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RAPID COMMUNICATION

S-(N-METHYLCARBAMOYL)-N-ACETYLCYSTEINE: A URINARY METABOLITE OF THE HEPATOTOXIC EXPERIMENTAL ANTITUMOUR AGENT N-METHYLFORMAMIDE (NSC 3051) IN MOUSE, RAT AND MAN.

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N-Methylformamide (NMF, NSC 3051) is an antineoplastic agent in mice[1,2]. In clinical trials in which the potential of NMF for the therapy of human cancers was evaluated, manifestations of liver damage were observed[3,4,5]. The mouse appears to be particularly sensitive to the hepatotoxic properties of NMF[6,7] and results of mechanistic studies in mice suggest that a reactive metabolite of NMF is responsible for its hepatotoxicity[7,8]. Whereas NMF is metabolised *in vitro* only to a very minor extent by liver fractions or isolated mouse hepatocytes, it undergoes extensive metabolism *in vivo* in rodents[9]. Carbon dioxide, methylamine and N-hydroxymethylformamide have been identified as major metabolites of NMF[10]. In that study, a further metabolite was detected but not characterised. We now report the identification of a new urinary metabolite of NMF and suggest that its precursor(s) may well be responsible for the hepatotoxicity and/or the antitumour activity of NMF.

MATERIAL AND METHODS

Chemicals. The labelled isotopomers of NMF, N-methyl-[^{14}C]formamide and N-[^{14}C]-methylformamide, were prepared as previously described[11]. S-(N-Methylcarbamoyl)-N-acetylcysteine was prepared as follows: Methyl isocyanate (2.0 g, 35 mmol) was added to N-acetyl-L-cysteine (4.1 g, 25 mmol) in anhydrous pyridine at 0°C and the mixture was stirred at this temperature for 4 days with the exclusion of moisture. The solvent was evaporated under reduced pressure. The residue was dissolved in dichloromethane and extracted with water. The aqueous extract was finally freeze-dried to furnish S-(N-methylcarbamoyl)-N-acetylcysteine (3.7 g, 67%) as a very hygroscopic white solid, MPt 65°C.

Drug administration and metabolite isolation and identification. Details of the i.p. administration of NMF or [^{14}C]-NMF (five daily doses of 400 mg/kg) to male CBA/CA mice (18-25 g) and male Wistar rats (300-350 g) and of the subsequent urine collection have been described previously[10,12]. Human urine samples were obtained from seven patients who received NMF (i.v. or p.o., dose range 300 mg/m^2 to 1200 mg/m^2) as a single dose. Urine samples were collected every 6 hr during a 24 hr period after drug administration. Details of the patients' age, sex and malignancies and of the formulation of NMF have been reported previously[4]. Urine samples were frozen after collection and pooled prior to analysis. After addition of 1M HCl to achieve pH1, samples were freeze-dried. The residues were suspended in methanol and subjected to TLC analysis using silica gel 60 coated plates (0.2 mm or 2 mm thickness, Merck AG, Darmstadt, W. Germany). Plates were developed in butan-1-ol:water:methanol (8:2:1 v/v). Thioesters were detected by spraying the plates either with 1M aqueous NaOH followed by Ellman's reagent[13] or with chloroplatinate reagent[14]. [^{14}C]-Labelled metabolites were detected as described by Kestell *et al.*[10]. On preparative TLC, the area of silica at the Rf value of the material which gave a reaction with Ellman's reagent after alkaline hydrolysis was removed from the plate and eluted with methanol. The solvent was evaporated at room temperature. The light brown residue was freeze-dried to remove traces of butan-1-ol. The metabolite isolated as described above or authentic S-(N-methylcarbamoyl)-N-acetylcysteine were dissolved in methanol (5 ml). HCl gas was bubbled through the solutions for 30 min. The solutions were left standing overnight at room

temperature, after which the solvent was evaporated under reduced pressure. The material was purified by HPLC with methanol:water (3:1 v/v) as eluant, and a FRAC-100 fraction collector (LKB, Croydon, U.K.). The eluate was concentrated under reduced pressure at 40°C. HPLC analysis was performed using a Waters trimodular system (Waters Associates, Northwich, U.K.), a Waters RCM-100 radial compression unit and a C_{18} 5 μm reverse-phase column. Radioactivity in the column eluate was detected by an HPLC ESI-Panax 506C radioactivity monitor (Rotherol and Mitchell Ltd., Ruislip, U.K.); u.v. detection was achieved with a Waters λ_{max} 480 LC spectrophotometer set at 205 nm. The eluant (either 0.01M octylamine in water adjusted to pH6 with concentrated HCl:methanol, 3:1 v/v; or water:methanol, 3:1 v/v) was pumped through the column at a flow rate of 1 ml/min. ^{1}H-NMR spectra were obtained at 400 MHz using a Bruker WH400 spectrometer with D_2O or borate buffer pH8/D_2O as solvents. Mass spectra were determined on a VG 7070 mass spectrometer in the chemical ionization mode, using 2-methylpropane as reagent gas. Spectra were run at a scan rate of 1 sec/decade and were processed on a VG 2035 data system.

RESULTS

TLC analysis of samples obtained by freeze-drying pooled urine of mice or rats which had received NMF or $^{[14]}$C-NMF or of patients who had been treated with NMF afforded a chromatographic band (Rf 0.30) which gave a positive reaction with the chloroplatinate reagent and with Ellman's reagent subsequent to alkaline hydrolysis. This material was absent in control urine samples. On HPLC analysis the material extracted from the TLC plate afforded one predominant peak detected by either its uv absorbance or monitoring of its radioactive label. The retention time of the metabolite was indistinguishable from that of authentic S-(N-methylcarbamoyl)-N-acetylcysteine.

The 400 MHz ^{1}H-NMR spectra of S-(N-methylcarbamoyl)-N-acetylcysteine at pH8 and of the metabolite isolated from patients' urine are shown in Fig. 1. In the spectrum of the reference compound the protons of the acetyl group resonate as a 3H singlet at δ2.01 ppm and the N-methyl moiety gives rise to a 3H singlet at δ 2.78 ppm. The remaining protons of the molecule, the cysteine α-CH and β-CH_2, form an ABX system. The magnetic inequivalence of the β-methylene protons arises from the prochirality of the CH_2 in the presence of a chiral centre. The α-H appears as δ4.40 ppm (1H, dd, J 7.6 Hz and 4.2 Hz), β-H as δ3.16 ppm (1H, dd, J 14.3 Hz and 7.6 Hz), β'-H as δ3.43 ppm (1H, dd, J 14.3 Hz and 4.2 Hz). The geminal coupling constant of 14.3Hz and the two vicinal coupling constants (7.6 Hz and 4.2 Hz) are similar to the corresponding J values previously reported by us for other mercapturic acids[15]. At this alkaline pH, slow degradation to unidentified products was observed. In the ^{1}H-NMR spectrum of the metabolite (Fig. 1), the general pattern is very similar and the coupling constants are identical to those of the synthetic material.

Both isolated metabolite and authentic reference compound were treated with methanolic HCl and thus converted to a derivative which migrated to Rf 0.70 on TLC analysis. The chemical ionisation mass spectra of the esterified products are shown in Fig. 2 and show excellent congruity. Each spectrum has principal ions at m/z 235, 178, 136 and 60 which are due to (M+H) ions of the complete methyl ester molecule, N-acetylcysteine methyl ester (loss of MeNCO), cysteine methyl ester (subsequent loss of ketene) and acetamide respectively. Although the spectra presented in Figs. 1 and 2 were obtained with samples isolated from patients' urine, the material isolated from the urine of mice and rats afforded spectra almost identical to those shown in the figures.

DISCUSSION

Studies of the mechanism by which NMF causes hepatotoxicity in mice have accrued strong evidence for the contention that it is metabolised to a reactive species responsible for toxicity[2,7,8,9,16]. However the identification of carbon dioxide, methylamine and the pharmacologically innocuous N-(hydroxymethyl)formamide[17] as metabolites of NMF *in vivo*[10] gives but little indication as to the nature of this species. NMF itself is not hydrolysed to methylamine and formic acid by liver fractions *in vitro*. One might speculate that products of oxidation of NMF such as N-methylcarbamic acid (HOCONHMe) or the potent toxin

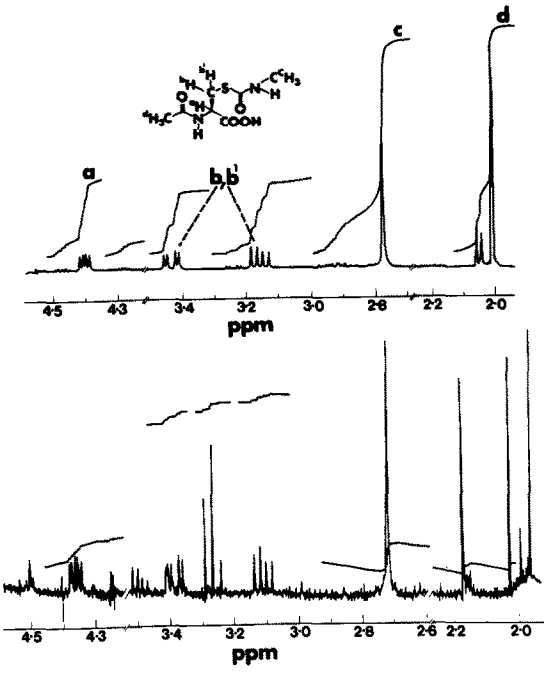

Fig. 1 Salient features of the ^{1}H-NMR spectra with D_2O of S-(N-methylcarbamoyl)-N-acetyl-cysteine (top panel) and the metabolite which was isolated by preparative TLC from the urine of patients who received NMF (bottom panel). The presence of alkaline buffer in the solution of authentic sample caused some degradation as shown by the signals near δ2.92, 3.23, 4.28 and 4.48 ppm. The chemical shifts of the synthetic mercapturic acid were found to be markedly sensitive to changes in pH in aqueous solution. It may be that the borate buffer used to mimic the pH of the solution of the isolated metabolite was not of precisely the same pH or ionic strength thus causing the small discrepancies in chemical shifts. The ^{1}H-NMR spectrum of an extract of control urine obtained by preparative TLC in the same way in which the metabolite was isolated showed resonances at δ2.17, 3.24, 3.27 and 3.29 ppm which can also be seen in the lower spectrum.

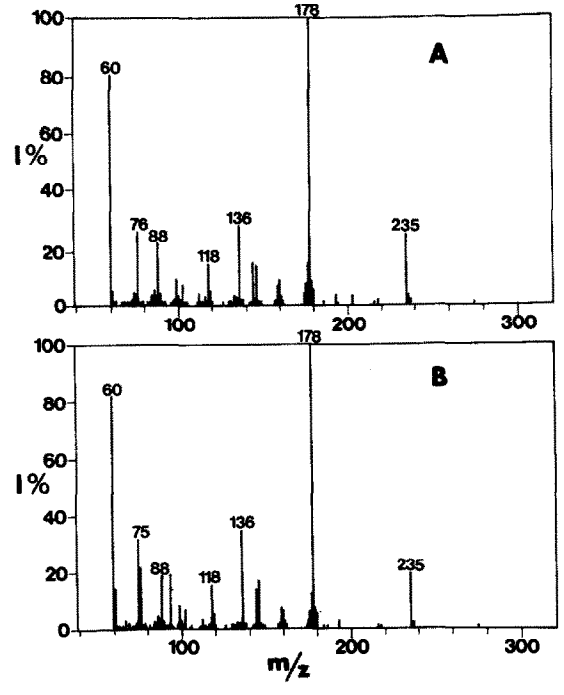

Fig. 2 Mass spectra of the methyl esters of (A) authentic S-(N-methylcarbamoyl)N-acetyl cysteine and (B) the metabolite isolated from the urine of patients who had received NMF.

methyl isocyanate (MeNCO) are the immediate precursors of the observed methylamine. Alternatively, these or other oxidation products of NMF would be able to form conjugates. In this report, the identification of such a conjugate, S-(N-methylcarbamoyl)-N-acetyl-cysteine, as a metabolite of NMF in the urine of mice, rats and men is described. Hydrolysis of N-methylthiocarbamates, such as this metabolite, indeed leads to methylamine and carbon dioxide. It is highly likely that the mercapturic acid characterised in this study results from metabolic modification of the corresponding S-(N-methylcarbamoyl)glutathione generated in the liver. The formation of this latter compound from NMF formally involves an oxidation at the formyl carbon of NMF, but the mechanistic route for this novel metabolic step is yet an enigma. It is noteworthy that N-alkylmonothiocarbamate compounds are electrophiles and carbamoylating agents and S-(N-alkylcarbamoyl)cysteines, compounds structurally related to the novel metabolite of NMF described here, have been reported to be potently cytotoxic[18].

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REFERENCES

1. D.A. Clarke, F.S. Philips, S.S. Sternberg, R.K. Barcley and C.C. Stock. Proc. Soc. Exp. Biol. Med. **84**, 203 (1953).
2. A. Gescher, N.W. Gibson, J.A. Hickman, S.P. Langdon, D. Ross and G. Atassi. Br. J. Cancer **45**, 843 (1982).
3. W.P. Laird Myers, D.A. Karnofsky and J.H. Burchenal. Cancer **9**, 949 (1956).
4. J.G. McVie, W.W. Ten Bokkel Huinink, G. Simonetti and R. Dubbelman. Cancer Treat. Rep. **68**, 607 (1984).
5. D.S. Ettinger, D.W. Orr, A.P. Rice and R.C. Donehower. Cancer Treat. Rep. **69**, 489 (1985).
6. S.P. Langdon, D. Chubb, A. Gescher, J.A. Hickman and M.F.G. Stevens. Toxicology **34**, 173 (1985).
7. H. Whitby, A. Gescher and L. Levy. Biochem. Pharmacol. **33**, 295 (1984).
8. P. Pearson, A. Gescher and E.S. Harpur, unpublished.
9. C. Brindley, A. Gescher, E.S. Harpur, D. Ross, J.A. Slack, M.D. Threadgill and H. Whitby. Cancer Treat. Rep. **66**, 1957 (1982).
10. P. Kestell, A. Gescher and J.A. Slack. Drug. Metab. Dispos. **13**, 587 (1985).
11. M.D. Threadgill and E.N. Gate. J. Labelled Cpds. & Radiopharmaceuticals. **20**, 447 (1983).
12. P. Kestell, L. George and A. Gescher. Biochem. Soc. Trans. **13**, 893 (1985).
13. C.B. Glaser, H. Maede and J. Meienhofer. J. Chromatog. **50**, 151 (1970).
14. E.A. Barnsley, A.E.R. Thomson and L. Young. Biochem. J. **90**, 588 (1964).
15. S.J. Addison, B.D.M. Cunningham, E.N. Gate, P.Z. Shah and M.D. Threadgill. J. Chem. Soc. Perkin Trans. 1 75 (1985).
16. V. Scailteur and R. Lauwerys. Arch. Toxicol. **56**, 87 (1984).
17. P. Cooksey, E.N. Gate, A. Gescher, J.A. Hickman, S.P. Langdon and M.F.G. Stevens. Biochem. Pharmacol. **32**, 3037 (1983).
18. L. Nemeth, S. Somfai-Relle, B. Kellner, J. Sugar, R. Bognar, J. Farkas, J. Balint, I. Palyi, K.Toth, Z. Szentirmay, Z. Somosy and E. Pokorny. Arzneim.-Forsch. **28**, 1119 (1978).