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Evaluation of proposed sulphoxidation pathways of carbocysteine in man by HPLC quantification

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Summary. A quantitative study has been made of the metabolism of S-carboxymethyl-L-cysteine (CMC) and its sulphoxides in volunteers by HPLC. Precolumn derivatization was applied prior to gradient reversed phase HPLC separation and fluorescence detection. For CMC and its metabolites containing a primary amino group the reagent 9-fluorenylmethylchloroformate was used. The other metabolites of CMC were derivatized at their carboxylic group with 1-pyrenyldiazomethane to give stable fluorescent products.

Urine samples were collected for 8 h after oral administration of 1.125 g CMC to 33 healthy volunteers. Elimination of CMC in urine as sulphoxides did not account for more than 1% of the dose in any of the volunteers. Thus, CMC-sulphoxide metabolites are not quantitatively important. Recovery of the original substance in 8-hour urines ranged from 10 to 30% and a further 2 to 20% was recovered as the metabolite thiodiglycolic acid.

Oral doses of 0.19, 1.125, and 2.25 g CMC in a second group of 12 healthy volunteers did not reveal dose dependence of the urinary excretion of the sulphoxides or of thiodiglycolic acid.

Serum concentration-time-curves of CMC, (S)- and (R)-CMC sulphoxide were measured in a group of 9 healthy volunteers. The CMC sulphoxides in serum reached 1.5% of the parent substance after 4 hours. The ratio of CMC to its sulphoxide metabolites was similar in serum and urine. Pharmacogenetic polymorphism of sulphoxidation was not confirmed by the specific HPLC methods used.

Key words: Carbocysteine, pharmacogenetics, drug metabolism, sulphoxidation, pharmacokinetics

Proposed impact of sulphoxidation polymorphism

Among studies on oxidative drug metabolism, including carbon and nitrogen oxidation reactions, oxidation of sulphur in drugs has been less extensively investigated. Early investigations in humans into the metabolism of the mucolytic drug S-carboxymethyl-L-cysteine (carbocysteine, CMC) revealed that, in addition to the parent substance, thiodiglycolic acid was excreted in urine as major metabolite [2]. Subsequently a complex pattern of urinary metabolites after oral CMC was reported [3]. Huge interindividual variation was described in the oxidation of the sulphide moiety of CMC and its metabolites [1]. A scheme of these reactions [4] is shown in Fig. 1.

In a study in 200 healthy volunteers, the urinary metabolites of CMC were summarised as the ratios of the reduced (sulphides) and the oxidized (sulphoxides) metabolites. The frequency distribution of this ratio failed to show an antimode on inspection, but mathematical analysis suggested the influence of a genetic polymorphic trait [1]. Subsequent family studies by the same authors confirmed the heritability of this trait. S-Oxidation of S-carboxymethyl-L-cysteine appears, therefore, to be a highly

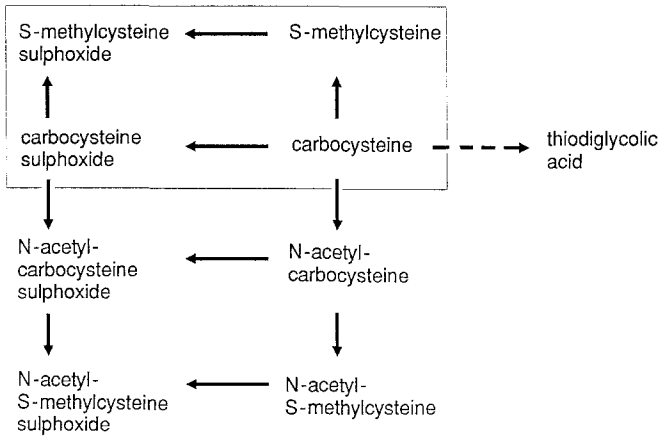

Fig. 1. Metabolism of carbocysteine as proposed by Waring et al. [3]. All compounds shown in the scheme have been synthesized and quantified in the present study, but only the original substance CMC, the metabolite thiodiglycolic acid and small amounts of CMC-sulphoxide could be detected in urine from healthy volunteers. The four substances in the box contain an amino group and would be detected after fluorescent derivatization with 9-fluorenyl-methylchloroformate. The other compounds are carboxylic acids and were derivatized with 1-pyrenyldiazomethane

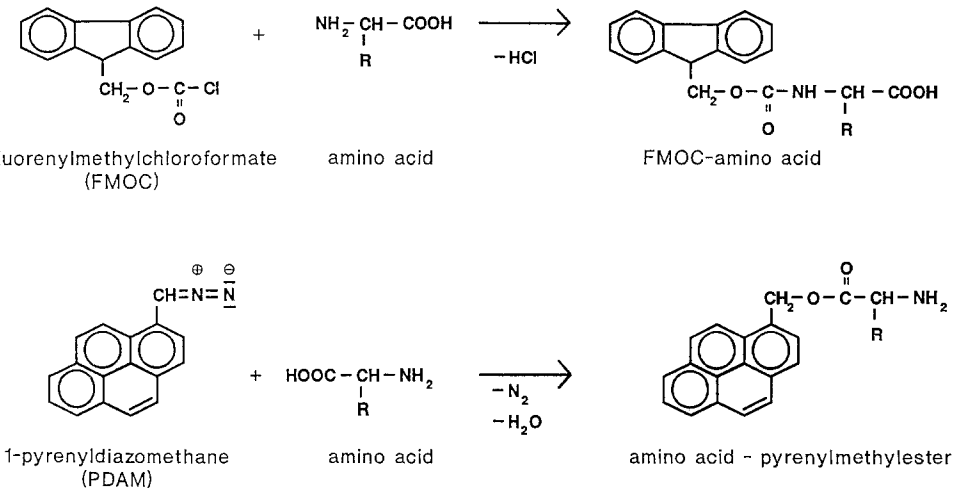

Fig. 2. Reactions of the analytical derivatizations used to detect CMC and all amino-group containing metabolites with the fluorescent reagent 9-fluorenylmethoxycarbonylchloride. Metabolites which are deaminated or acetylated were derivatized with the aryldiazoalkane 1-pyrenyldiazomethane to yield stable and highly fluorescent 1-pyrenyl methylester derivatives. As all compounds in Fig. 1 contain carboxylic groups they would all be derivatized by the diazomethane derivative, but the HPLC separation was superior if the basic amino groups were derivatized

interesting polymorphism from the pharmacogeneticist's viewpoint. This view has been strengthened as several pharmacotherapeutic and general medical implications of the trait have been described: individuals with low sulphoxidation capacity were markedly overrepresented among patients with severe adverse effects of D-penicillamin [5], and the occurence of food allergies has been correlated with slow disposition of CMC [6]. In addition, severe diseases, such as primary biliary cirrhosis [7], have been associated with a low extent of formation of sulphoxide metabolites of CMC.

Methods of measuring sulphoxidation in humans

The cited reports on sulphoxidation polymorphism have relied on urine analysis using paper chromatography [1] and detection by means of the chloroplatinic acid reagent of Toennis and Kolb [8]. However, the detection of sulphoxides with chloroplatinic acid, known as a probe for certain reducing compounds, is not well understood. The paper chromatographic measurements are time consuming and have only moderate sensitivity, precision, and separating power. Therefore, techniques for HPLC quantitation of sulphoxidation have been developed, involving precolumn derivatisation with dimethylaminoazobenzene-sulphonyl chloride (DABSCl) prior to reversed phase chromatography [9]. Derivatisation of zwitterionic compounds, such as CMC and its sulphoxides, greatly improves the chromatographic separation and detection sensitivity.

In the present report, CMC and its sulphoxides, as well as the presumed metabolite methylcysteine and its sulphoxides, were analysed by a procedure using the reagent 9-fluorenylmethylchloroformate (FMOC) based on a method developed for amino acid analysis [10], which has the advantage of more simple derivatization conditions and great sensitivity extending to low nanogram-range concentrations. The (R)- and (S)-enantiomers of the sulphoxides were completely separated [11]. All metabolites containing a carboxylic group could be derivatized by the aromatically substituted diazomethane derivative 1-pyrenyldiazomethane [12].

Subjects and methods

Volunteers

Following informed consent, 1.125 g CMC (three capsules of Transbronchin™; Homburg, Frankfurt, FRG) was given to 33 healthy volunteers, in the morning, after emptying the bladder (14 f, 19 m; median age 30 y, range 22 to 50 y). Urine was collected for 8 h and was immediately frozen at −20 °C in small aliquots.

The same dose was given to 9 volunteers (4 f, 5 m, aged 24 to 40 y) after an overnight fast for the serum kinetics study, with blood sampling predose and 1, 2, 3, 4, 5, 6, 7, and 8 h after dosage. Blood was left for 30 min at room temperature and then serum was kept frozen at −20 °C until analysis.

For the dose dependency study, 12 volunteers (4 f, 8 m, aged 23 to 33 y) each received doses of 0.19, 1.125, and 2.25 g CMC, in randomized sequence, with a 1 week interval between each dose.

Materials

Pure carbocystein (CMC) and methylcysteine came from Sigma (Deisenhofen, Germany), thiodiglycolic acid was from Aldrich (Steinheim, Germany). CMC-(R,S) sulphoxides, methylcysteine (R, S) sulphoxides, N-acetyl-CMC, N-acetyl-CMC-(R, S) sulphoxides, N-acetyl-methylcysteine, and N-acetyl-methylcysteine-(R, S) sulphoxides were kindly provided by E. Merck AG (Darmstadt, Germany). Optically pure carbocysteine (−) (S)- and (+) (R) sulphoxides were purified from a racemic mixture by fractional crystallization [13] synthesized by hydrogen peroxide oxidation of carbocysteine.

The acetylated derivatives were synthesized from the corresponding amines using acetic anhydride and the products were purified by ion exchange chromatography. The identity and purity of all substances was tested by melting point analysis, mass spectrometry, NMR spectroscopy, and polarimetric analysis in case of the optical enantiomers.

HPLC quantification of CMC and metabolites

CMC and all presumed metabolites that contained an amino group, were detected by precolumn derivatization with 9-fluorenylmethylchloroformate (FMOC; Merck, Darmstadt, Germany) and subsequent gradient reversed phase chromatography with fluorescence detection (Fig. 2). All acetylated or deaminated metabolites were derivatized using the fluorescent reagent 1-pyrenyldiazomethane (PDAM; Molecular Probes, Oregon, USA) and then chromatographed.

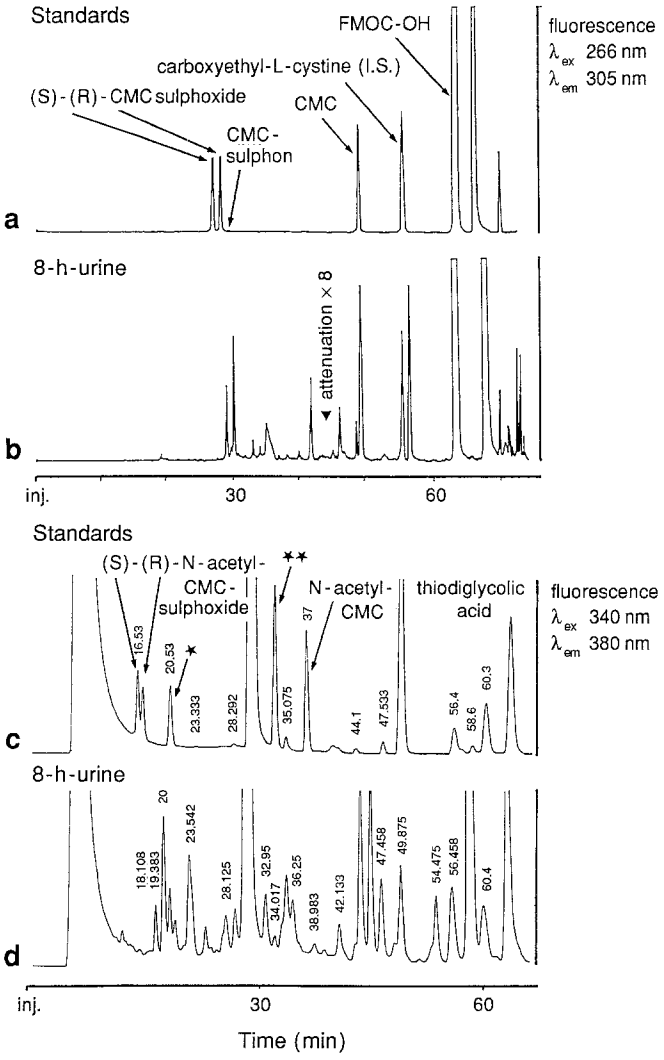

Fig. 3a. Chromatogram of CMC, CMC sulphoxides and the internal standard carboxyethylcystein derivatized with FMOC. The elution position of CMC sulphone is also indicated, although it was not selected. **b** Chromatograms of an 8-hour-urine sample after 1.125 g CMC p.o. A 50 µl urine sample was derivatized with FMOC and analysed in the same manner as the standards in chromatogram A. Note the change in recorder attenuation between the CMC sulphoxides and CMC. **c** Chromatograms of N-acetyl-CMC, N-acetyl-CMC sulphoxides, thiodiglycolic acid derivatized with 1-pyrenyldiazomethane. * Denotes a second PDAM-derivative formed from both enantiomers of N-acetyl-CMC sulphoxide, ** is the peak of a second PDAM-derivative always formed from N-acetyl-CMC. **d** Chromatogram of an 8-hour-urine sample after 1.125 g CMC p.o. The sample was derivatized with PDAM as described under Materials and Methods and chromatographed using the same gradient as the standards in chromatogram C. No N-acetyl-CMC or N-acetyl-CMC sulphoxides could be detected

For derivatization with FMOC 50 µl carboxyethylcysteine was added to each urine or serum sample as the internal standard (5 µg for 50 µl urine and 5 ng for 50 µl serum). Then, 0.5 M sodium borate buffer 400 µl, pH 7.7, and 400 µl 10 mM FMOC in acetone were added and derivatization was completed at room temperature after 1 min. The reaction was stopped by addition of 5 M ammonium acetate 50 µl, pH 7.7. Gradient reversed phase chromatography was performed on 4.6 × 250 mm HPLC columns filled with Hypersil™ octadecylsilane derivatized silica (5 µm particle size; Shandon, U.K.). A low pressure mixing gradient HPLC system from Perkin Elmer was used, with a shallow binary gradient of 25 mM ammonium acetate, pH 3.8 and 4% dimethyl formamide as initial eluent, followed by gradient elution with acetonitrile. On injection, columns were equilibrated with 10% acetonitrile and after injection a linear gradient from 10 to 35% acetonitrile in 60 min with a flow rate of 0.8 ml · min^{-1} was started. A Shimadzu RF-530 fluorescence detector was set at an excitation wavelength of 266 nm and an emission wavelength of 305 nm. Linear calibration curves were obtained between 0.2 and 600 µg · ml^{-1} for CMC, and between 10 ng · ml^{-1} and 20 µg · ml^{-1} for (R)- and (S)-CMC sulphoxides added to blank urine samples. On repeated analysis of the same sample the coefficient of variation was 6%.

For derivatization of compounds containing carboxylic groups with PDAM 5 µg N-acetyl-L-alanine was added as internal standard to 50 µl urine or plasma. Then 50% 2-propanol 400 µl in 50 mM phosphate-buffer, pH 4, 20 mM octadecylsulphonic acid 400 µl in 2-propanol, and 400 µl PDAM, dissolved in ethyl acetate, were added. The reaction was allowed to proceed for 2 h at 40 °C. Gradient reversed phase chromatography was done on 4.6 × 160 mm HPLC columns filled with Hypersil™ (3 µm particle size; packed by Shandon, Frankfurt, FRG). The first eluent was 15 mM triethanolamine adjusted with phosphoric acid to pH 2.4, 5% tetrahydrofurane, and 20% methanol. The second eluent was acetonitrile. Eluent flow was 0.8 ml · min^{-1} and elution was done with a shallow linear gradient from 20% to 35% acetonitrile in 70 min. The quantitation limit for the N-acetylated CMC metabolites in urine samples was 5 µg/ml of urine. Addition of 50 µg · ml^{-1} of N-acetyl-CMC, N-acetyl-CMC sulphoxide and thiodiglycolic acid to 10 different blank urine samples gave standard deviations of 5.5, 4.7, and 6.5, respectively. The coefficient of variation for repeated analysis of the same sample on different days was 9%. Chromatograms of the pure reference compounds and of a typical urine sample of a volunteer who took 1.1 g carbocysteine are illustrated in Fig. 3.

Kinetics and statistical analysis

Maximum concentrations (C_{max}) and times of maximum concentrations (t_{max}) in Table 1 are the measured maximum values. The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule without extrapolation beyond the last measured value. Apparent terminal elimination half lives ($t_{1/2}$) were determined using the serum concentrations obtained at 6 h and later after dosing. Total clearance (CL = dose/AUC) after oral administration could only be obtained in relation to the systemic availability (f). Data are given as median and range between first and third quartiles, since a normal distribution could not be assumed. Statistical significance was tested by the Wilcoxon matched pair signed rank test.

Results

The new HPLC methods described here have been employed in three studies: urinary recovery of CMC and its metabolites in 8-hour urine, the 8-hour serum kinetics of CMC and its sulphoxide metabolites, and the dose dependence of sulphoxide formation.

Metabolites of CMC recovered in urine

The frequency distribution of the urinary recovery of CMC, thiodiglycolic acid and CMC sulphoxides (sum of both enantiomers) obtained in 33 healthy volunteers are shown in Fig. 4.

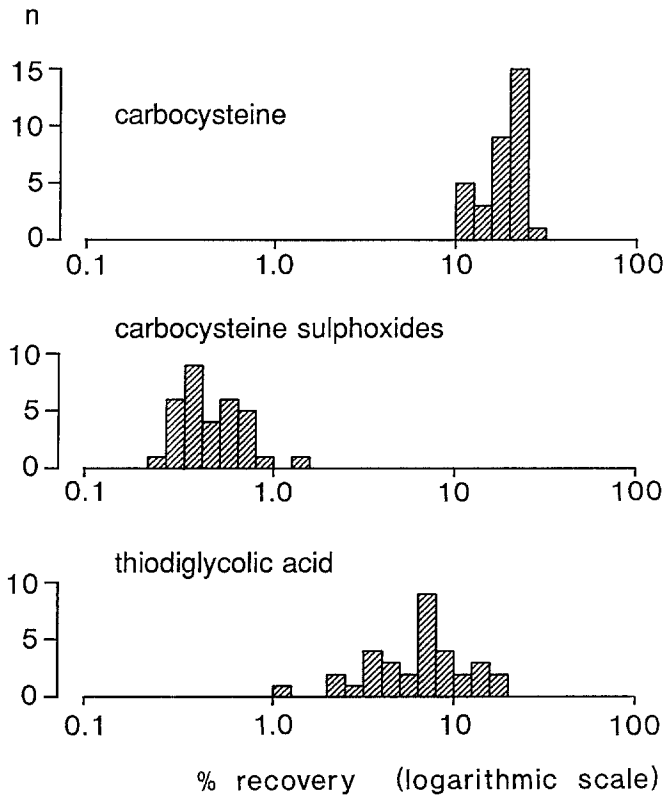

Fig. 4. Percentage recovery after 1.125 g CMC p.o. in 8-hour urine samples from 33 healthy volunteers of CMC, CMC sulphoxides, and thiodiglycolic acid on a logarithmic scale. The quantities of methylcysteine, methylcysteine sulphoxides, N-acetyl CMC and N-acetyl CMC sulphoxides were below the quantification limit of 5 µg/ml

N-acetyl-CMC, N-acetyl-CMC sulphoxides, and methylcysteine could not be detected in any of the 33 urine samples. As the quantitation limit for these compounds was 5 $\mu g \cdot ml^{-1}$, less than 0.5% of the given dose might have been excreted in urine as N-acetyl-CMC or N-acetyl-CMC sulphoxides. The mean recoveries of CMC, CMC sulphoxides, and thiodiglycolic acid in 8-h-urine relative to the dose of 1.125 g was 19.1%, 0.5%, and 7.7%, respectively (values calculated on a molar basis). Thus, from the given dose of CMC 20 to 45% could be recovered in urine, mainly as original CMC, followed by thiodiglycolic acid and a very small amount of CMC sulphoxides.

In the 33 persons studied, recovery of CMC and sulphoxides did not cover a very wide range and no extreme outliners were seen, as is typical of polymorphism in drug metabolism (Fig. 4).

Kinetics of CMC and its sulphoxides in serum

Serum concentration-time curves for CMC (Transbronchin™) have previously been described [14]. In this study, specific concentration-time curves are given for carbocystein (R)- and (S)-sulphoxide enantiomers. Peak levels of the (R)- and the (S)-sulphoxides of carbocysteine appeared about 4 h after the oral dose of carbocystein (Fig. 5). In all 9 subjects they were seen about one hour later than the parent substance. A kinetic evaluation of these three curves is given in Table 1.

The maximum concentration of carbocysteine sulphoxide reached only 1.5% of the maximum concentration of carbocysteine, indicating, in parallel with the urine data, a very low degree of sulphoxide formation from carbocysteine. Similarly, the AUC of the sum of both sulphoxide enantiomers reached only 1.7% of the AUC of the parent substance CMC. The R-enantiomer of the CMC sulphoxides predominated in serum and also in urine. The maximum serum concentrations and AUC were significantly ($P < 0.05$) higher for the (R)-CMC sulphoxide, while the elimination half lives were not significantly different.

As can be seen in Fig. 5, at 8 h after oral administration measurable quantities of CMC and its sulphoxides were still found in plasma. The concentrations, however, were already low and the urine sampling period of 8 h appeared to have been sufficiently.

Dose dependency of urinary recovery

Each of 12 volunteers received an oral dose of 0.19, 1.125, and 2.25 g of CMC in a random sequence. Urinary recoveries are illustrated in Fig. 6. Recovery of CMC (as median and range) increased slightly from 15.8 (10–23)% after the 0.19 g dose to 19 (10–30)% at 2.25 g (not statistically significant). In contrast, recovery of the CMC sulphoxides tended to decrease from 0.5 (0.25–0.7) to 0.35 (0.25–0.55)% of dose. The quotient of the sum of both enantiomers of the sulphoxides to CMC was 38 at the lowest dose (0.19 g), in contrast to 60 at the highest dose of 2.25 g (significant, $P < 0.05$).

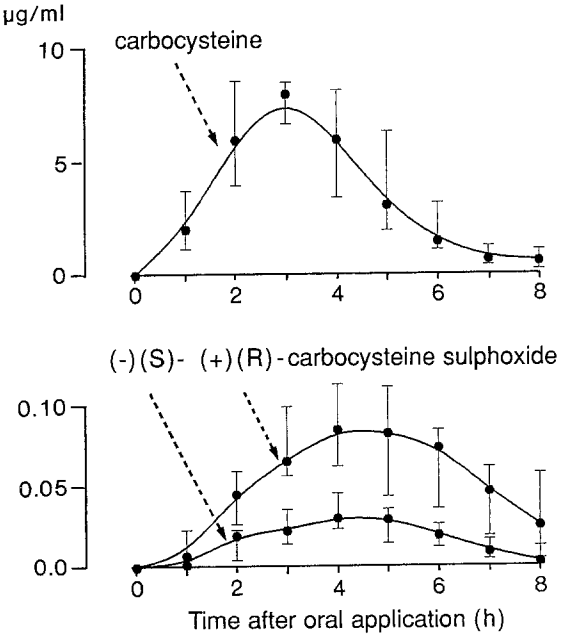

Fig. 5. Serum concentration-time curves of CMC and its (+) (R)- and (−) (S)-sulphoxides following 1.125 g CMC p.o. in 9 healthy volunteers (averaged concentrations at each time point are given as medians and first and third quartiles)

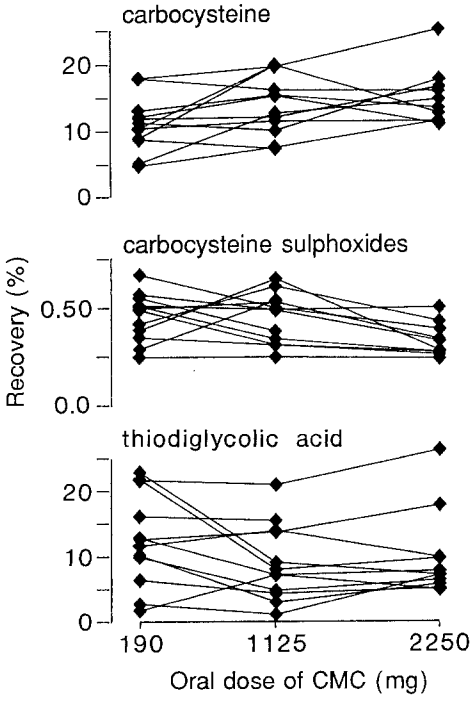

Fig. 6. Dose dependency of urinary recovery of CMC, CMC sulphoxides, and thiodiglycolic acid in 12 volunteers. The percentage recoveries of the metabolites has been calculated on a molar basis

Discussion

The extent and interindividual variability of the urinary excretion of sulphoxide metabolites of carbocystein described in a series of preceeding publications [1, 3–7] was not confirmed. Those studies had assessed the capacity of CMC sulphoxidation, mostly as the summarised ratio of sulphide/sulphoxide metabolites. According to Waring [4], the excretion of CMC sulphoxides varied between 1% and 14.2% of the given dose in 20 volunteers. This was around tenfold more than the average recovery of 0.5% CMC sulphoxide (relative to the given dose) in the present study (Fig. 4 and 6). The group of 33 volunteers tested seems large enough to conclude that these discrepancies can not be due to statistical uncertainty. A similar finding of very low levels of CMC sulphoxides in urine was reported in a previous study employing a different quantification method [9].

The high resolving power of the HPLC methods is illustrated by their ability to separate the optical isomers of the sulphoxides and the fluorescence detection gave high sensitivity. Thus, the results obtained by analysis of urine were confirmed by analysis of serum kinetics. In serum, as in urine, none of the subjects tested had more than one twentieth of CMC sulphoxides relative to the concentration of CMC. The serum peak level of the CMC sulphoxides in all cases occurred 1 to 2 h later than that of the parent compound CMC. This might indicate that the sulphoxides have been formed in the human body rather than by chemical oxidation during sample storage. The serum concentrations of all three compounds depicted in Fig. 5 had decayed to low levels 8 h after CMC ingestion, indicating that an 8 h urine collection should suffice to reflect sulphoxidation capacity. In contrast, a recent report [15] claims that considerable amounts of (unspecified) sulphoxides were eliminated mainly after a lag time of 8 h, as determined by paper and thin layer chromatography.

The differences from the previous studies (e.g. [1]) might have come from use of a test dose of 1.125 g CMC, whereas the British workers had employed 0.75 g. An intermediate sulphoxidizer would appear as a poor sulphoxidizer on saturation of the sulphoxidizing enzymes (R. H. Waring, personal communication). However, Fig. 6 shows that doses as low as 0.19 g or as high as 2.25 g failed to vary the ratio of CMC to its sulphoxides or to its secondary metabolite thiodiglycolic acid. Using the sulphoxidation index introduced by Mitchell et al. [1], all volunteers in Berlin were extremely inefficient sulphoxidizers in contrast to the 30% of poor sulphoxidizers in the groups studied by the British authors [1],

Only 20 to 50% of the dose was recovered as CMC and metabolites. This should not be surprising as CMC metabolites may enter general amino acid degradation pathways. Animal studies with radiolabeled CMC have been started to provide a complete picture of carbocysteine degradation and to identify further metabolites.

Thiodiglycolic acid as a metabolite of carbocysteine was first reported by Turnbull et al. [2] using gas chromatography. Thiodiglycolic acid seems to be formed by the enzymes of intermediary metabolism, as significant transamination by incubation of CMC with glutamate-oxalacetate transaminase could be measured, and thiodiglycolic acid was formed in the presence of ketoglutarate dehydrogenase and succinyl-CoA-synthetase (unpublished observations).

By a completely different analytical approach, using ^{13}C-NMR analysis of urine after oral dosing of ^{13}C-labeled CMC to healthy volunteers, Meese et al. [16] obtained very similar results to the measurements presented here. Furthermore, in those studies [16] the sulphoxide of thiodiglycolic acid was measured in urine as a quantitatively relevant metabolite of CMC. Woolfson et al. [17] described an HPLC method with electrochemical detection for quantifying CMC, (R)- and (S)-CMC sulphoxides, methylcysteine, and its sulphoxides. As described here,

Table 1. Serum kinetics after 1.1 g carbocystein p. o. in 9 healthy volunteers. Median and first and third quartiles are given as evaluated individually for each volunteer

	Carbocysteine	Carbocysteine (−) (S)-sulphoxide	Carbocysteine (+) (R)-sulphoxide
C_{max} [μg·ml^{-1}]	8.2 (6.4–11.7)	0.035 (0.022–0.05)	0.09 (0.069–0.15)
t_{max} [h]	3 (2–4)	4 (3–5)	4 (3–5)
AUC (0–8 h) [h·μg·ml^{-1}]	32.6 (13.4–38.5)	0.144 (0.09–0.178)	0.42 (0.29–0.61)
$t_{1/2}$ [h]	1.18 (0.62–1.51)	0.87 (0.56–2.15)	1.10 (0.79–3.36)
CL/f [ml·min^{-1}]	530 (460–875)	–	–

only low levels of sulphoxides were detected in a small number of urine samples from humans who had received carbocysteine orally.

In conclusion, an unexpectedly low extent of sulphoxidation of CMC was observed in the present study. This indicates that some compounds included in the sulphoxidation index [1] may have been misidentified. The relevance of the proposed pharmacogenetic polymorphism of sulphoxidation appears questionable and the interesting correlations claimed with certain diseases require further investigation.

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