Journal of Chromatography, 414 (1987) 399–404
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3462

Note

Identification of N-acetyl-S-(N-methylcarbamoyl)cysteine, a human metabolite of N,N-dimethylformamide and N-methylformamide

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(First received June 3rd, 1986; revised manuscript received October 9th, 1986)

The biotransformation of N,N-dimethylformamide (DMF) in animals and man proceeds via enzymatic oxidation to give stable carbinolamines, e.g., N-methyl-N-hydroxymethylformamide (DMF-OH) and N-hydroxymethylformamide (MF-OH), which are excreted in urine [1–3]. In gas chromatographic (GC) analysis, DMF-OH and MF-OH decompose to N-methylformamide (MF) and formamide (FA), respectively, the latter compounds being detected as volatile products [4]. In addition, a small amount of MF, present in rat urine, does not result from spontaneous decomposition of DMF-OH, but is formed in an alternative metabolic path [2]. Recently, methylamine has been found by GC as a metabolite of MF in mice [5].

Exposure of the organism or cell cultures to DMF or MF is known to induce changes in the level of glutathione (GSH) [2,6,7]. Furthermore, enhanced excretion of unidentified urinary thioethers has been observed in mice [8], rats [2] and man [9]. This paper reports on the identification of a conjugate of DMF and MF with GSH and its GC analysis through a volatile solvolytic product.

0378-4347/87/$03.50

EXPERIMENTAL

Chemicals

DMF and the ion exchanger (Ionenaustauscher III, 0.35–0.60 mm) were obtained from Merck (Darmstadt, F.R.G.). Silufol thin-layer chromatography (TLC) plates and silica gel were obtained from Kavalier (Votice, Czechoslovakia). N-Acetylcysteine was purchased from Fluka (Buchs, Switzerland). N-Methyl isocyanate was prepared from potassium cyanate and dimethyl sulphate [10], N-acetylcysteine methyl ester by esterification of N-acetylcysteine with diazomethane and diazomethane from N-nitroso-N-methyl-*p*-toluenesulphonamide.

Spectral methods

Mass spectra were obtained on a Jeol D-100 mass spectrometric (MS) system at 75 eV. The samples were introduced via a direct probe inlet at 95°C or in a GC–MS coupling column (SE-30, 3% on Chromosorb W, 2 m×3 mm I.D., temperature programmed from 30 to 170°C at 5°C/min). ^{1}H and ^{13}C NMR spectra were measured on a Varian XL-200 spectrometer (200.058 MHz for ^{1}H and 50.309 MHz for ^{13}C, Fourier transform mode). The chemical shifts are expressed in δ (ppm) with reference to tetramethylsilane.

DMF exposure

A 25-year-old male volunteer inhaled vapour over a liquid in a beaker for 6 h. MF was inhaled over a warm (40°C) liquid to increase the vapour pressure. Urine samples were collected for seven days and stored at 0°C until taken for analysis. The total concentration of thioethers in urine was determined spectrophotometrically [9,11].

GC analysis of ethyl N-methylcarbamate (1, Fig. 1)

A urine sample (1 ml) was mixed with ethanol (2 ml) in a glass-stoppered tube. Powdered anhydrous potassium carbonate (1.5 g) was added and the mixture was shaken for 2 min. After centrifugation at 500 *g* for 2 min, 1 μl of the organic layer was sampled and analysed by GC. The analyses were carried out using a Chrom 5 gas chromatograph (Laboratorní přístroje, Prague, Czechoslovakia) equipped with a nitrogen-selective detector and a glass column (240 cm×3 mm I.D., packed with 5% potassium hydroxide and 10% Carbowax 20M on silanized Chromosorb W, 80–100 mesh). Nitrogen was employed as the carrier gas at a flow-rate of 50 ml/min. Hydrogen and air were supplied to the detector at 16 and 400 ml/min, respectively. The temperatures of the injector, column and detector were 230, 170 and 290°C, respectively.

Isolation of the metabolite

Human urine collected after exposure to DMF or MF was saturated with ammonium sulphate (100 g per 100 ml of urine) and extracted twice with an equal

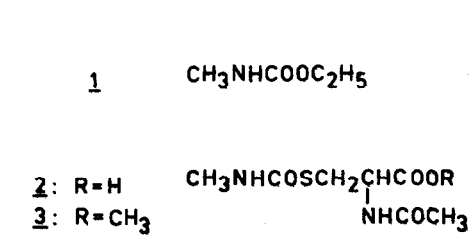

Fig. 1. Chemical structures of ethyl N-methylcarbamate (1), the sulphur-containing urinary metabolite of N,N-dimethylformamide (2) and N-acetyl-S-(N-methylcarbamoyl)cysteine methyl ester (3).

volume of ethyl acetate. The urine was then acidified (2 *M* sulphuric acid, 1:10) and extracted twice with ethyl acetate (1:1). The extracts obtained after acidification (1600 ml) were combined and concentrated to ca. 0.5 ml. The concentrate was chromatographed on silica gel (Silikagel L 40/100; elution with benzene–acetone–acetic acid, 3:2:1). Instead of this mixture containing benzene it is possible to use an alternative system, chloroform–methanol–acetic acid (18:4:1). After evaporation of the solvent in vacuo at 50°C, the residue was taken up with water and further purified on a strongly basic anion exchanger (Ionenaustauscher III) in the Cl^- form. Elution with 20-ml aliquots of 0.001, 0.01 and 0.1 *M* hydrochloric acid, followed by 1 *M* hydrochloric acid, afforded fractions containing the metabolite, which were combined, saturated with ammonium sulphate and extracted with ethyl acetate. The organic layer was dried with sodium sulphate and the solvent was evaporated in vacuo to give an oily product 2 (Fig. 1), which was characterized by NMR spectroscopy. ^{13}C NMR ($CDCl_3+CD_3OD$), δ 22.95+23.00 (q, CH_3CO), 28.26+28.41 (q, CH_3NH), 31.37+31.49 (t, SCH_2), 53.90+53.98 (d, CH), 172.22, 172.29, 172.64 (s, COS + $COOH$ + $COCH_3$); ^1H NMR, δ 2.19 (s, $COCH_3$), 2.82 (s, CH_3NH), 3.27, 3.14 (m, J=15.0, 7.4, 4.5 Hz, SCH_2), 4.62 (m, J=7.4, 4.5 Hz, CH). R_F=0.30 (Silufol, chloroform–methanol–acetic acid, 18:4:1).

The isolated compound was dissolved in ethyl acetate and treated with an excess of ethereal diazomethane. After evaporation of the solvents, a light yellow solid 3 (Fig. 1) was obtained (m.p. 79–82°C), R_F=0.44 (Silufol, chloroform–methanol, 5:1), which was characterized spectroscopically. ^1H NMR ($CDCl_3$), δ 2.02 (s, $COCH_3$), 2.85 (d, J=4.8 Hz, CH_3NH), 3.32 (m, SCH_2), 3.74 (s, $COOCH_3$), 4.71 (m, CH), 6.28 (bd, CH_3NH), 7.08 (bd, J=6.7 Hz, $NHCOCH_3$); ^{13}C NMR, δ 22.89 (q), 31.21 (t), 52.54 (q), 53.32 (d), 167.24 (s), 170.41 (s), 170.70 (s).

Isolation of ethyl N-methylcarbamate (1) after alcoholysis

Human urine collected after exposure to DMF was mixed with ethanol (1:1) in a separating funnel. Powdered potassium carbonate (130 g per 100 ml of urine) was added in one portion and the mixture was shaken vigorously. The separated

organic layer (total amount 3 l) was concentrated in vacuo (1:30), diluted with distilled water (9:1) and extracted with ethyl acetate (1:2, three times). The ethyl acetate extract was concentrated and purified on a silica gel column (Silikagel L40/100; elution with chloroform–methanol, 4:1). After evaporation of the solvent, an odorous liquid 1 resulted, which was characterized by mass and ^{1}H NMR spectrometry. Mass spectrum, *m/z* (relative intensity) 103(26), 76(7), 75(41), 74(27), 60(6), 59(14), 58(100), 57(7), 46(6), 45(31), 44(30), 43(9), 42(2), 41(7), 39(7), 38(1), 32(7), 31(30), 30(60), 29(71), 28(39), 27(37), 26(8), 15(31), 14(3); ^{1}H NMR ($CDCl_3$), δ 1.24 (t, CH_2CH_3), 2.79 (d, $NHCH_3$), 4.11 (q, CH_2CH_3). The corresponding GC peak of 1 was followed in the eluate in order to detect the product during purification.

Synthesis of reference compounds

Ethyl N-methylcarbamate (1). Ethyl chloroformate was added dropwise at 20°C to an excess of an ethanolic solution of methylamine. The mixture was stirred for 20 min, poured into water and the product was extracted with diethyl ether. The ethereal solution was dried with sodium sulphate, the ether was distilled off and ethyl N-methylcarbamate was distilled in vacuo, b.p. 70°C per 15 Torr.

N-Acetyl-S-(N-methylcarbamoyl)cysteine methyl ester (3). Methyl isocyanate (2 mmol) was added at 0°C to a solution of N-acetylcysteine methyl ester (1 mmol) in acetonitrile (3 ml). The mixture was stirred at 20°C for 8 h, the solvent was evaporated in vacuo and the residue was purified on a silica gel column (elution with chloroform–methanol–acetic acid, 18:4:1). Trituration with diethyl ether gave a white solid, which was recrystallized from dichloromethane–diethyl ether to yield 156 mg (67%) of 3, m.p. 89–91°C.

RESULTS AND DISCUSSION

The GC analysis of the urinary extracts obtained from a man exposed to a large dose of DMF vapour revealed the presence of an unknown volatile component (1) in addition to MF and FA (Fig. 2). The new component was formed when the post-exposure urine was treated with ethanolic potassium carbonate, but was absent after a standard non-basic work-up [4, 12]. Analysis by GC–MS and molecular formula determination by accurate mass measurements (*m/z* 103.0631; $C_4H_9NO_2$ requires 103.0633) pointed to the structure of ethyl N-methylcarbamate (1). This was confirmed by the ^{1}H NMR spectrum of an isolated sample of 1 and by comparison of the GC, MS and NMR data with those of a reference compound.

As the presence of 1 in post-exposure urine coincided with an increased total concentration of urinary thioethers, determined spectrophotometrically, it is probable that 1 was formed from a primary metabolite of DMF during the basic work-up. On treating the post-exposure urine with potassium carbonate in methanol or 1-propanol a different volatile product was detected in each instance, with

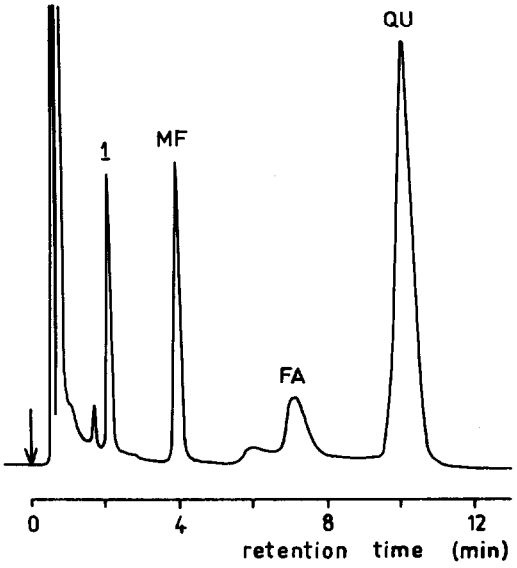

Fig. 2. Chromatogram of the ethanolic extract of a urine sample collected over a period of 29–33 h after the start of exposure to DMF. Peaks: 1 = ethyl N-methylcarbamate; MF = N-methylformamide; FA = formamide; QU = quinoline (internal standard). For chromatographic conditions, see Experimental.

GC retention times shorter (methanol) or longer (propanol) than that of 1. This indicates that the N-methylcarbamoyl moiety of 1 is present in the primary metabolite in an active form and undergoes solvolysis in the basic medium.

The sulphur-containing urinary metabolite of DMF (2) was isolated by combined chromatography on silica gel and an ion-exchange resin, and analysed by NMR spectroscopy. The ^{1}H NMR spectrum showed two three-proton signals of an acetyl and a methylamino group, and an ABX system corresponding to a CH_2CH grouping. Further, the ^{13}C NMR spectrum revealed the presence in 2 of three distinct carbonyl groups. Metabolite 2 was then converted into a methyl ester, 3, and the latter was analysed by MS. The mass spectrum of 3 (Fig. 3) displays a weak molecular ion at *m/z* 234 and abundant fragments at *m/z* 177 ($C_6H_{11}NO_3S$), 133, 118, 88, 76, 60 and 43, which are typical of an N-acetylcysteine derivative [13]. The final structural proof was achieved by comparing the R_F values and spectral data of 3 with those of a synthetic standard, N-acetyl-S-(N-methylcarbamoyl) cysteine methyl ester. Hence, the urinary metabolite 2 was identified as N-acetyl-S-(N-methylcarbamoyl) cysteine.

TLC analysis of urinary extracts after exposure to MF revealed the presence of a metabolite which had R_F values identical with those of 2 and afforded 1 on basic ethanolysis. It is therefore very probable that this human metabolite of MF and 2 are identical. Recently, N-acetyl-S-(N-methylcarbamoyl) cysteine was identified as an MF metabolite in mouse, rats and man [14].

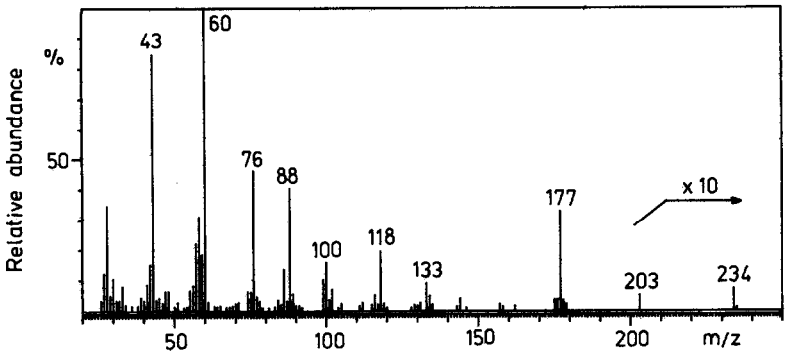

Fig. 3. Mass spectrum of N-acetyl-S-(N-methylcarbamoyl)cysteine methyl ester (3).

In conclusion, the isolation of the mercapturic acid 2 indicates that conjugation with GSH is an important metabolic pathway of DMF and MF in man. The solvolytic work-up, followed by the GC determination of 1, can be used with advantage for the easy indication of 2 in post-exposure urine. In contrast to non-specific tests for total thioethers that give non-zero levels in controls, our method makes it possible to monitor 2 specifically with an undetectable normal level ($n=10$). Quantitative determination of 1 and 2, together with more detailed toxicological data, will be reported in a forthcoming paper.

ACKNOWLEDGEMENTS

The authors thank Dr. I. Linhart and Mr. J. Šmejkal for stimulating discussions, Mrs. H. Nohová for skillful technical assistance, Dr. H. Maloňová for the spectrophotometric measurements and Dr. V. Šedivec and Dr. J. Flek for reading the manuscript.

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