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NMR study of whole rat bile: the biliary excretion of 4-cyano-N,N-dimethyl aniline by an isolated perfused rat liver and a liver *in situ*

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Abstract: The structure of two biliary metabolites of 4-cyano-N,N,-dimethyl aniline (CDA) contained in whole rat bile have been studied in detail by NMR at 400 MHz. A 4-cyano-N-methyl glutathione-N-aniline conjugate was identified as a biliary metabolite of CDA using relatively simple ^{1}H NMR techniques. Isotopically ^{13}C labelled CDA was used to generate ^{13}C labelled xenobiotic conjugates. Our use of $^{1}H/^{13}C$ heteronuclear NMR techniques, in particular a ^{13}C-selective HMQC–TOCSY experiment, allowed a N-β-glucuronide conjugate, a previously unknown biliary metabolite of CDA, to be identified. Bile samples obtained from both the isolated perfused rat liver and the rat liver *in situ* were analysed.

Keywords: *Isolated perfused rat liver; bile; NMR; HMQC; HMQC–TOCSY; ^{13}C selective.*

Introduction

Using radiolabelling and routine analytical methods during xenobiotic metabolism studies it is possible that metabolites formed from a given compound may remain unidentified. This is because fragments of the compound not containing a radiolabel are not isolated and analysed. Compound metabolites may also prove difficult to identify because of their instability during isolation [1]. Provided that molecules are present in sufficient quantities, and contain NMR sensitive nuclei, NMR has the potential to examine all molecules in a given solution. NMR analysis of unpurified biological samples potentially could be a method for analysing xenobiotic metabolites that are not accessible during conventional drug metabolism studies. Although this potential has yet to be realised it has been demonstrated that significant information can be obtained during drug metabolism studies from examining whole biological fluid by NMR [2]. Our studies provide an extension of the use of NMR in this field.

The metabolism of 4-cyano dimethyl aniline (CDA) has been studied in detail by others as part of a collaborative study for the UK Environmental and Mutagenic Society [3, 4] in which it was compared with that of dimethyl amino azo benzene (DMBA). The biotransformation of DMBA and the biochemical mechanisms by which it is carcinogenic have also been studied in detail [3]. CDA is an analogue of this compound and the differences in the metabolism of these two compounds has been considered important for understanding the carcinogenic properties of these compounds. The biotransformation of CDA is thought to produce twelve distinct urinary metabolites [4, 5]. CDA was considered to be a suitable candidate for study within an isolated rat liver perfusion system because 25% of an administered dose at 18.5 mg kg^{-1} body weight appears in urine as mercapturic acid derivatives [4] (metabolite C — 10% and B — 15%). This type of xenobiotic metabolite is first formed in the liver as a glutathione conjugate before being excreted into the biliary tract. Enterohepatic circulation results in a glutathione conjugate then appearing in urine as a mercapturic acid derivative [6].

Our work describing the identity of the biliary excretory products of cefoperazone and

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benzyl chloride [7] allowed us to develop an experimental protocol for the analysis of xenobiotic metabolites in whole bile samples. We present here an NMR study of the biliary metabolites of 4-cyano-N,N,-dimethyl aniline (CDA). In contrast to other compounds we have studied CDA undergoes numerous metabolic changes before it is excreted in the urine, of which most are thought to occur in the liver. Our inspection of its biliary metabolites revealed that an expected glutathione conjugate and a previously unknown glucuronic acid conjugate are excreted into bile.

Isolated rat liver perfusion experiments can be criticized because they do not reflect precisely the *in vivo* situation. It is possible, therefore, that they may provide misleading results. We have addressed this issue by conducting *in situ* experiments: the results from these experiments clearly show that the biliary excretion of xenobiotics that occurs in the whole animal situation was duplicated in our rat liver perfusion experiments.

The work we present here also further explores the use of heteronuclear NMR experiments as analytical tools for characterizing xenobiotic metabolites in a whole biological fluids. During this work ^{13}C isotopic labelling of CDA was used and unconventional ^{1}H-^{13}C heteronuclear NMR experiments using 'rectangular' ^{13}C selective pulses were also devised. These techniques present a novel approach for the analysis of xenobiotic metabolites by NMR and represent a diversification of the use of ^{13}C isotopic labelling for the structural analysis of xenobiotic metabolites.

Experimental

Rat liver perfusion experiments

Bile samples were obtained from isolated perfused rat livers by the methods outlined in the preceding publication [7]. Bile samples were collected over a dry ice/ethanol mixture at hourly intervals over 5 h. CDA or $(^{13}CH_3)_2$-CDA (Shell Research Centre, Sittingbourne, Kent, UK) was administered at dose levels of 18.5 mg kg^{-1} body weight as a solution in corn oil (0.5 ml). This dose level was the same administered during whole body experiments in which the urinary metabolites of CDA were identified [3, 4].

Obtaining bile samples from a rat liver in situ

Sprague–Dawley rats (250–300 g) were chosen as liver donor animals. They were maintained on a standard laboratory diet (Diet 41B, Quest Ltd, Canterbury, UK) and allowed water *ad libitum*, and were starved overnight prior to use. Donor animals were maintained under di-ethyl ether anaesthesia during surgical procedures. Injectable anaesthetics were not used as they were found to be excreted into the biliary tract in large amounts.

The surgical procedure for obtaining bile samples from a rat liver *in situ* were as follows. The abdomen of the animal was swabbed with 95% ethanol and the peritoneal cavity opened by a small incision along the mid-abdominal line to reveal the liver and lower abdomen. The intestine were moved sideways from the lower liver to reveal the hepatic artery and bile duct. A cannula (40 cm, portex polyethylene tubing i.d. 0.28 mm; o.d. 0.61 mm) was inserted into the bile duct and firmly ligated. A puncture in the skin of the rat was then made close to the shoulders. The open end of the cannula was passed behind the rib cage and through this opening. The abdominal opening was then closed. During a recovery period the bile duct cannula was fitted into a harness that secured the cannula to the shoulders of the animal. The rat was then placed in a holding cage and allowed free access to food and water. The cannula was secured to allow bile samples to be collected.

Bile samples were collected for a control period of 30 min. CDA was administered at dose levels of 18.5 mg kg^{-1} body weight as a solution in corn oil (0.5 ml). An oral gavage was used to administer the dose directly into the stomach. 0–1, 1–3, 3–6 and 6–24 h bile samples were collected over ice.

Sample preparation for NMR analysis

Bile samples were exchanged twice with D_2O (99.8%), containing a standard amount (ca 0.5 mM) of sodium 3-trimethyl silyl propionate-2,2,3,3,-d_4(TSP) by lyophilization and were stored dry at −10°C. Samples were dissolved in 0.5 ml 100% D_2O for NMR analysis.

NMR experimental parameters

All NMR spectra were acquired on a Bruker AM 400 spectrometer using 5-mm dual probe and 5-mm sample tubes.

One-dimensional ^{1}H spectra

One-dimensional ^{1}H spectra were acquired

with a spectral width of 4800 Hz (−1 to 11 ppm) using 8,192 real data points. The acquisition time per transient was 1.27 s and 128 transients were sufficient for most analyses. A recycle time of 2.27 s was used per transient; this period was extended to 4 s for spectra on which integration were performed to allow for full ^{1}H relaxation. Integration was used to calculate the concentration of CDA metabolites in bile. This was achieved by comparing peak areas of aromatic resonances with that of the internal standard TSP peak. Presaturation (1 s) was used to remove the HDO resonance during acquisition of spectra; the minimum power pulse that produced complete saturation of the HDO signal was used. Free induction decays (FIDs) were zero filled to 16 384 data points and mild Gaussian apodization was applied prior to their Fourier transformation.

Two-dimensional ^{1}H–^{1}H COSY

Two-dimensional double quantum filtered ^{1}H–^{1}H COSY [8] were acquired with a spectral width of 4800 Hz (−1 ppm to 11 ppm) in both dimensions. The f_2 dimension was acquired with 2048 real data points. The f_1 dimension contained data points from 256 experiments. Sixty-four transients were acquired per experiment with an acquisition time of 0.32 s and a recycle time of 1.32 s. The incremental delay of this experiment did not exceed 0.016 s. Prior to Fourier transformation spectra the f_2 and f_1 dimension were zero filled to 4096 and 512 data points, respectively. Fourier transformation was carried out applying Gaussian apodization in both dimensions.

One-dimensional ^{13}C spectra

^{13}C spectra were acquired at 100 MHz with a spectral width of 29 500 Hz (−5 ppm to 290 ppm) using WALTZ 16 decoupling [9]. Spectra contained 32 768 real data points and had an acquisition time of 0.28 s. A relaxation period of 2 s was used between acquisitions. The number of transients per spectrum was extended until adequate signal to noise was obtained, usually 256 transients. Spectra were zero filled to 65 536 data points and mild Gaussian apodization applied prior to Fourier transformation.

J-modulated spin–echo spectra

Spectra were acquired with 32 768 data points over a spectral width of 29 500 Hz (−5 ppm to 290 ppm) with an acquisition time of 0.28 s. A relaxation period of 2 s was used between scans and spectra were acquired with a recycle time of 2.28 s per transient. The evolution delay in this experiment was set to 0.0068 s to optimize for a coupling of 147 Hz. Spectra were acquired with 256 transients and processed after zero filling to 65 536 data points. Mild Gaussian apodization was applied prior to Fourier transformation.

Two-dimensional ^{1}H–^{13}C correlation HMQC spectra

Spectra were acquired using methods described by Muller [10] using phase cycling devised by Cavanagh and Keeler [11].

Spectra were acquired with 2048 data points in the f_2 dimension covering a spectral width of 6400 Hz (−2 to 14 ppm). The f_1 dimension contained data points from 128 experiments covering a spectral width of 10 000 Hz (−5 to 95 ppm). Ninety-six transients were acquired for each experiment. The acquisition time per transient was 0.32 s and a recycle time of 1.32 s was used. The evolution delay in this experiment was optimized for small ^{1}H–^{13}C coupling and was set to 0.050 s. Prior to Fourier transformation f_2 and f_1 dimensions were zero filled to 4096 and 256 data points, respectively. Fourier transformation was carried out applying Gaussian apodization in both dimensions.

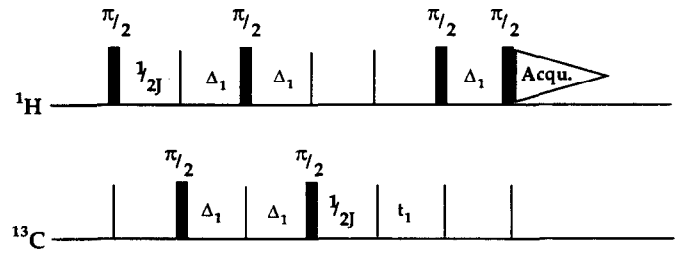


Figure 1
HMQC–COSY pulse sequence.

^{13}C-selective one-dimensional ^{1}H–^{13}C correlation HMQC NMR experiments

The pulse sequence used to acquire these spectra is shown in Fig. 1. It was based upon work published by Wollborn and Leibfritz [12] and constructed by Miss C.-W. Chung (Dept of Chemistry, University of Cambridge) who also devised the appropriate phase cycling. Δ_1 was a delay of 3 μs and t_1 was the incremented delay. 'Rectangular', selective ^{13}C pulses were generated on a Bruker AM 400 spectrometer by placing a 24 dB attenuator in the ^{13}C transmitter cable and by use of low power carbon pulses. The merits and disadvantages of using this type of selective pulse have been discussed by Kessler [13]. Our use of ^{13}C selective pulses to produce one-dimensional versions of a two-dimensional heteronuclear NMR experiments derives from work by Crouch [14] and Poppe [15]. HMQC–TOCSY spectra were acquired using a pulse sequence that was an adaptation of that shown above in which the final proton pulse of the experiment was replaced by a 32 pulse TOCSY sequence [16].

Spectra were acquired with a spectral with of 4800 Hz (−1 to 12 ppm) containing 8192 real data points. The acquisition time per transient was 1.27 s and transients were acquired with a recycle time of 2.42 s FIDs were zero filled to 16 384 data points and Gaussian apodization applied prior to their Fourier transformation.

Results and Discussion

A ^{1}H NMR spectrum of the 0–1 h bile samples obtained from a perfusion experiment after dosing with CDA (18.5 mg kg^{-1} body weight) is shown in Fig. 2. Four aromatic doublets in this spectrum were not present in a control bile spectrum and were presumably produced by CDA biliary metabolites. In a ^{1}H–^{1}H COSY spectrum of a 0–1 h bile sample these aromatic signals appeared as two sets of coupled doublets. These results reveal that two CDA metabolites were present in our bile samples, neither of which had undergone aromatic hydroxylation or sulphonation reactions. These reactions would result in loss of

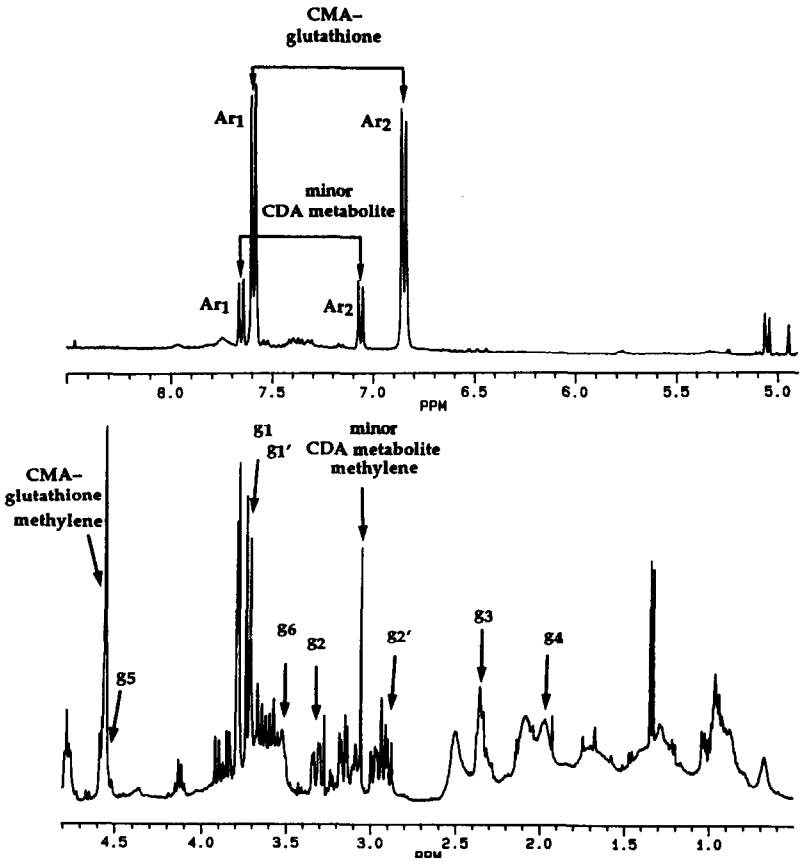
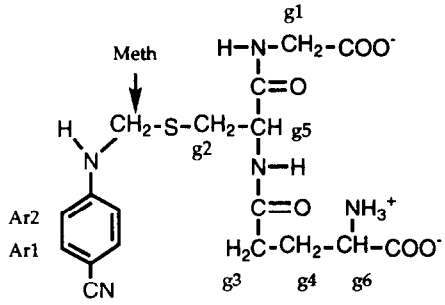


Figure 2
400 MHz ^{1}H NMR spectrum of a whole bile sample obtained from a perfused rat liver between 1 and 2 h after dosing with CDA (18.5 mg kg^{-1} body weight). Assignments for CMA-glutathione (I) and the minor CDA metabolite are shown.

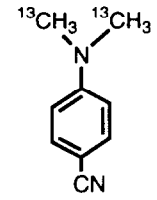
aromatic protons and would remove from a ^1H–^1H COSY spectrum the crosspeaks we observed. The two CDA metabolites will be referred to as the major and minor CDA metabolites for the purposes of this discussion.

Over a 5 h perfusion experiment the appearance of aromatic resonances remained as four doublets and indicated that substantial amounts of only two CDA metabolites were excreted into bile. By integration of these resonances it was possible to calculate the concentrations of the major and minor metabolites contained in bile samples (Table 1). The concentration of the major metabolite found in 1–2 h bile sample represents a ca 90 fold increase in concentration above that found in the circulating perfusate. Several other resonances appeared in bile spectra. Their appearance and chemical shifts indicated that a glutathione conjugate was present. Over several repeat experiments slight variations in the proportions of CDA biliary metabolites indicated that the glutathione resonances were associated with the major CDA metabolite. A further resonance, a dominant singlet at 4.55 ppm, was also associated with this metabolite. The presence of this signal in the absence of a further singlet at ca 2.5–3.0 ppm (an indicator of an N-dimethyl group) together with an association with glutathione, provided us with sufficient data to identify the major CDA metabolite as a 4-cyano-N-methyl glutathione-N-aniline conjugate (CMA-glutathione [1]). An assignment of the resonances associated with CMA-glutathione is made in Fig. 2 and Table 2. Confirmation of the assignments of conjugated glutathione was made by identification of the appropriate crosspeaks in a ^1H–^1H COSY spectrum (Fig. 3). During our ^{13}C isotopic labelling studies of CDA ^{13}C coupling from CDA to glutathione occurred in CMA-glutathione providing experimental evidence for the linkage between CMA and glutathione. Identification of the minor CDA metabolite contained in bile was not possible using ^{12}C-CDA. Apart from a doublet at 5.05 ppm no other resonances associated with this metabolite could be identified, largely because of the complexity of bile spectra.

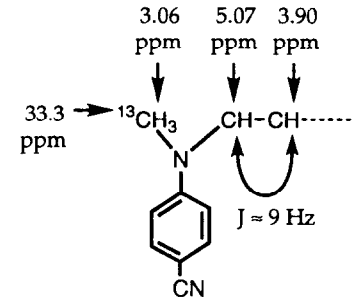
Bile samples were not collected over ice during our preliminary attempts to study CDA biliary metabolites. Bile samples obtained



I CMA–glutathione



II ^{13}C– CDA



III Minor CDA metabolite

I – assignments for CMA-glutathione are in FIGS. 2 & 3.
III – assignments for the minor CDA metabolite are in FIG. 6.

Table 1
Concentrations of CDA metabolites found in rat bile*

Sample	Mass (gm)	Amount of metabolite (μmole)		Concentration of metabolite in bile (mM)	
		Major	Minor	Major	Minor
Control	0.27	—	—	—	—
0–1 h	0.95	3.5	0.8	3.7	0.8
1–2 h	0.69	9.0	2.2	13.1	3.3
2–3 h	0.60	4.8	1.3	8.1	2.2
3–4 h	0.44	0.9	0.1	2.1	0.2
4–5 h	0.26	0.3	—	1.0	—

*The total amounts of CDA metabolites found in bile samples was: major metabolite 18.5 μmole (52.5% of CDA dosed); minor metabolite 4.4 μmole (11.5% of CDA dosed).

Table 2
Assignments of proton resonances for the CMA-glutathione conjugate and the minor CDA metabolite in bile

Proton	CMA-glutathione conjugate (Major metabolite) (ppm)	Minor CDA metabolite (ppm) incomplete
Ar_1	7.76	7.64
Ar_2	6.83	7.05
Methylene	4.55	3.05
g_1	3.73	—
g_1'	3.70	—
g_2	3.17	—
g_2'	2.91	—
g_3	2.35	—
g_4	1.96	—
g_5	4.54	—
g_6	3.55	

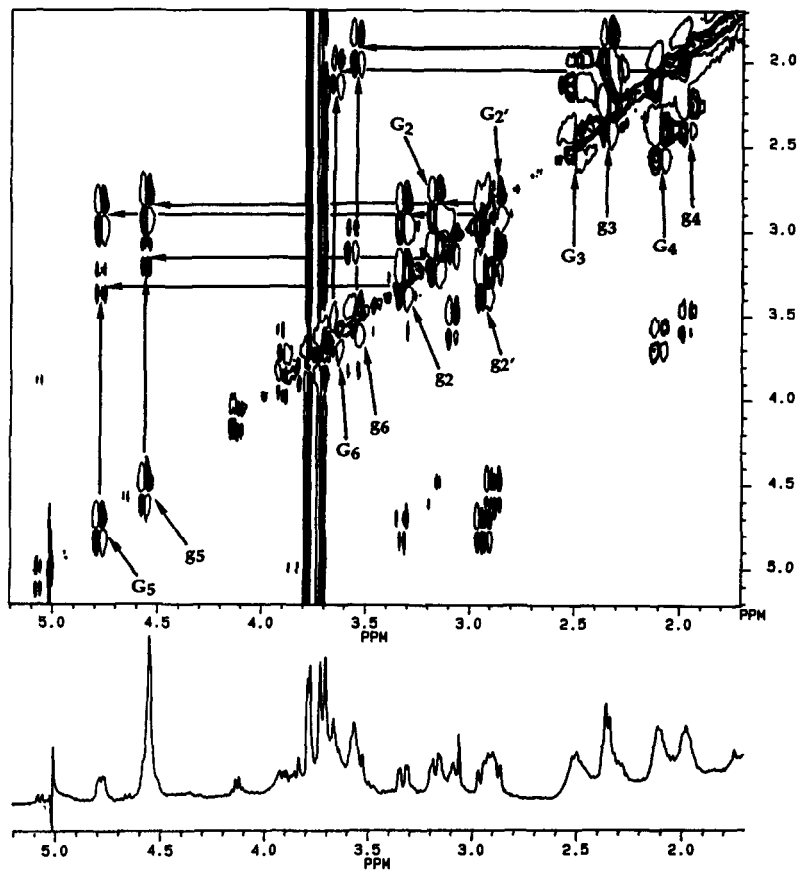


Figure 3
Contour plot of a 400 MHz ^1H–^1H COSY spectrum obtained from a whole bile containing CDA metabolites. Assignments for CMA-glutathione (I) and oxidized glutathione (G_1–G_5) are shown.

from these experiments produced ^1H spectra that contained a number of resonances associated with the dosing of CDA, none of which could be used to identify any particular CDA metabolite. On freezing bile samples as they were produced it subsequently became apparent that CMA-glutathione was unstable in whole bile. Our preliminary studies had produced bile samples that contained a mixture of CMA-glutathione and its breakdown products.

The use of isolated perfused liver experiments in xenobiotic metabolism studies can be criticized because they cannot reflect precisely

the *in vivo* situation. It is possible that an isolated perfused liver may not be as metabolically competent as a liver *in situ* and our isolated rat liver perfusion experiments may, therefore, have provided misleading results. Our experimental approach can also be criticized for a further reason. Dosing of a perfused liver at the same levels that are administered during *in vivo* experiments exposes a perfused liver to higher concentrations of xenobiotic than occur in a the blood during whole body experiments and for this reason our experiments may again have provided misleading results.

To counter the above arguments biliary CDA metabolites excreted by a rat liver *in situ* and from an isolated perfused liver exposed to CDA at low dosage levels were examined by NMR analysis. Spectra obtained from a perfused rat liver dosed at 2 mg kg^{-1} body weight showed resonances in the aromatic region at the same characteristic chemical shift as those associated with high dose levels (18.5 mg kg^{-1} body weight) of CDA. The CDA metabolites observed in low dose experiments were, therefore, the same as those observed at high dose levels. The proportions of major and minor metabolites found in bile samples were also comparable at both dose levels. This indicating that the capacity for metabolism of CDA in an isolated, perfused rat liver is also comparable at low and high doses of CDA.

Figure 4 contains the aromatic regions of spectra that were obtained from bile samples collected over 24 h from a rat liver *in situ*. Spectra obtained from bile collected over the first 6 h post-dosing contain the familiar pattern of aromatic resonances that are indicative of the major and minor CDA metabolites. Spectra of bile samples collected after 6 h post-dosing show no resonances associated with CDA metabolites. These results demonstrate that the metabolism of CDA we have highlighted for the isolated perfused rat liver also occurs in the whole animal situation. Aromatic resonances in Fig. 4 show that decay of CMA-glutathione also occurred in bile samples from *in situ* experiments.

The biliary excretion of CDA metabolites observed in isolated liver perfusion experiments does not, however, reflect precisely the CDA excretory profile observed for *in situ* experiments, even though both types of experiments produced exactly the same biliary metabolites. The major difference between

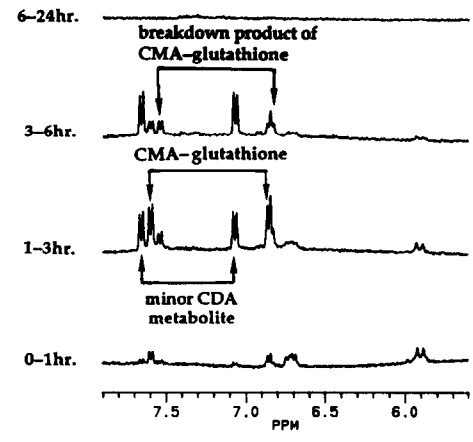


Figure 4
400 MHz ^1H NMR spectra of whole bile obtained from a rat liver between *in situ* over 24 h after orally dosing with CDA (18.5 mg kg^{-1} body weight). Aromatic resonances produced by CMA glutathione and the minor CDA metabolite have been assigned. Degradation of CMA glutathione, which produced extra aromatic signals, has occurred in the bile samples used to produce these spectra.

these two studies was *in situ* experiments produced bile samples containing higher proportions of the minor CDA metabolite. The release of significant amounts of CDA metabolites during perfusion experiments also occurred at an earlier stage; during the first 2 h after dosing, in contrast to *in situ* experiments where this occurred over a longer period of up to 6 h after dosing. As CDA must travel from the intestine to the liver before undergoing biliary excretion in whole body situation these observations are not surprising. Our observations demonstrate that perfusion experiments produce qualitatively useful results and show that it is only possible to obtain accurate quantitative data describing the metabolism of xenobiotics *in vivo* by conducting whole body experiments.

To study the structure of the minor CDA metabolite and the breakdown products of CMA-glutathione we conducted ^{13}C isotopic labelling studies (II). A ^{13}C NMR spectrum of a 0–1 h bile sample containing ^{13}C labelled CDA metabolites is shown in Fig. 5. This spectrum contains two resonances produced by CMA-glutathione and the minor CDA metabolite.

During studies of the metabolism of antipyrine in an isolated perfused rat liver [17] a ^{13}C resonance of 84.5 ppm was used to identify formaldehyde hydrate as a biliary metabolite of this compound; the formaldehyde was produced as a product of antipyrine demethyl-

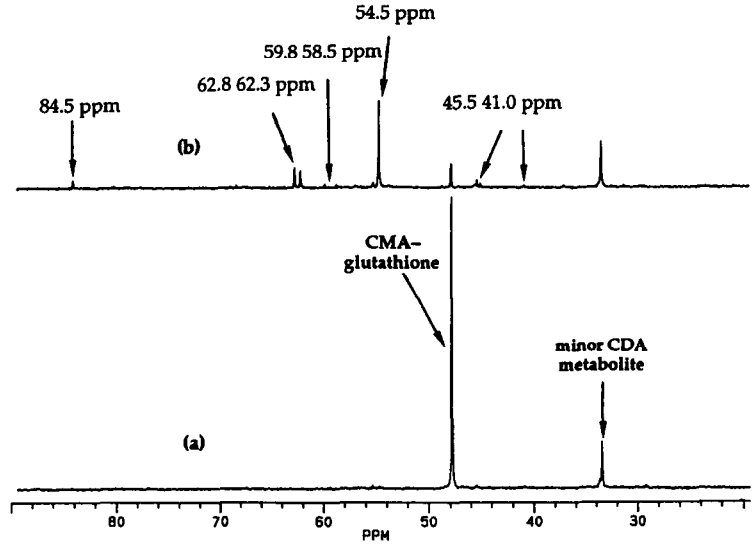


Figure 5
100 MHz ^{13}C spectra obtained from bile samples (a) before and (b) after decay of CMA glutathione. ^{13}C labelled CDA was used as a dose compound to produce bile samples for these experiments.

ation. Demethylation occurs to CDA as the first step of its metabolism in rats. In the light of the above observations it would not have been surprising after dosing a perfused liver with (^{13}C) CDA to observe a ^{13}C resonance produced by formaldehyde hydrate in bile spectra. A resonance at 84.5 ppm was, however, not observed and the release of substantial amounts of formaldehyde into the bile duct clearly does not occur from the biotransformation of CDA: it may be, of course, that any formaldehyde produced is rapidly oxidized to CO_2 and lost from view.

^{13}C NMR spectra were acquired during the degradation of ^{13}C-labelled CMA-glutathione in bile. Spectra were obtained at 30 min intervals over a 14 h incubation at 310 K incubation. They show that the intensity of the ^{13}C methylene resonance at 47.7 ppm produced by CMA-glutathione was rapidly lost during the incubation period. Bile samples incubated for longer than 14 h showed that a resonance at 47.7 ppm completely disappeared from bile spectra. In contrast, the ^{13}C signal from the minor CDA metabolite did not change in intensity during this period, demonstrating the stability of this metabolite. Several resonances appear in ^{13}C bile spectra during the degradation of CMA-glutathione. Since CMA-glutathione contains only a single ^{13}C label then each of these resonances was presumed to represent a single decay reaction product.

The first and largest resonance prduced as a result of decay reactions was at 54.5 ppm. This was the only breakdown product to appear during the first 3 h of incubation, and it could be formed directly from CMA-glutathione. Several other resonances (58.5, 59.8, 62.3, 62.8 and 84.5 ppm) appeared in bile spectra after ca 3 h of incubation. Resonances at 58.5 and 59.9 ppm appeared only transiently in bile spectra. Bile samples incubated for over 14 h did not contain these signals. Resonances at 62.3 and 62.8 ppm continued to increase in magnitude during the incubation and must be produced by stable reaction products. The appearance in bile spectra of a ^{13}C resonances at 84.5 ppm after ca 5 h incubation indicated that formaldehyde hydrate was produced by breakdown reactions. Further low intensity signals also appeared at 45.5 and 41.0 ppm during incubation.

The degradation of benzyl-glutathione in bile we have already described [7]. Our studies of CMA-glutathione produced ^{13}C spectra containing resonances dispersed over ca 50 ppm after the breakdown of this metabolite. In contrast the breakdown of benzyl-glutathione shifted ^{13}C resonances less than 0.5 ppm. It is clear from these observations that the degradation of these two xenobiotic metabolites occurs by very different mechanisms even though both metabolites are glutathione conjugates. Attempts to investigate the structure CMA-glutathione degradation reactions using

two-dimensional $^{1}H-^{13}C$ correlation HMQC spectra produced limited correlation data and was hindered by the lack of sensitivity at 400 MHz of this NMR technique. The precise nature of these reactions, therefore, still remains largely unknown.

The remainder of this discussion will now describe the experiments we employed to elucidate a significant part of the structure of the minor CDA metabolite. This metabolite we discovered to be novel for the biotransformation of CDA in rats. The experimental techniques contained in the following sections are an extension to the $^{1}H-^{13}C$ multinuclear NMR techniques that were used for the study of the biliary metabolites of benzyl glutathione [7]. Again these NMR techniques are novel and represent a further diversification of the use of heteronuclear NMR experiments in a xenobiotic metabolism study.

Acquiring ^{13}C-selective one-dimensional ^{1}H spectra is an alternative method of obtaining data contained in two-dimensional ^{1}H-^{13}C correlation HMQC spectrum. ^{13}C selective experiments can be used to examine molecules at low concentrations that could not be studied in two-dimensional experiments without excessively long acquisition periods. This is because ^{13}C-selective one-dimensional experiments produce a single spectrum with sufficient signal to noise to be useful whilst two-dimensional experiments require many more spectra to be acquired. The number of individual acquisitions used for a two-dimensional acquisition can, thus, be used to collect correlation data for a single ^{13}C resonance, rather than for all the signals produced by a single sample.

^{13}C selective techniques are only successful if individual ^{13}C resonances produced by a sample can be selectively excited. A ^{13}C spectrum of decayed bile (Fig. 5) contained four resonances which, apart from resonances at 62.3 and 62.8 ppm, are separated by at least 700 Hz. This separation was sufficient to allow resonances at 54.5 and 33.3 ppm to be excited separately by low power pulses. Having found that we could produce selective excitation of particular resonances we applied these pulses to extended $^{1}H/^{13}C$ heteronuclear NMR pulse sequences. Figure 6 contains spectra that reveal the chemical shift of protons coupled to the long-range ^{13}C coupled protons (3.83 ppm) of the minor CDA metabolite. These spectra were produced using a ^{13}C-selective HMQC–TOCSY pulse sequence. ^{1}H resonances at 3.06 and 5.07 ppm were first observed as crosspeaks in two-dimensional HMQC spectra correlated to a ^{13}C resonance at 34.4 ppm. A ^{1}H resonance(s) at 3.83 ppm was observed in both ^{13}C-selective one-dimensional HMQC–COSY and –TOCSY spectra. These spectra showed that this resonance was a homonuclear coupled partner to a resonance at 5.07 ppm. These results allowed part of the minor CDA metabolite structure to be formulated (III). The incomplete structure was sufficient to identify the minor CDA metabolite as a N-β-glucuronic acid conjugate. Xenobiotic glucuronic acid conjugates have been studied in purified solution [18] and in bile [19] by NMR and the chemical shift of the glucuronic acid anomeric proton and its homonuclear coupled partner are characteristic for this type of conjugate. For the minor CDA metabolite these resonances appear close to the expected chemical shifts (5.07 and 3.83 ppm, respectively) and the anomeric proton (5.07 ppm) has the 9 Hz coupling expected of the anomeric proton of a β-glucuronide.

Identifying the minor CDA metabolite as a biliary glucuronic acid conjugate is novel for the metabolism of CDA. Firstly, the minor CDA metabolite still contains a ^{13}C labelled N-methyl group although conjugation at the nitrogen atom of its amine group has occurred during its biotransformation. Within rats CDA is entirely demethylated before undergoing this type of reaction. The minor metabolite should, therefore, not contain a ^{13}C label. Secondly, the minor CDA metabolite is conjugated but is not the product of N-acetylation reactions. We have shown this to be true by demonstrating that the long-range ^{13}C coupled proton (5.07 ppm) of this metabolite is coupled to another proton. If the minor CDA metabolite had undergone N-acetylation then this group would not possess such a homonuclear coupling and would not produce the TOCSY transferred resonance observed in Fig. 6. Previous studies [4, 5] have indicated that CDA at its amine group only undergoes N-acetylation during its biotransformation. Interestingly we also see no sign of conjugated biliary metabolites which are substituted in the aromatic region.

Bile samples obtained from isolated perfused livers and *in situ* experiments showed that the minor CDA metabolites was excreted into bile in both experiments. Our results have, therefore, shown that a metabolite that has not

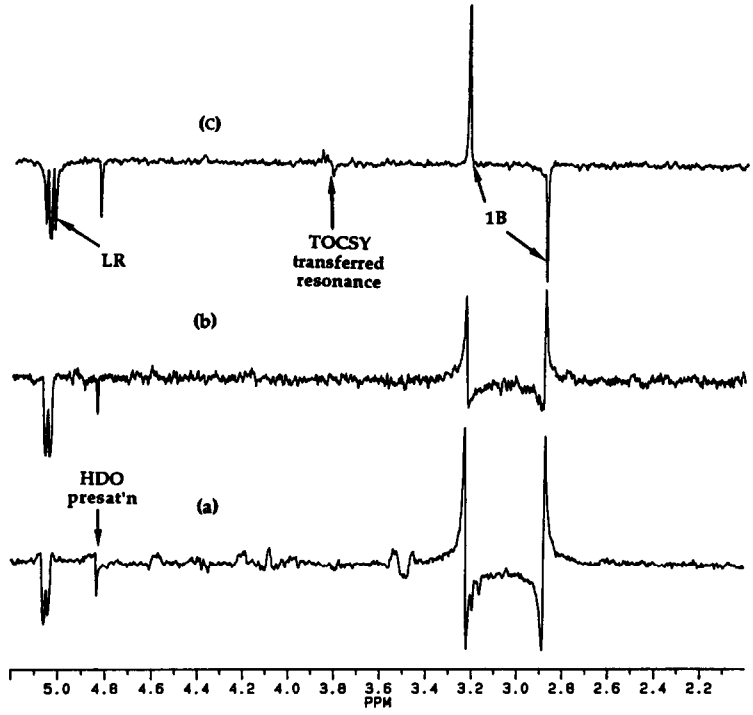


Figure 6
400 MHz ^{1}H NMR spectra obtained from a whole bile sample containing ^{13}C labelled CDA metabolites. (a) ^{1}H detected HMQC spectrum; (b) ^{1}H detected HMQC spectrum obtained using a ^{13}C selective pulses (33.3 ppm); (c) ^{1}H detected HMQC–TOCSY spectrum obtained using a ^{13}C selective pulse (33.3 ppm). LR — resonance of long-range ^{13}C coupled proton. 1B — resonance of one-bond ^{13}C coupled protons.

been isolated from urine samples in previous studies of the metabolism of CDA [4, 5], is formed as part of its biotransformation in rats. A reason for the minor CDA metabolite not appearing as a urinary metabolite maybe that glucuronide conjugates are typically readily deconjugated by β-glucuronidase enzymes that are present within the gut. If reabsorbed the deconjugated metabolite would be expected to undergo further biotransformations before appearing in the urine.

Conclusions

The biotransformation of CDA in rats involves several reactions. We have shown that this compound is excreted as two conjugated metabolites into the biliary tract of rats. By collecting bile samples from rat livers *in situ* the formation of these biliary products in the whole body situation was confirmed. These experiments demonstrated that isolated organ perfusion experiments are a qualitatively valid approach for studying the biotransformation of CDA in the rat liver.

The biliary metabolites of CDA were found to be CMA-glutathione and another metabolite whose partial structure we were able to identify as III. The use of ^{13}C isotopic labelling in xenobiotic biotransformation studies is most useful when labelling is located close to area of interest in the molecular structure. For the minor CDA metabolite the ^{13}C coupling from its N-methyl group was insufficient to allow heteronculear NMR experiments to identify its entire structure. The partial structure of the minor metabolite we revealed is, however, sufficient to identify it as a β-glucuronic acid conjugate. As such it is novel for the biotransformation of CDA in rats.

We have shown that CMA-glutathione is unstable in whole rat bile. Our data was sufficient to show that these reactions produce at least eight products. The reaction path by which this metabolite is degraded could not be identified from our NMR results. Our ^{13}C labelling studies produced a wide chemical shift dispersion for these breakdown products, much wider than we have observed for the breakdown of benzyl-glutathione in rat bile [7].

It is clear that these two xenobiotic-glutathione conjugates undergo different degradative reactions in bile. The significance of the structural modification of xenobiotic glutathione conjugates in bile *in vivo* remains to be studied.

NMR has the potential to examine molecules within intact cells. Our use of heteronuclear NMR experiments was used to confirm the structure of CMA-glutathione and the minor CDA metabolite as biliary products of CDA. These NMR techniques could, however, be applied to *in vitro* cell systems in which ^{13}C labelled compounds undergo biotransformation. It remains to be seen under these circumstances whether NMR could reveal the mechanisms by which particular xenobiotic metabolites are formed *in vivo*.

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