Tracing the Human Metabolism of Stable Isotope-Labelled Drugs by *ex vivo* NMR Spectroscopy
A Revision of *S*-Carboxymethyl-L-cysteine Biotransformation

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A direct structural identification, and quantitative assessment below the 50 nmol/ml level, of the full pattern of renally excreted metabolites is made possible by ^{13}C NMR measurements of untreated urine samples when stable isotope-labelled (^{13}C) drug analogues are administered to humans. The full potential of the new *ex vivo* NMR approach is exemplified by a study, for a group of volunteers, of *S*-carboxymethyl-L-cysteine metabolism. The metabolic sulphoxidation pathway of *S*-carboxymethyl-L-cysteine in man, accepted so far, needs to be profoundly revised on the basis of the ^{13}C NMR results.

Introduction

It is a well-established fact that the metabolism of numerous drugs is subject to pronounced interindividual variation, part of which is genetically determined. It would be desirable, therefore, in testing drug metabolism to have a panel of volunteers which is statistically significant for the patients eventually to be treated. So far, data on human metabolism have been derived almost exclusively from analogues incorporating radioactive labels. The number of volunteers, however, who may be included in a screening with such compounds is for ethical reasons limited to the absolute minimum [1]. Radioisotope studies also afford, primarily, an overall balance of matter. Structural assignments by necessity require chromatographic work-up, and correlation of the retention times of peaks, which have been indicated as radioactive, with those of authentic material. Even so, the procedure provides no absolute proof of a metabolite structure since chromatographic coincidence can rarely be excluded with certainty. Reliable quantification is further hindered when certain metabolites are constituents also of the regular diet, or of endogenous origin [2].

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Many of these problems can be overcome if the drugs to be investigated are labelled with the naturally occurring, non-radioactive isotopes ^{13}C and ^{2}H in lieu of the commonly used radioisotopes ^{14}C and ^{3}H. The NMR spectrometer can then be employed as detector for these magnetically active nuclei [3]. From individual chemical shifts, ^{13}C or ^{2}H spectra might provide direct structural proof of the respective metabolites. Interference from food constituents or endogenous material thus is excluded since the ^{13}C or ^{2}H label can be incorporated chemically with $\geq 99\%$ enrichment.

Methods

Materials

S-Carboxy-[^{13}C]methyl-L-cysteine (**1b**), *S*-[^{13}C]-methyl-L-cysteine (**5b**), several of the unlabelled and all of the ^{13}C labelled compounds (series **a** and **b,** respectively, Scheme 1) were prepared as described recently [4]. *S*-Carboxymethyl-L-cysteine (**1a**), *S*-methyl-L-cysteine (**5a**), and thiodiglycolic acid (**9a**) were used as received (Fluka, Neu-Ulm, F.R.G.).

Instrumentation

Proton-decoupled ^{13}C NMR spectra were recorded on Bruker CXP or AMX 300 spectrome-

ters (75.5 MHz; 30 °C, 10 mm tubes) with standard PFT techniques. For regular, non-lyophilized samples, 2000–5000 transients were aquired (1.5–4 h; 20–30° pulses; repetition time 2–3 s; LB = 1.5). For samples where resolution of extremely closely spaced resonances was required, *e.g.* in the 40 ppm region (see text), acquisitions were run for up to 24 h. A substantial reduction in spectrometer time is expected with special techniques; work in this direction is in progress in collaboration with Bruker.

Additional quantitative measurements were carried out in a similar way in a standard 10 mm ^{13}C probe head with a Bruker WP 80 (20.1 MHz; 35 °C; 10 mm sample tubes). About 100,000 FIDs were accumulated without relaxation delay during 24 h (45° pulses; repetition time 0.7 s; spectral width 5000 Hz).

Sample preparation

Aliquots of the untreated urine samples (1 vol.-% corresponding to 3–10 ml) from the respective collection periods were adjusted to pH 7.0 by adding a few drops of 0.1 N NaOH, diluted with 1 M phosphate buffer (pH = 7.0, 1–4 ml) and D_2O (0.2–1.5 ml) as internal lock substance, and finally spiked with acetonitrile (104 µl, 2.0 mmol; internal standard, $\delta(CH_3)$ 3.70 ppm relative to sodium 2,2,3,3-[$^2H_4$]-3-(trimethylsilyl)propionate). For long-term measurements (> 5 h), 1 mg/ml of sodium azide was added to prevent bacterial growth.

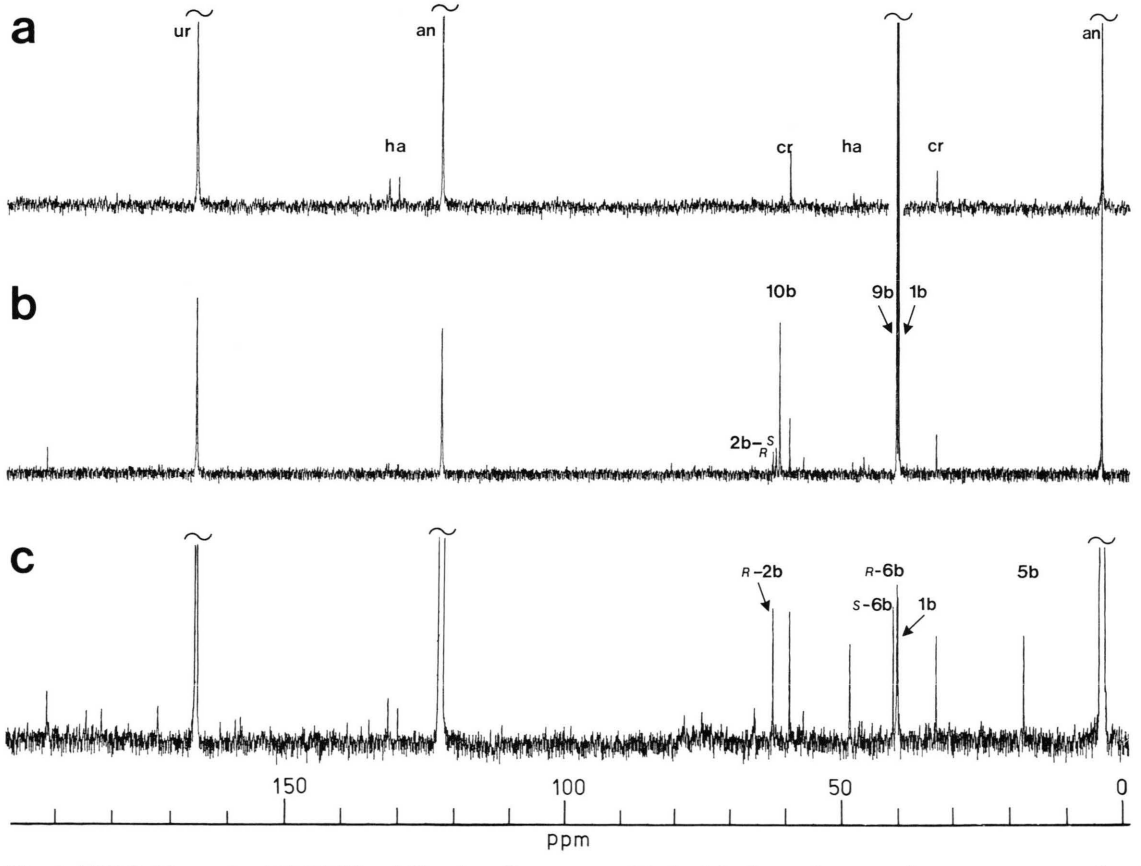


Fig. 1. ^{13}C NMR spectra (75.5 MHz, shift values [in ppm, see Methods] of prominent peaks or resonances of enriched carbons are given) of a) native human blank urine, an = acetonitrile, internal standard (3.70, 122.30), cr = creatinine (33.08, 59.27), ha = hippuric acid (46.80), ur = urea (165.58); b) a urine collected during 0–8 h after administration of 750 mg of **1b** (**1b:** 40.03, **9b:** 40.31, **10b:** 61.08); c) a blank sample spiked with 50 nmol/ml each of isomerically pure (*R*)-**2b** [62.26, (*S*)-**2b:** 61.72], **5b** (17.52), and a mixture (*ca.* 1:1) of (*R*)-**6b** (40.16) and (*S*)-**6b** (40.95) (see Scheme 1). – The configurational prefixes (*R*) and (*S*) refer to the sulphoxide moiety of L-cysteines [4].

Under these conditions the salt neither reduced sulphoxides nor influenced the spectral pattern.

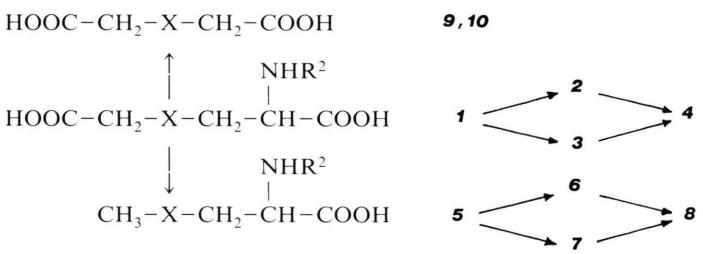
Studies with humans

Aqueous suspensions of finely powdered *S*-carboxy-[^{13}C]methyl-L-cysteine [**1b,** 375 mg (2.08 mmol) or 750 mg (4.17 mmol)] or *S*-[^{13}C]-methyl- L-cysteine [**5b,** 136 mg (1.00 mmol)] were administered orally to healthy volunteers (**1b:** $n = 15$; **5b:** $n = 1$). Urine was collected in fractions over 24 h according to the usual protocol [5], and kept frozen (−20 °C) until *ex vivo* NMR analysis.

Results and Discussion

In order to test the feasibility of the NMR approach, we have first measured the ^{13}C NMR spectrum of untreated human urine. Significant signals in this "native" spectrum (Fig. 1a), apart from those of the added standard CH_3CN, arise from urea, creatinine, and hippuric acid only. In comparison, Fig. 1b shows the ^{13}C spectrum of a human urine sample, likewise untreated and collected from 0−8 h after administration of the mucolytic agent *S*-carboxymethyl-L-cysteine (**1b,** see Scheme 1), labelled in one methylene group with ≥99% ^{13}C. The two prominent signals in the spectrum are due to unchanged **1b** and thiodiglycolic acid (**9b,** see Scheme 1) which in this study was identified for the first time as the main metabolite of **1.** These peaks, pertaining to the applied drug, by far surpass, in their intensity, all signals of endogenous material (*cf.* Fig. 1a). *Ex vivo* metabolites of a ^{13}C labelled drug thus appear clearly set off from the background of the multitude of endogenous compounds (biological noise) in the renal excretions. The samples from all fifteen volunteers displayed similar spectral patterns. After 8 h, for instance, most of the parent drug has been eliminated whereas residual amounts of **9b** and **10b** are still excreted after 24 h (data not shown).

Additional smaller signals are due to further metabolites formed and/or excreted on the <10% level. Among these, the sulphoxide **10b** has been identified for the first time with the NMR method; it is present in the 0−8 h sample with 1−6% of the applied dose. In contrast, no trace can be detected of the metabolites (in this case ^{13}C-labelled) postulated in a previous study [6], *e.g. S*-methyl-L-cysteine (**5b**), *N*-acetyl-*S*-methyl-L-cysteine (**7b**), or



Scheme 1.

a, b[a]	R^1	R^2	X[b]	
1	HOOC−^{n}CH_2	H	S	
2	HOOC−^{n}CH_2	H	SO	
3	HOOC−^{n}CH_2	$COCH_3$	S	
4	HOOC−^{n}CH_2	$COCH_3$	SO	
5	^{n}CH_3	H	S	
6	^{n}CH_3	H	SO	
7	^{n}CH_3	$COCH_3$	S	
8	^{n}CH_3	$COCH_3$	SO	
9			S	
10			SO	

[a] **a:** n = $^{12}CH_2/^{12}CH_3$ (natural abundance); **b:** n = $^{13}CH_2/^{13}CH_3$ (≥99% enriched).

[b] Sulphoxides **2, 4, 6,** and **8** consist of a mixture of diastereoisomers. The *S*-alkyl cysteines have the natural L configuration.

the respective sulphoxides **6b** and **8b.** Even *S*-carboxymethyl-L-cysteine-*S*-oxide (**2b**), the proposed main metabolite, is detected only in minute amounts (≤2% of the dose in the 0–8 h period). The absence of significant amounts of these compounds is established, beyond any doubt, by adding 50 nmol each of authentic ^{13}C-labelled material (Fig. 1c). The spectral trace in Fig. 1c presents clear evidence that metabolites can be identified unequivocally, and characterized structurally, by ^{13}C NMR at a level of less than 1% of the applied dose. The spectrum also demonstrates the sensitivity of the ^{13}C NMR probe for slight structural differences, as evidenced by the well-resolved signals for the two diastereoisomeric *S*-oxides **2b** [$\delta(^{13}CH_2SO)$ 62.26 (*R*), 61.72 (*S*)] and **6b** [$\delta(^{13}CH_3SO)$ 40.95 (*S*), 40.16 (*R*)].

The NMR method thus allows, after administration of a ^{13}C-labelled drug, simultaneous detection, with concomitant structural characterization, of all renally excreted biotransformation products which retain the label and are excreted with at least 0.1–1% (depending, of course, on applied dose and volume intake before and during the sampling period). The biological samples can be measured directly, without any discriminative manipulations, which is especially advantageous in the case of chemically sensitive and/or volatile structures. Metabolites with the ^{13}C label in a methyl or methylene group furthermore allow for quantitative evaluation; this is an unexpected bonus and has been checked very carefully with authentic labelled material [7]. Thus, the mean 24 h urinary excretion was balanced as follows (% of the dose): parent drug **1** (≈20%), major metabolite **9** (≈20%), minor metabolites **10** (≈13%) and **2** (≈1%, sum of diastereoisomers). This balance, as well as an individual total recovery of up to 70%, has been verified by methodologically independent measurements with newly developed HPLC [8] and GC/MS [9] techniques.

Chemical reactions, *e.g.* for obtaining additional confirmation of individual metabolite structures, can be performed directly on the native sample in the NMR tube. Thus, all thioethers are transformed quantitatively into the respective sulphoxides if an approximately stoichiometric amount of H_2O_2 is added to the samples shown in Fig. 1b and 1c. Under the prevailing mild reaction conditions, the sulphoxides formed are not in turn oxidized further to the corresponding sulphones [4]. If these samples or untreated urines are lyophilized, and the measurements repeated in D_2O, one observes selective "loss" of those carbon resonances that originate from methylene or methyl groups, adjacent to the sulphoxide groups (not shown), due to slow H/D exchange [4].

In a complementary experiment, *S*-methyl-L-cysteine (**5b**), with the ^{13}C label in the methyl group, was applied orally (136 mg, 1.00 mmol). The renal excretions, collected for three successive 4 h periods, showed no trace of either unchanged drug **5b** or its *N*-acetyl derivative **7b,** but rather the signals of a mixture of the diastereoisomeric *S*-methyl-L-cysteine-*S*-oxides **6b** [(+)-(*S*):(−)-(*R*) = 30:70]. This result finally refutes the biotransformation pathway proposed for **1a** [6], with *S*-methyl-L-cysteine (**5a**) as primary metabolite in the human organism (see Scheme 1). In this case, significant amounts of **6b,** or the corresponding mercapturates **7b, 8b,** would be expected in the urine samples, on the basis of the findings for authentic **5a** [10]. There is no trace, however, of the respective signals in the ^{13}C spectra. On the basis of the stable isotope methodology reported here, a genetically determined polymorphism of *S*-carboxymethyl-L-cysteine sulphoxidation in respect to the formation of cysteinyl sulphoxides [5] cannot be verified.

Recent results indicate that the NMR approach is not restricted to carbon. When synthetic, and selectively deuteriated analogues of the alkaloid *l*-sparteine [11] are administered to rats and humans, the parent drug, and its metabolites excreted in the urine, can be monitored directly by ^2H NMR [12]. The pronounced differences in regio- and stereoselectivity, observed for the biotransformation, clearly demonstrate *ex vivo* NMR spectroscopy of stable isotope-labelled drugs as a powerful tool for rapidly uncovering metabolite patterns and species differences in drug metabolism.

Acknowledgements

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[1] Patients, children, and young females are for obvious reasons generally excluded from studies with radiolabelled drugs. As a rule, metabolism studies with radioisotopically labelled analogues are restricted to single, healthy individuals. Thus, inter-individual variations in drug metabolism, as reported for **1 a** [5], cannot be recognized.

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