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RAPID COMMUNICATION

IDENTIFICATION OF THE "MAJOR" POLYMORPHIC CARBOCYSTEINE METABOLITE AS *S*-(CARBOXYMETHYLTHIO)-L-CYSTEINE

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The metabolism of the mucolytic agent carbocysteine (*S*-carboxymethyl-L-cysteine, CMC) has attracted substantial interest during recent years [1]. It has been reported that sulphoxidation of CMC and its cysteinyl metabolites constitutes a major pathway of biotransformation. Moreover, it has been suggested that CMC sulphoxidation exhibits a genetic polymorphism expressed in two phenotypes in the population, the poor sulphoxidizers (PS) and the extensive sulphoxidizers (ES) [2]. This sulphoxidation polymorphism has been linked to certain idiopathic diseases (*e.g.* primary biliary cirrhosis) and the toxicity of sulphur-containing drugs (*e.g.* D-penicillamine, sodium aurothiomalate) [1]. However, more detailed metabolic studies on CMC and a carbon-13 labelled analogue (*S*-carboxy-[^{13}C]methyl-L-cysteine, ^{13}C-CMC) have demonstrated the absence of significant amounts of any of the putative cysteinyl sulphoxide metabolites in the urine. These studies which were based on HPLC [3-5], ^{13}C NMR [5,6], and gas chromatography/mass spectrometry (GC/MS) [7] demonstrated that, besides unchanged carbocysteine, thiodiglycolic acid (di-[carboxymethyl] sulphide, TDGA) and its sulphoxide (TDGA-SO) were the major biotransformation products of CMC in man [8]. Approximately 6-8 hr after administration of the drug, urinary excretion of an additional novel metabolite (1) was observed by TLC after sulphur-selective visualization [9]. This particular metabolite 1 of yet unknown structure was the only urinary component exhibiting polymorphic character present after oral administration of CMC [10]. About 7.5-10% of the two populations excreted only marginal amounts of 1, and a more than two-thousand fold difference in the urinary excretion of metabolite 1 has been estimated by TLC [10,11]. The objective of this study was to isolate and identify this metabolite.

MATERIALS AND METHODS

Instrumental analyses. All ^{13}C NMR spectra were acquired as described recently [6]. Electron impact (70 eV) mass spectra were recorded with a Hewlett-Packard 5890A GC equipped with a 12m HP-1 (dimethylpolysiloxane gum) capillary column (i.d. 0.2 mm) and coupled to an HP-5970 mass selective detector. For high-resolution GC/MS (R = 5000) an AutoSpec instrument (VG Analytical) was used.

Chemicals. *S*-(Carboxymethylthio)-L-cysteine and other chemicals were prepared as described elsewhere [12,13].

Studies. An aqueous suspension (750 mg, 4.1 mmol) of CMC and the carbon-13 labelled analogue *S*-carboxy-[^{13}C]methyl-L-cysteine (^{13}C-CMC) [13] was administered orally on separate occasions to two male volunteers according to the original protocol [2] and urine was collected in fractions up to 72 hr. The samples were stored frozen (-20°) until analyzed.

Urine work-up. For the purification of the metabolite of yet unknown structure urine samples were adjusted to pH 7, lyophilized and redissolved in a small volume of water (*cf.* Fig. 1A). The samples were then applied at 5° on a cation exchange resin column (Dowex 50Wx8, H^+-form, 100-200 mesh). The columns were first washed with distilled water until neutrality and then eluted with 1*N* aqueous ammonia. After evaporation *in vacuo* the residues were redissolved in water and applied on strongly basic ion-exchange resin columns (Dowex 1x8-400, OH^--form, 200-400 mesh). The columns were washed with water, and the amino acids were eluted at 5° with 1*N* aqueous acetic acid. The eluates were concentrated in vacuum and filtered through solid phase extraction columns (octadecylsilane bonded to silica gel). Aliquots of these samples (*cf.* Fig. 1B) were used for chemical reactions and GC/MS analysis.

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HPTLC analysis. Pre-coated silica gel 60 F254 plates and a mobile phase consisting of a mixture of *n*-propanol/glacial acetic acid/water (60:25:15, v/v%) were used for HPTLC analyses. Divalent sulphur-containing spots were visualized on pink background with the iodoplatinate dipping reagent [14]; R_F = 0.43 (CMC, white spot), 0.51 (**1**, yellow spot).

RESULTS AND DISCUSSION

TLC analysis revealed that the major proportion of **1** was excreted between 12-16 hr after drug intake. The colour reaction with ninhydrin, which disappeared after pre-treatment of lyophilized samples with acetic acid anhydride, and a strong reaction with a sulphur-selective dipping reagent suggested the presence of a sulphur-containing free amino acid. Furthermore, the metabolite was slightly less polar than CMC, was readily oxidized with H_2O_2, and reacted with borohydride under the conditions reported for cleavage of disulphides [15].

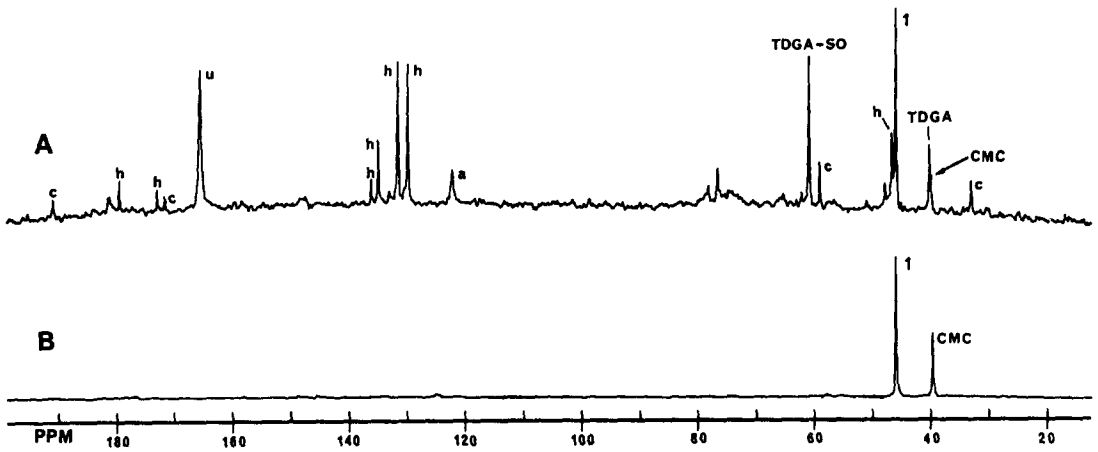

Fig. 1 ^{13}C NMR spectra (20.1 MHz) of human urine samples (pH adjusted at 7.0; internal standard: acetonitrile, **a**, $\delta[CH_3]$ = 3.07 ppm (not shown), δ[CN] = 122.44 ppm) after administration of ^{13}C-CMC. **A**: Lyophilisate of a 12-16 hr urine sample. **B**: Purified 8-24 hr pool sample (for details see *Materials & Methods*). Natural abundance resonances (δ-values in ppm) of endogenous compounds are assigned as creatinine (**c**, 33.1, 59.2, 171.8, 191.2), hippuric acid (**h**, 46.8, 130.0, 131.7, 135.1, 136.3, 173.2, 179.7), and urea (**u**, 165.7). The $^{13}CH_2$ signals of labelled CMC (40.0), and metabolites TDGA (40.3), **1** (46.1), and TDGA-SO (61.1) are indicated.

Administration of ^{13}C-CMC allowed for more specific NMR monitoring of the metabolic profile [6]. A typical ^{13}C NMR spectrum of a lyophilized 12-16 hr urine sample is shown in Fig. 1A. Metabolite **1**, whose time course paralleled the TLC assay, exhibited a chemical shift similar to that of dithiodiglycolic acid. By combining TLC and NMR to monitor the efficiency of the chromatographic purification procedures, pooled urine samples of the 8-24 hr collection periods after ^{13}C-CMC administration were worked up as described in *Materials & Methods*. Fig. 1B illustrates the selectivity of the method employed. Additional NMR measurements using the DEPT pulse sequence (not shown) confirmed the methylene multiplicity of the ^{13}C label of **1**. Furthermore, *in situ* competition reactions revealed that **1** is more readily oxidized by H_2O_2 than sulphoxides, or even thioethers such as CMC and TDGA. Since a single oxidation product of carbon-13 labelled **1** that had a chemical shift superimposable with that of sulphoacetic acid (δ = 61.8 ppm) was observed by NMR, one moiety of the assumed disulphide consists of the carboxymethylthio group.

For a complete structural elucidation of the disulphide, the amino acid fraction of the chromatographic work up (sample shown in Fig. 1B) was derivatized in two steps (diazomethane/pentafluoropropionic acid anhydride) and subjected to GC/MS. In addition, the same work up procedure was employed for samples obtained after administration of unlabelled CMC. Comparison of the mass spectra of the gas chromatographically separated derivatives revealed that only two components of the CMC and ^{13}C-CMC study differed in their molecular ion by one a.m.u., namely the derivatives of CMC and **1**. The corresponding mass spectrum of labelled **1** is depicted in Fig. 2A. High-resolution MS of the fragments shown in Fig. 2A proved a molecular composition of $C_9{}^{13}CH_{12}F_5NO_5S_2$ (calc. for $M^{+\cdot}$ *m/z* 386.011, found *m/z* 386.010). In view of the straightforward fragmentation pattern and in comparison with the retention time as well as the mass spectrum of the derivative of the chemically prepared authentic disulphide (Fig. 2B), the structure of **1** can unequivocally be assigned as *S*-(carboxymethylthio)-L-cysteine.

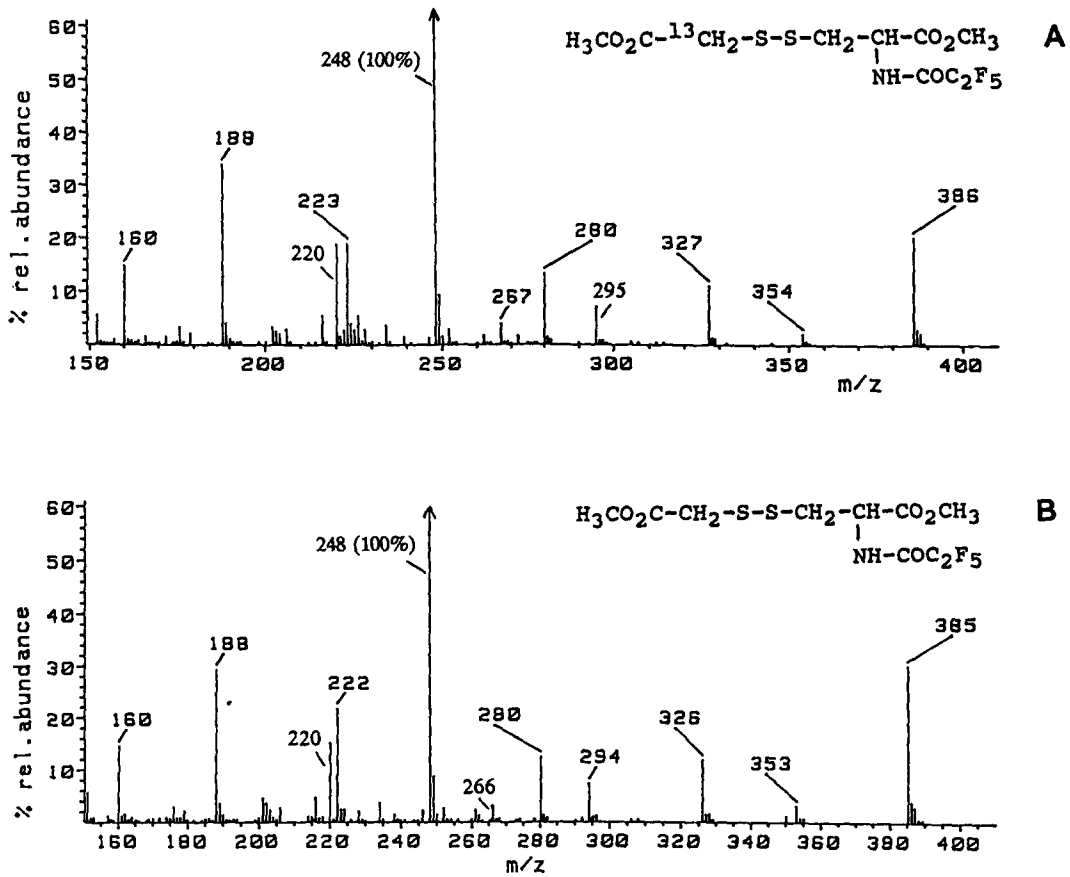

Fig. 2 Mass spectra of the *N*-pentafluoropropionyl dimethyl ester derivatives of the carbon-13 labelled metabolite 1, isolated after administration of ^{13}C-CMC (Fig. 2A), and of authentic unlabelled *S*-(carboxymethylthio)-L-cysteine (Fig. 2B).

$HO_2C-CH_2-(S)_n-CH_2-CH-CO_2H$
NH_2

Structure of CMC (n=1) and metabolite 1 (n=2)

It is interesting to note that the mixed disulphide metabolite 1 has been identified as an endogenous compound in human urine [16-19] and normal values of 0.64 μmol/L [17] and 6.8-14.3 μmol/L [18] have been reported. Its biosynthesis most likely proceeds via oxidative transamination of L-cystine to furnish *S*-(2-oxo-2-carboxyethylthio)cysteine which is then decarboxylated to yield 1 [17]. Large amounts of 1 were also formed when pig liver or kidney homogenates were incubated with L-cystine in the presence of thioglycolic acid (TGA) [17]. Similarly, the "PS" phenotype of CMC is fully converted to the "ES" phenotype when *N*-acetyl-L-cysteine is co-administered in the CMC test [20]. These observations and the retention of the carbon-13 label in 1 after administration of ^{13}C-CMC indicate that TGA is an intermediate metabolite in the biotransformation of CMC. TGA could either be formed by ß-thionase hydrolysis of TDGA [21] or by direct cleavage of CMC by cysteinyl conjugate ß-lyases [22]. Re-conjugation of TGA with glutathione or, more directly, with cysteine may finally lead to the formation of 1. Since 7.5-10% of the subjects studied [10,11] excreted only traces of 1, it is possible that the formation of this disulphide metabolite might be polymorphic. However, in spite of its intense colour reaction in the TLC assay, ^{13}C NMR analysis [6] showed that metabolite 1 only amounts to about 6% of the dose administered during its excretion period of 6-48 hr. Therefore, it is very unlikely that 1 contributes to a substantial degree to the elimination of CMC from the organism.

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