HPLC-ANALYSIS OF S-CARBOXYMETHYLCYSTEINE AND ITS SULPHOXIDE METABOLITES

J. Brockmöller, Z.J. Simane*, I. Roots
Institute of Clinical Pharmacology
Klinikum Steglitz
Free University of Berlin,
Hindenburgdamm 30, D-1000 Berlin 45
**E. Merck AG, Darmstadt, FRG*

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I. INTRODUCTION

Genetic polymorphisms in xenobiotic metabolism have a major influence on the therapeutic efficiency and the occurrence of side effects of certain drugs /1/. Moreover, certain diseases, such as cancer /2/, have been associated with genetically polymorphic enzyme activities in foreign compound metabolism.

Activity of enzymatic oxidation of certain sulphides in humans has a wide range of interindividual variation, most likely reflecting a genetic polymorphism of sulphoxidizing enzyme activities /3/. S-Carboxymethyl-L-cysteine (carbocysteine, CMC) is used as a test substance for determination of this sulphoxidation phenotype in man. /3/. Figure 1 shows the structure of this substance and part of its metabolic pathway, as proposed by Waring and Mitchell /4/. An association between impaired sulphoxidation and D-penicillamine toxicity has been described /5/. Recently, sulphoxidation phenotype was also correlated with primary biliary cirrhosis /6/.

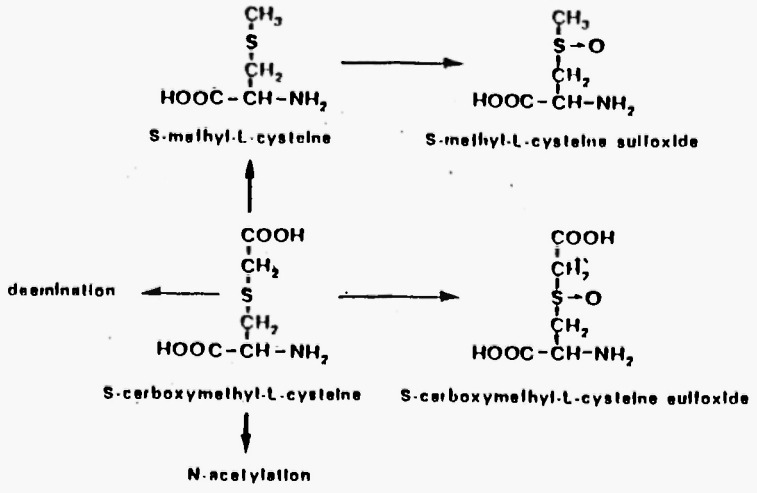

Fig. 1: Metabolic pathways of S-carboxymethyl-L-cysteine /4/.

Pharmacogenetic phenotyping requires a reliable method for *in vivo* determination of the respective enzyme activities and the test substrate should be devoid of serious side effects. An elegant example of non-invasiveness is the determination of N-acetyltrans-

ferase activity by urine analysis of caffeine metabolites /7/: test substance is a cup of coffee. Test substrate for sulphoxidation phenotyping is the similarly harmless mucolytic agent carbocysteine. Currently, its urinary metabolites are analysed by paper-chromatography /5/. This manuscript describes the development of an HPLC method for determination of sulphoxidation activity utilizing precolumn derivatization amino acid analysis methods. Several reagents are currently used for this purpose, including *ortho*-phthaldialdehyde (OPA), dimethylaminoazobenzene sulfonyl chloride (DABS-Cl), and dimethylaminonaphthalene-5-sulfonyl chloride (DANS-Cl). These reagents were compared for analysis of carbocysteine and its metabolites. In our hands DABS-Cl was the most suitable reagent for this purpose. Direct quantitation is possible for CMC, CMC sulphoxide, the decarboxylated derivative S-methylcysteine (MC), and its sulphoxide. The respective sulfones can be equally well determined, but have been found only in trace amounts in biological fluids in this study. Analysis of the four acetylated derivatives would be possible after hydrolysis.

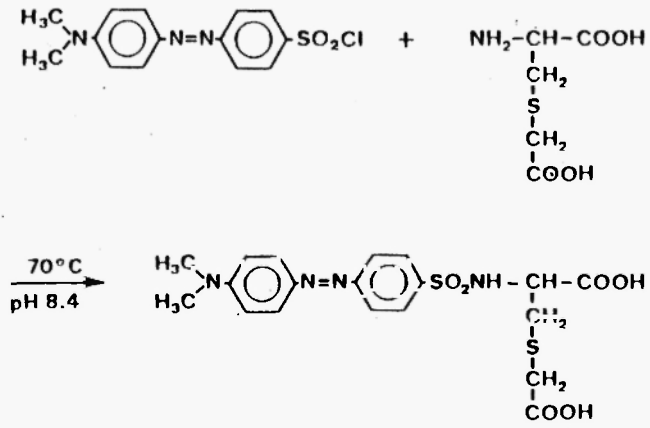

Fig. 2: Precolumn derivatization of carbocysteine using DABS-Cl.

Recently, reports on HPLC-quantitation of CMC have appeared for pharmacokinetic measurements of CMC serum levels /8/, but do not allow quantitation of CMC-metabolites. But also a similar approach as in the present manuscript has been reported, applying

electrochemical detection after precolumn derivatization with ortho-phthaldialdehyde /9/.

II. MATERIALS AND METHODS

2.1 Chemicals

DABS-Cl and o-phthaldialdehyde were obtained from Pierce (USA), DANS-Cl was from Serva (Heidelberg, FRG), acetonitrile, acetone and dimethylformamide were Lichrosolv™-grade from Merck (Darmstadt, FRG). All other chemicals (analytical grade) were from Merck. Transbronchin™ (Degussa Pharma, Frankfurt, FRG) was the CMC brand used for in vivo tests. Reference substances including CMC, CMC-sulphoxide, methylcysteine, methylcystein-sulphoxide and their acetylated derivatives were synthesized by standard methods.

2.2 Analysis by high performance liquid chromatography

All separations were done on reversed-phase columns filled with Hypersil™ ODS, 5 μm particle size (from Shandon, UK). A low pressure mixing HPLC system (Series 400 from Perkin-Elmer) was used equipped with an autosampler (ISS 100 from Perkin-Elmer). Eluent A was 25 mM sodium acetate pH 4.25 including 4% dimethylformamide. Eluent B was acetonitrile. The elution gradient was: 0-1% eluent B in 10 minutes, 15-25% eluent B in 40 minutes and 25-40% eluent B in 25 minutes followed by a 10 min equilibration step (0 % eluent B) before injection of the next sample.

2.3 Derivatization-procedure using DABS-Cl

The method of Chang /10/ has been modified for analysis of plasma and urine samples. Urine samples are made up to pH 8 with 5 N KOH, 100 μl samples are dried, dissolved in 200 μl 50 mM sodium bicarbonate (pH 8.4) and derivatized by addition of 400 μl acetone containing 5 mg/ml DABS-Cl. Derivatization was done for 10 min at 70°C. Reaction was stopped by dilution with 2 volumes of a mixture of 40% ethanol, 30% acetone and 30% distilled water. Derivatives were stable for at least 3 days.

2.4 Precision

Calibration was performed by adding standards to blank urine samples. Linear regression curves were obtained between 5 and 500 μg/ml of CMC and its sulphoxides. Standard deviation at each concentration tested was below 7% at different days.

2.5 In vivo sulphoxidation phenotyping

Healthy volunteers (members of our institute, cf. table 1) took in the morning 3 capsules of CMC (a total of 1125 mg). Urine was collected for 8 hours. No other drugs were allowed, however all volunteers had their usual coffee intake of one to two cups.

III. RESULTS

3.1 Evaluation of derivatization parameters

Influence of derivatization conditions and sample parameters (such as pH and salt concentration) on derivatization yields was investigated for urine or plasma samples and these conditions are given in Materials and Methods. Maximum yield of derivatization was obtained by use of 2 mg (= 6.2 μmol) of reagent for analysis of 100 μl urine samples: Reagent must be present in excess over CMC and metabolites, but also over all other nucleophiles present in the sample, which might react with DABS-Cl at pH 8.4. Reaction temperature and time were similarly determined and pH-value of the reaction must be slightly basic, as only the unprotonated amino group can react. Therefore, urine samples being sometimes rather acidic, were adjusted to pH 8 prior to derivatization.

3.2 Evaluation of optimal conditions for high performance liquid chromatographic analysis

The acetate buffer for chromatography of DABS-Cl derivatized amino acids /10/ was modified for analysis of CMC and its sulphoxides. A rather acidic pH of 4.25 was found optimal for separation of CMC and its sulphoxides and a shallow elution

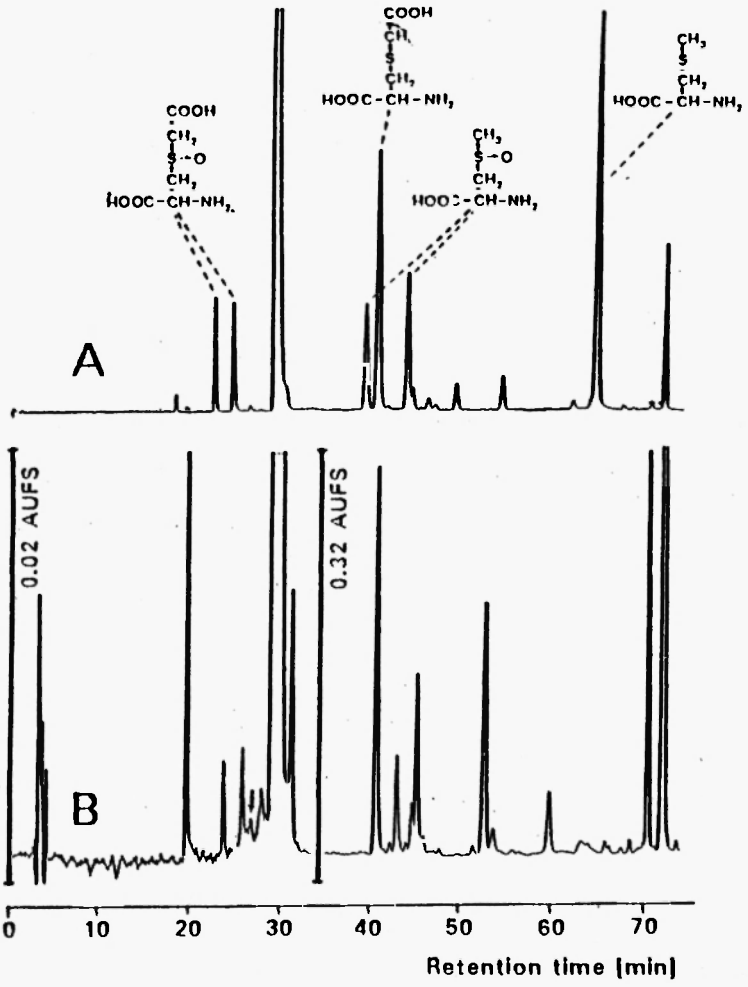

Fig. 3: A: Reversed phase chromatogram of CMC and three of its amino group containing metabolites (100 µg of each). Note, that the two sulfoxides appear as double peaks, representing the optical isomers formed by sulfoxidation.
B: Chromatogram of a urine sample (8 h collection after ingestion of 1.1 g CMC). Note the change in the recorder attenuation. The peak indicated by an arrow is carboxymethylcysteine sulfone, as tested by chromatography of this sulfone after chemical oxidation. Reliability and precision of these determinations was confirmed by repeated analysis and also by a further determination of each urine sample after addition of a standard mixture containing 25 µg of CMC, methylcysteine and their sulphoxides.

gradient was applied. Under these conditions, no interfering substances were seen at the positions in the chromatograms, at which CMC and its sulfoxides have been identified. All endogenous amino acids eluted later than CMC and its sulphoxides. For methylcysteine and its sulphoxides a better separation from some interfering peaks was obtained at pH 5.4.

3.3 Evaluation of alternative methods

Two other reagents widely used in analysis of biological amines, namely *ortho*-phthalaldehyde/2-mercaptoethanol and dansylchloride have been tested and gave essentially equivalent results. Also by use of these reagents, the two optical enantiomers of the sulphoxides could be well separated, but separation from some interfering peaks was less satisfactory. Moreover, derivatives after *o*-phthaldialdehyde derivatisation were not stable.

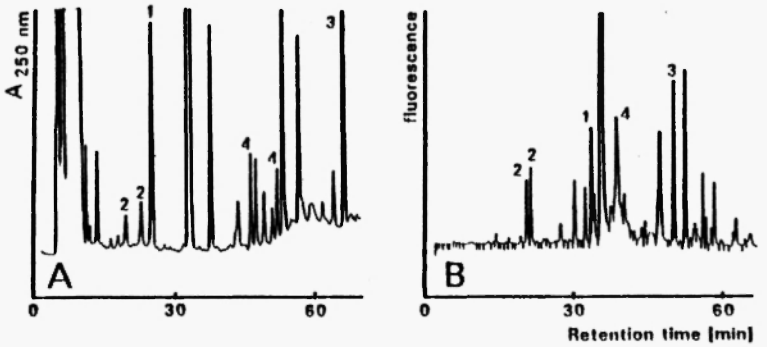

Fig. 4: A. Analysis of a blank urine sample spiked with 30 μg of CMC /1/, CMC sulphoxide /2/, methylcysteine /3/ and methylcysteine sulphoxide /4/ after derivatization with DANS-Cl. This chromatogramm was recorded using UV-absorbance (250 nm) monitoring.
B. Analysis of a similar sample using derivatization with o-phthaldialdehyde/2-mercaptoethanol and fluorescence detection (λex = 340 nm; λem = 450 nm).

3.4 Application of the HPLC-method to sulphoxidation-phenotyping

Chromatograms of these urines all looked similar, containig a large amount of unchanged drug, about 3-6% of carboxymethyl-

cysteine sulphoxide relative to the excreted CMC, and no notable amounts of methylcysteine or methylcysteine sulphoxide. A typical chromatogram is shown in figure 3b. There was no significant difference in the amounts of R- and S-carboxymethylcystein sulphoxide.

TABLE 1

Application of the carbocysteine-test for sulphoxidation phenotyping using the HPLC-procedure. 1125 mg (3 capsules) of CMC were given orally.

Volunteer	% recovery of dose as CMC and CMC sulphoxide in urine	CMC-sulfoxide % CMC + CMC sulfoxide	Total urine volume ml
Female 38 y.	45	3.3	575
Female 30 y.	41	4.2	394
Female 37 y.	56	5.0	1580
Female 25 y.	38	2.6	340
Female 41 y.	37	3.4	475
Female 23 y.	30	2.5	300
Male 31 y.	51	2.9	375
Male 33 y.	39	2.9	380
Male 26 y.	35	2.9	400
Male 30 y.	20	4.4	490
Male 46 y.	50	3.9	680

IV. DISCUSSION

By the HPLC-method described here, quantitation is possible for CMC and three of its metabolites. In addition to previous methods, the two optical isomers formed by in vivo sulphoxidation are quantified separately and by this HPLC method quantitation is also possible for the sulfones. Detection by this method is based on labelling of the amino groups, therefore not all metabolites were detected, which can be quantified by paper chromatography and platinic chloride visualization. No extraction steps are included in the DABS-Cl method.

The optical isomers were found in urine of persons tested so far in nearly equal amounts. But these in vivo sulfoxidation measurements

may be a sum of different processes and chirality of sulphoxidation of CMC has not yet been investigated by in vitro experiments. Further in vitro studies seem at present very necessary to see, whether sulphoxidation of CMC (and oxidation reactions of its metabolites methylcysteine, N-acetylcysteine and N-acetyl-S-methylcysteine) can be referred to a single enzyme.

We expect, that measurement of carboxymethylcysteine and carboxy-methylcysteine sulphoxide will be sufficient for determination of sulphoxidation phenotype, but this should be confirmed in a larger reference collective. Surprisingly, only relatively small amounts of sulfoxide have been found in the persons tested. This contrasts to the findings of Waring et al. /3/. Therefore, further studies are necessary to determine, whether saturation kinetics exists for sulphoxidation in man. Futhermore, differences in sulphoxidation capacity due to ethnic differences and due to certain environmental factors might affect the outcome of sulfoxidation phenotyping.

Special care has to be taken to avoid artificial chemical sulphoxidation as well as sulphoxide reduction during sample storage, derivatisation, or during chromatography. Use of 2,2'-thiodiethanol is currently tested for this purpose.

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