 84(54), 59(11)
N,N-Dimethylaspartic acid*	189	189(2), 131(9), 130(100), 116(28), 98(30), 88(20), 71(12), 56(9)
N,N-Dimethylglutamic acid*	203	203(2), 172(8), 145(10), 144(100), 116(8), 85(9), 84(69), 70(8), 56(6)

*Substance was found in the organic acid fractions.
**Molecular weight.

GC retention behaviour as the corresponding products from the reference amino acids. The spectra of the methyl esters of N-methylleucine, N,N-dimethyltyrosine, N,N-dimethyltryptophan [4], N,N-dimethylphenylalanine, N-methylglutamic acid [7] and N,N-dimethylglutamic acid [7, 11] have been previously published.

As is to be expected, the phenolic —OH group in the two tyrosine reaction products is methylated. The —SH group in the cysteine derivatives is transformed into $-SCH_3$ by diazomethane as well.

Proof for artifact production

The question of whether the N-methylated and N,N-dimethylated amino acids identified in the course of the analysis of organic acids are exclusively artifacts or in part biochemical N-methylation products of amino acids, was answered by the experiment with deuterated substances. The mass spectra of N-methylvaline methyl ester in a urinary acid fraction treated with regular reagents (Fig. 8) and of the corresponding product in a urinary acid fraction reacted with deuterated reagents, in particular deuterated diazomethane (Fig. 9), exemplify the findings. By GC—MS scanning of the N-methylvaline peak within the chromatogram of the organic acids treated with deuterated reagents, the characteristic fragment *m/e* 102 is not seen at all. Instead, a shift mainly to *m/e* 108 is observed. This shift by six masses originates from $-C^2H_3$ in the carbomethoxy group and $-C^2H_3$ (or $-CH^2H_2$ plus ^2H) at the nitrogen. The accompanying fragments *m/e* 107 and *m/e* 109 in Fig. 9 demonstrate that the exchange of deuterium for hydrogen is not uniform at the $-NH_2$ group. For the second characteristic fragment of N-methylvaline, i.e. $M-COOCH_3$, a shift is observed from *m/e* 86 (Fig. 8) to *m/e* 89 (Fig. 9). From these observations it can be concluded, that N-methylvaline was not originally present in the urine. It is an artifact from diazomethane and not of biochemical origin. The same result was found in the deuterium experiment for other N-methylated and N,N-dimethylated amino acids.

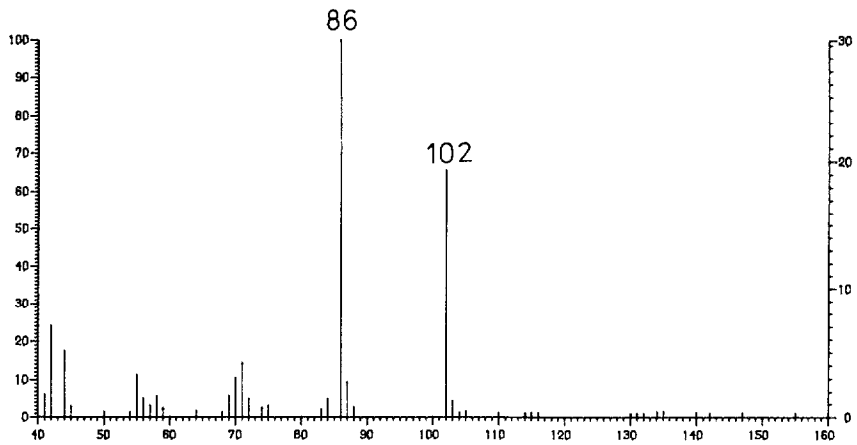

Fig. 8. Mass spectrum of N-methylvaline detected in fraction 3d of the organic acid methyl esters prepared with regular reagents.

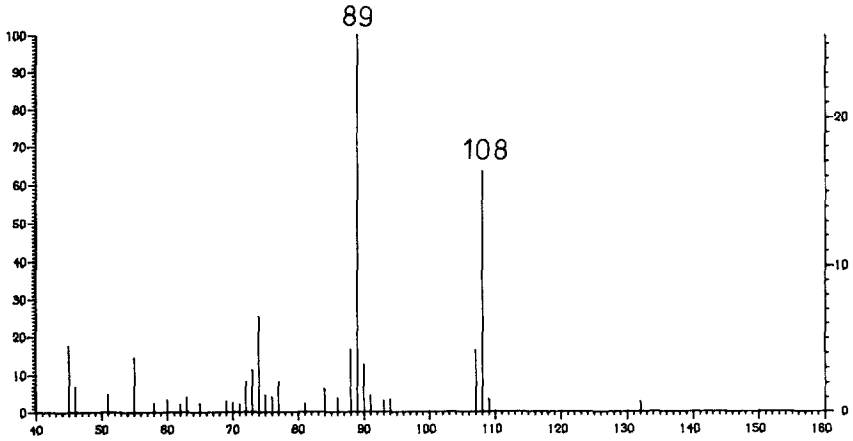

Fig. 9. Mass spectrum of N-methylvaline detected in fraction 3d of the organic acid methyl esters prepared with deuterated reagents.

The high reactivity of diazomethane, which is one of the reasons for its use as derivatizing agent in organic acid analysis, is at the same time its limitation. The investigator has to be aware, especially when he analyses lower-concentrated constituents, that a number of substances, such as the described amino acid derivatives, are artifacts from the procedure.

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