Pergamon

0731-7085(95)01264-8

Journal of Pharmaceutical & Biomedical Analysis, Vol. 13, No. 6, pp. 723–734, 1995
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0731-7085/95 $9.50 + 0.00

NMR study of whole rat bile: the biliary excretion of cefoperazone and benzyl chloride by an isolated perfused rat liver

DAVID A. RYAN,*† JEREMY K.M. SANDERS,† GERALD C. CURTIS‡ and HELEN HUGHES‡

† *University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK*
‡ *Department of Biochemistry, University of Wales College of Cardiff, PO Box 903, Cardiff CF1 1ST, UK*

Abstract: ^{1}H NMR spectroscopy at 400 MHz has been applied to the analysis of whole bile samples produced by the isolated perfused rat liver. Using relatively simple NMR experiments biliary excretory products of cefoperazone and benzyl chloride were identified as cefoperazone itself and a benzyl-glutathione conjugate, respectively. Our use of ^{13}C isotopic labelling demonstrates how ^{1}H/^{13}C heteronuclear NMR techniques can be used to produce uncrowded and informative spectra from whole bile. From the use of a HMQC–COSY experiment the structure of a benzyl-glutathione conjugate contained in whole bile was confirmed.

Keywords: *Isolated perfused rat liver; bile; NMR; HMQC; HMQC–COSY.*

Introduction

The biochemical mechanisms by which xenobiotics exert adverse effects within the body are diverse, and the toxicity of particular compounds cannot always be predicted from their structure, but must be determined experimentally. Isolated organ perfusion techniques allow an organ to be maintained in a viable state outside of the body and can be used to elucidate xenobiotic biotransformation reactions that occur within particular organs. The bile duct is a major excretory route for removing xenobiotics from the mammalian body, especially for glutathione conjugated xenobiotics. We have used isolated rat liver perfusion experiments as a routine method for obtaining bile samples and thus, as a technique for investigating the biliary excretion of particular xenobiotics.

NMR analysis, primarily one-dimensional ^{1}H and ^{13}C spectroscopy, of xenobiotic metabolites is generally carried out to confirm their structure after these molecules have been isolated from a biological matrix [1]. Several xenobiotic metabolites have been found to be unstable to extraction from biological samples, such as bile, using routine chromatographic methods [2]. NMR is non-destructive and as such is an analytical method that can examine molecules regardless of their stability to extraction techniques. It may prove to be a method of choice for the analysis of xenobiotic metabolites that prove difficult to purify. We describe here structural NMR studies of xenobiotic metabolites contained in whole rat bile.

NMR has been used to examine metabolic processes in whole organs and cellular systems at least since the early 1980s. Work conducted by Cohen [3] provides an example of this work and describes an NMR study of liver metabolism. Reviews of the application of NMR in metabolism studies are provided by London [4] and Malet-Martino [5]. Recent studies have been successful in examining by NMR the structure of xenobiotic metabolites in urine [6]. This area of research has proven fruitful in providing experimental evidence for organ-specific toxicity by particular xenobiotics [7]. One-dimensional ^{1}H NMR studies of xenobiotic metabolites contained in whole rat bile have been conducted during an investigation of the biotransformation of paracetamol and *para*-aminophenol [8, 9]. Studies of xenobiotic metabolites in bile using ^{13}C and ^{19}F NMR spectroscopy have also been conducted [10,

* Author to whom correspondence should be addressed. Present address: Dept. of Drug Metabolism and Environmental Chemistry, Hazleton Europe, Otley Road, Harrogate, North Yorkshire, HG3 1P4, UK.

11]. Our studies have been directed towards expanding the role of NMR in providing valid toxicological data in this research field.

We have used two compounds, cefoperazone (II) and benzyl chloride (III), as model compounds with which to develop our experimental methods. Cefoperazone, a widely used antibiotic that does not undergo biotransformation in the liver, was used to develop an approach for obtaining useful one-dimensional ^{1}H NMR spectra from bile samples. We then used benzyl chloride, a compound that is readily converted to a glutathione conjugate in the liver, to inspect a conjugated xenobiotic metabolite in bile. During this work two-dimensional ^{1}H NMR techniques and isotopic ^{13}C labelling of benzyl chloride were employed; unconventional ^{1}H–^{13}C heteronuclear NMR experiments were also devised. These techniques present a novel approach for the analysis of xenobiotic metabolites by NMR and provide an excellent example of how ^{13}C labelling of a xenobiotic can be used for the structural analysis of its metabolites.

Experimental

Rat liver perfusion experiments

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* prior to use.

Procedures for performing isolated liver experiments were essentially those described by Curtis [12], first developed from methods by Ross [13]. In brief by introducing Ringers solution through a cannula placed in the lower inferior vena cava our procedures allowed a rat liver *in situ* to be flushed of blood. The liver was then excised and placed within a perfusion apparatus. The essential features of the perfusion apparatus used allowed an isolated liver to be continually supplied with oxygenated perfusate at a constant temperature and head pressure. The entire perfusion apparatus was housed within a thermostatically controlled cabinet (37°C). A blood transfusion filter (SQ40S) was included in the apparatus. This was essential for removing fibrin clots formed within the perfusate.

The toxicity of cefoperazone (Sigma C-4292) and benzyl chloride (Fisons CAS100-44-7, $[CH_2]$-^{13}C labelled MSD MS3139) to a perfused liver appears to be unknown prior to our studies. Benzyl chloride was administered by directly adding it to circulating perfusate, whilst cefoperazone was administered as an aqueous solution (*ca* 1 ml). Dose levels that reduced the flow of perfusate through the liver, or its production of bile, in comparison to control experiments by 25% or more, were reduced. Dose level of 100 mg kg^{-1} body wt were used in our final experiments for cefoperazone and benzyl chloride.

Perfusate preparation

The perfusate composition was that described by Curtis [8] and consisted of 7.5 g of bovine serum albumin (Sigma A-7906 Lot. 10H0249) and 250 mg of glucose per 250 ml of Krebs–Ringer saline that contained bovine red blood cells at a final haemocrit of 25–35%. Bovine blood was collected fresh from a local abattoir. Red blood cells were prepared by centrifugation [14] and stored at 4°C. They were kept for a maximum of 7 days after isolation.

Bile sample collection and preparation

During perfusion experiments bile samples were collected over an ethanol/dry ice mixture to cause immediate freezing of bile on leaving the bile duct cannula. Bile samples were deuterium exchanged twice by lyophilization with D_2O (99.8%), containing a standard amount (0.5 mM) of Sodium 3-trimethyl silyl propionate-2,2,3,3,-d_4 (TSP) 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 a 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 8192 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 was to be performed. Presaturation (1 s) was used to remove the HDO resonance during acquisition of spectra; the minimum power that produced complete saturation of the HDO signal was used. Free induction decays were zero filled to 16,384 data

points and mild Gaussian apodization was applied prior to their Fourier transformation.

Two-dimensional ^1H–^1H COSY spectra

Two-dimensional double quantum filtered ^1H–^1H COSY[15] 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 processing spectra, the f_2 and f_1 dimensions 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

One-dimensional ^{13}C spectra were acquired at 100 MHz with a spectral width of 200 Hz (36–38 ppm) using WALTZ 16 decoupling [16]. Spectra contained 256 real data points and had an acquisition time of 0.64 s. A relaxation period of 2 s was used between acquisitions. Eight transients per spectrum provided adequate signal to noise. Free induction decays were zero filled to 512 data points and mild Gaussian apodization applied prior to Fourier transformation.

$^3J_{CH}$–HMQC–COSY spectra

$^3J_{CH}$–HMQC–COSY spectra were acquired using the pulse sequence shown in Fig. 1 which is based upon work published by Wollborn and Leibfritz [17]. It was constructed by Dr C.-W. Chung (University of Cambridge Chemical Laboratories, Lensfield Road, Cambridge, UK), who also devised the appropriate phase cycling. The delays Δ_2 and Δ_3 were set to 0.0035 s (1/2J) and 0.046 (13 × 1/2J), respectively to optimize the cancellation of protons with a ^1H–^{13}C coupling of 143 Hz. Δ_1 and Δ_4 were delays of 3 μs and t_1 was the incremented delay.

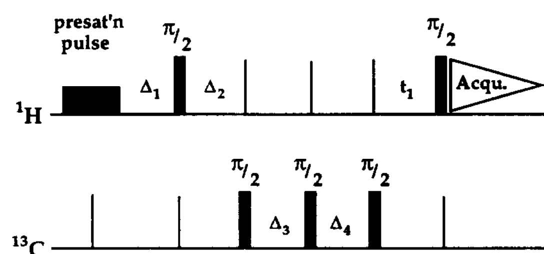


Figure 1
$^3J_{CH}$–HMQC–COSY pulse sequence.

One-dimensional $^3J_{CH}$–HMQC–COSY spectra were acquired with a spectral width of 4800 Hz (−1 to 11 ppm) using 8192 real data points. The acquisition time per transient was 1.27 s with 128 transients. A recycle time of 2.62 s was used per transient. Presaturation was used to remove the HDO resonance from spectra. Free inducation decays (FIDs) were zero filled to 16 384 data points and mild Gaussian apodization was applied prior to their Fourier transformation.

Two-dimensional $^3J_{CH}$-HMQC–COSY spectra were acquired with a spectral width of 4800 Hz (−1 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. Each experiment was acquired with 16 transients. The acquisition time per transient was 0.51 s and a recycle time of 1.32 s was used. The length of the incremented delay did not exceed 0.027 s. The f_2 and f_1 dimensions were zero filled to 4096 and 512 data points respectively before processing. Fourier transformation was carried out applying Gaussian apodization in both dimensions.

Results and Discussion

A typical ^1H NMR spectrum of a bile sample obtained from the control period of a perfusion experiment is contained in Fig. 2. Bile samples were twice exchanged with D_2O by lyophilization prior to NMR analysis to replace H_2O with D_2O. This procedure led to a reduction in the magnitude of the H_2O resonance which would otherwise dominate a bile spectrum. This approach together with the application of a presaturation pulse prior to acquisition enabled bile spectra to be acquired with limited interference from the HDO resonance of only ±0.05 ppm.

A large number of resonances appearing in the bile spectrum shown in Fig. 2 have previously been assigned [6, 9] and arise from acetate, alanine, cholesterol, glutamine, glycochenodeoxycholate, 3-hydroxybutyrate, isoleucine, lactate, leucine, phosphatidyl choline tryglyceride, saturated and unsaturated fatty acids, taurocholate and valine. The broad envelopes of resonances covering 2.6–0.6 and

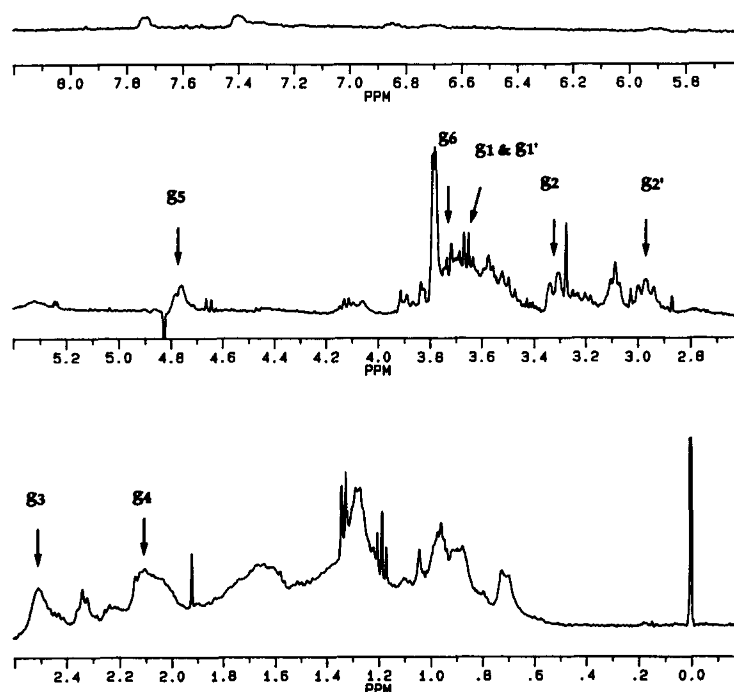


Figure 2
400 MHz ^{1}H NMR spectrum of whole bile. Assignments for oxidized glutathione (g_1–g_6) are shown. The HDO resonance at 4.83 ppm was suppressed by a presaturation pulse.

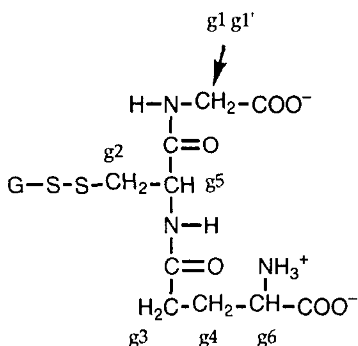
Table 1
Assignment of the proton resonances of oxidized glutathione and a benzyl-glutathione conjugate in bile

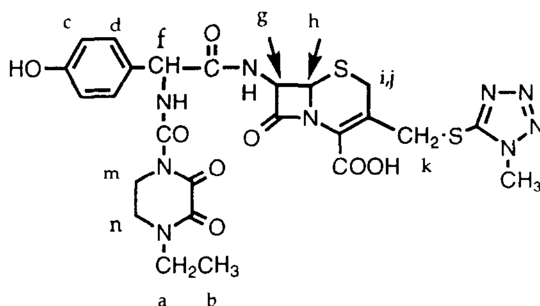
Amino acid of glutathione	Proton assignment	Oxidized glutathione* (bile pD 8.5) ppm	Benzyl-glutathione conjugate (bile pD 8.0) ppm
Glycine	g_1	3.67	3.77
	$g_{1'}$	3.65	3.69
Cysteine	g_5	4.76	4.46
	g_2	3.33	3.02
	$g_{2'}$	2.96	2.81
Glutamate	g_3	2.50	2.45
	g_4	2.12	2.09
	g_6	2.74	3.66

* Assignments made from spectrum contained in Fig. 2.

4.2–2.8 ppm are produced by bile acids and triglycerides. Their resonances appear as broadened signals because they are contained within structured micelles in bile and their free rotation is hindered. The methyl groups of bile acids and the terminal *N*-methyl groups of the triglyceride phosphatidyl choline appear in contrast as sharp signals in ^{1}H bile spectra as they are at the surface of micelles and can rotate freely. All other assigned resonances in bile spectra arise from small, freely mobile, organic molecules that are contained in the aqueous portion of bile and are, thus, sharp in appearance.

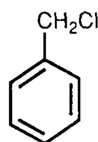
The concentrations of glutathione (GSH) in bile has previously been reported to be *ca* 1.5–2 mM [18, 19]. ^{1}H NMR spectra acquired from bile samples collected during our liver perfusion experiments contain prominent resonances from oxidized glutathione (I) (GSSG). An assignment of these signals in bile spectra, summarized in Table 1 and Fig. 2, has not previously been made. These assignments were confirmed by the presence of the relevant crosspeaks in a ^{1}H–^{1}H COSY spectrum acquired from an appropriate bile sample. Chemical shift values were always indicative of oxidized glutathione rather than its reduced form because the g_1 signal of glycine appeared as a doublet and because the separation of the g_2 and $g_{2'}$ signals of cysteine was 0.37 ppm, as opposed to 0.1 ppm for reduced glutathione



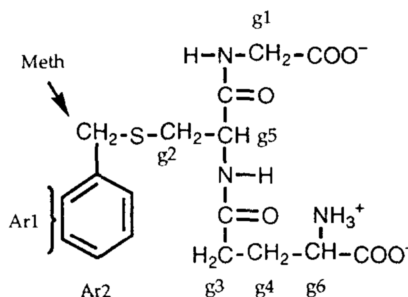


I Oxidised Glutathione (GSSG)

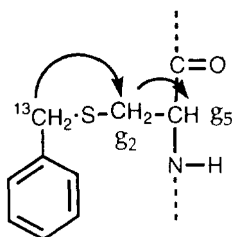
II Cefoperazone



III Benzyl Chloride



IV Benzyl glutathione



V

[20]. A comparison of the magnitude of the g_2 cysteine resonances with that of a TSP standard contained samples revealed that oxidized glutathione was present at approximately millimolar concentrations in bile. The observation of oxidized glutathione in the absence of its reduced form is surprising because this contrasts with Abbott and Meister's [19] report that showed rat bile contained five times the amount of reduced glutathione than oxidized glutathione. However our freeze drying of bile samples cannot be discounted as a possible mechanism by which reduced glutathione was transformed into an oxidized form.

Cefoperazone (II) is a commonly used antibiotic and provided us with a means of examining the biliary excretion of a compound that is not metabolized by the liver. A 0–1 h bile sample that was obtained after dosing a perfused liver with cefoperazone (100 mg kg^{-1}

body weight) produced the ^{1}H NMR spectrum shown in Fig. 3. It is clear from this spectrum that cefoperazone is excreted into the biliary tract unchanged. By integration of resonances C and D and that of the TSP standard the amounts of cefoperazone excreted into bile samples during a perfusion experiment were calculated. These results are summarized in Table 2. The total biliary excretion of cefoperazone amounted to 43% of the administered dose, the major proportion (40%) of this occurring within the first two hours after dosing. The concentration of cefoperazone found in the 0–1 h bile sample represents a *ca* 200 fold increase above the circulating perfusate antibiotic concentration. The ability of the biliary system to actively transport and concentrate xenobiotics is well known and our findings provide a clear visual demonstration of this. Our observations of the biliary excretion of cefoperazone indicate that ^{1}H NMR is a viable approach to studying xenobiotics in whole, unpurified bile.

Many xenobiotics that appear in bile first undergo biotransformation in the liver. Benzyl chloride (III) was used to study a xenobiotic that undergoes hepatic biotransformation. A ^{1}H NMR spectrum acquired from a bile sample

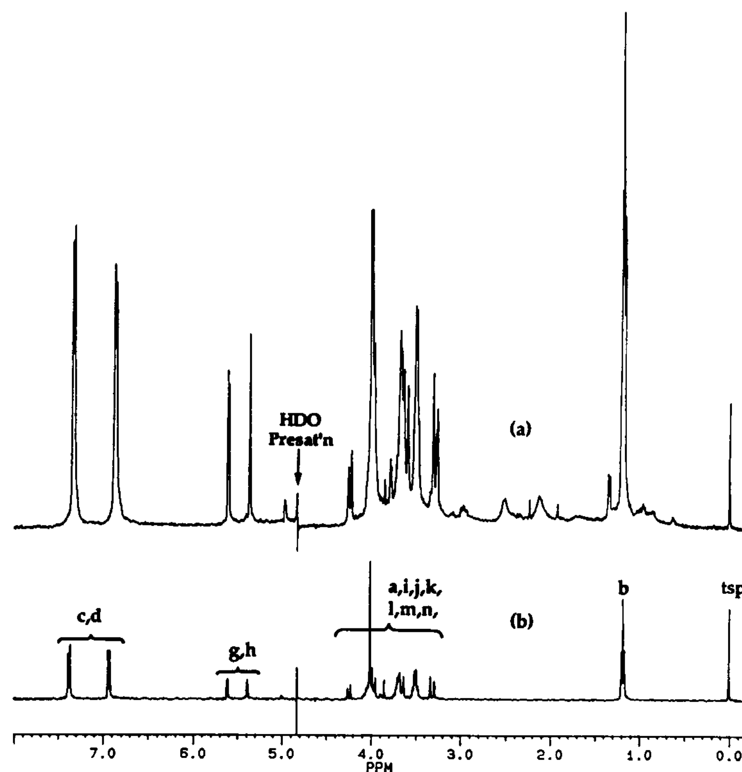


Figure 3
400 MHz ^{1}H NMR spectrum of (a) whole bile sample obtained from a perfused rat liver between 1 and 2 h after dosing with cefoperazone (100 mg kg^{-1} body weight). (b) A D_2O solution of cefoperazone.

Table 2
The amounts of cefoperazone excreted into bile at dose levels of 100 mg kg^{-1} body weight

Sample	Mass of bile sample (g)	Amounts excreted (μmoles)*	Concentration of cefoperazone in bile (mM)*	% of dose
Control	0.23	—	—	—
0–1 h	0.70	11	31	27
1–2 h	0.54	5	11	13
2–3 h	0.38	1.0	1.6	2
3–4 h	0.16	0.2	0.1	0.5
4–5 h	0.09	—	—	—
Total				43

*Calculated by integration of peak areas of aromatic resonances C and D of cefoperazone.

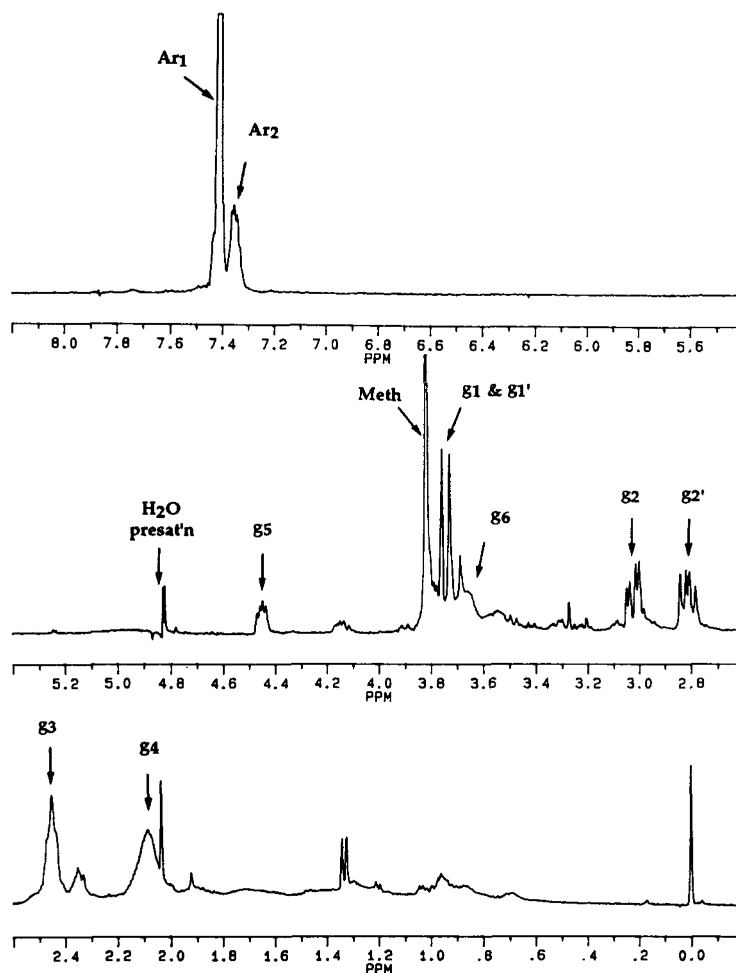


Figure 4
400 MHz ^1H NMR spectrum of whole bile obtained from a perfused rat liver between 0 and 1 h after dosing with benzyl chloride (100 mg kg^{-1} body weight). Assigned resonances are benzyl glutathione (IV).

obtained 0–1 h after dosing a perfused liver with benzyl chloride (60 mg kg^{-1} body weight) is contained in Fig. 4. The aromatic (7.42 and 7.36 ppm) and methylene (3.83 ppm) resonances associated with benzyl chloride can be easily seen in this spectrum together with a number of other resonances that appear at comparable intensities. The similarity between the chemical shift assignments of isolated glutathione conjugates reviewed by Feil [1] and the dominant resonances appearing in Fig. 3 identified the biliary metabolite of benzylchloride as the benzyl-glutathione conjugate (IV). A ^1H–^1H COSY spectrum (Fig. 5) obtained from an appropriate bile sample contained crosspeaks that confirmed our assignments, which are summarized in Table 1.

Bile samples obtained during an entire experimental period after dosing with benzyl chloride were consistent with the biliary excretion of only benzyl-glutathione by a perfused liver. The concentrations of this conjugate in bile samples were at first comparable with those of cefoperazone summarized in Table 2. However, the amounts of benzyl-glutathione conjugate excreted after the first hour of perfusion dropped more rapidly in comparison to the biliary excretion of cefoperazone. This may indicate toxification of the liver by benzyl chloride or a loss of glutathione available for xenobiotic biliary excretion.

^{13}C isotopic labelling provides a means by which compounds of interest can be discriminated from other components within a given sample. NMR has been used to examine in rat bile metabolites formed from ^{13}C isotopically labelled antipyine by a perfused rat liver [10]. In order to further explore the use ^{13}C labelling for the study of biliary metabolite structure we introduced ^{13}C-[CH_2]-benzyl chloride as a dose

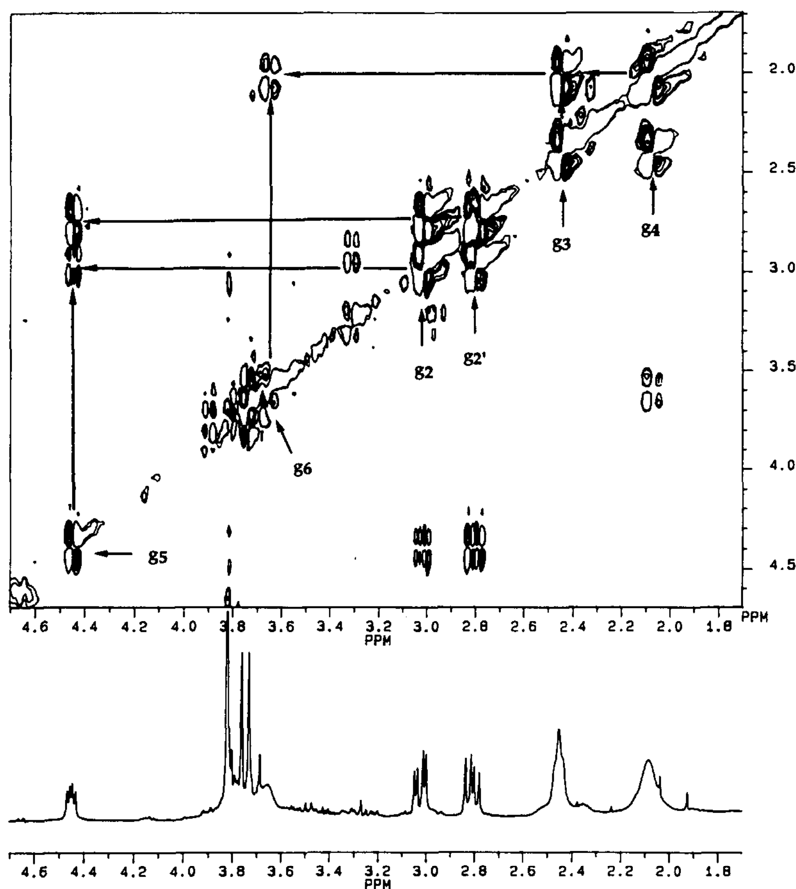


Figure 5
Two-dimensional contour plot of a 400 MHz ^1H-^1H COSY spectrum obtained from a whole bile sample containing benzyl gluathione (IV).

compound. The effect of this labelling regime on the appearance of ^1H resonances of benzyl-glutathione in bile can be seen in the spectra contained in Fig. 6. These spectra show that there is not only a large one bond ^{13}C coupling to the methylene protons in benzyl-glutathione but there is also the expected three bond coupling [21] across the sulphide linkage to the g_2 cysteine protons of glutathione. There is also a coupling to the aromatic benzyl protons. ^1H-^{13}C coupling constants are summarized in Table 3. The observation of ^{13}C coupling across the sulphide linkage to glutathione proves there is a covalent linkage between the benzyl and glutathione moieties present in our bile samples and confirms the structure of benzyl-glutathione as the biliary metabolite.

During our studies of benzyl-glutathione this conjugate was found to be unstable in bile if samples were not frozen during collection and until spectroscopic examination. ^{13}C spectra obtained before and after degradation showed that five resonances appear as a result of degradative processes indicating that there must be at least five products formed from the breakdown of benzyl-glutathione. The chemical shift dispersion of the ^{13}C signals was very narrow (0.4 ppm) indicating that the ^{13}C label in benzyl-glutathione is distant from these metabolite reactions. The enzymes γ-glutamyl transpeptidase and dipeptidase of the β-lyase pathway [22] are present in bile [19], and would release two products from benzyl-glutathione. As we observed five ^{13}C signals this pathway cannot fully account for the number of decay reaction products.

With the exception of the acyl migration of xenobiotic glucuronide conjugates [23] we are not aware of other reports of xenobiotic metabolites being unstable in bile. Our efforts to study these new reactions by NMR did not prove definitive and will be presented here only in part. Several of the NMR techniques we employed during this work, however, are novel and represent a diversification of the use of heteronuclear NMR experiments in a xeno-

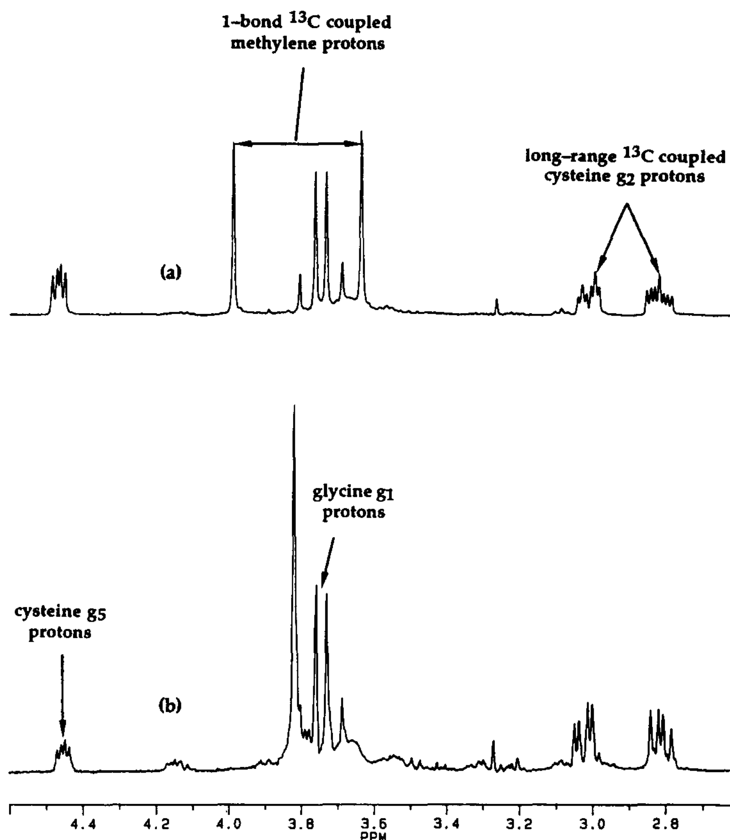


Figure 6
400 MHz ^{1}H NMR spectra of (a) ^{13}C-[CH_2] labelled benzyl glutathione contained in whole bile. (b) ^{12}C-[CH_2] benzyl glutathione contained in whole bile.

Table 3
Assignment of proton resonances and ^{1}H–^{13}C coupling constants of ^{13}C-[CH_2]-benzyl chloride in bile

Proton	Chemical shift (ppm)	^{13}C coupling (Hz)
Meth	3.82	141
g_2	2.81	5
$g_{2'}$	3.01	4
Ar_1	7.40	—*
Ar_2	7.35	—*

* ^{13}C couplings to aromatic protons could not be measured.

biotic metabolism study. They represent a useful analytical tool in the drug metabolism and will, hence, be described fully.

Two-dimensional ^{1}H–^{13}C correlation NMR spectra permit chemical shift correlations between ^{13}C and ^{13}C coupled ^{1}H resonances to be made [16]. This type of data can be important in determining molecular structures; for example, ^{13}C detected ^{1}H–^{13}C correlation spectra have been used to identify the structure of the biliary metabolites of 1,2 dibromo-3-chloropropane [24]. It is now routine to generate these spectra from a series of ^{1}H spectra by applying a recently developed Heteronuclear Multiple Quantum Coherence (HMQC) pulse sequence [25, 26]. These experiments allow one to observe selectively only those protons which are coupled to ^{13}C. They were used to produce simple spectra of the benzyl-glutathione and its decay products from whole bile samples.

It is possible to extend ^{1}H–^{13}C HMQC experiments to include either TOCSY (TOtal Correlation spectroscopY [27]) or COSY (COrrelated SpectroscopY [28]) pulse sequences as methods of magnetization transfer. These extended pulse sequences can result in resonances from the homonuclear coupled partners of 'long-range' ^{13}C coupled protons (V: g_2–g_5) appearing in spectra. These techniques can be described as HMQC–COSY or –TOCSY [29] experiments and allow the chemical shift of protons four bonds distant from a ^{13}C label to be inspected. These protons were thought to be closer to chemical changes

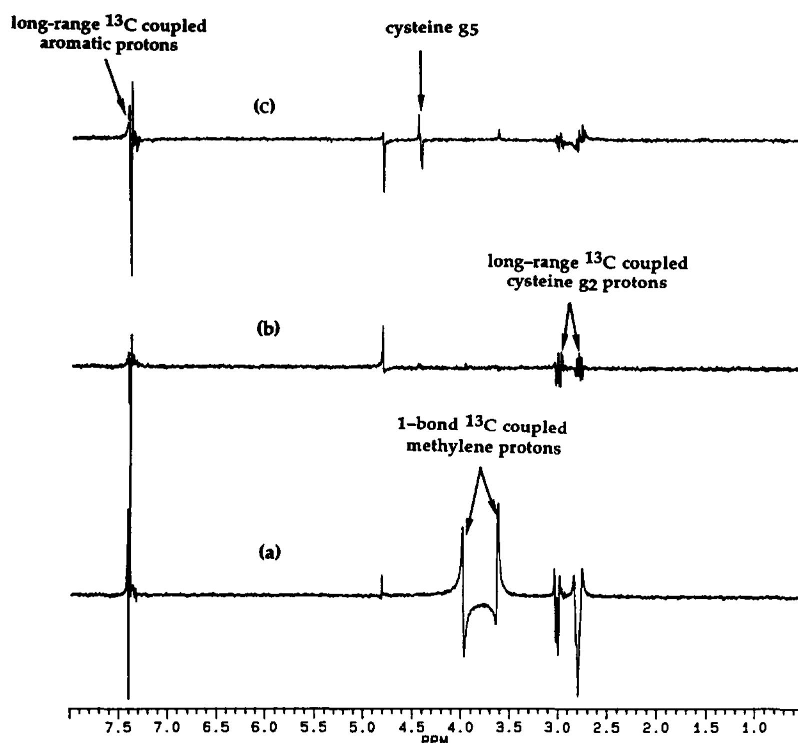


Figure 7
400 MHz ^{1}H NMR spectra obtained from a whole bile sample containing undegraded $[CH_2]$-^{13}C labelled benzyl glutathione. (a) ^{1}H detected HMQC spectrum (b) ^{1}H detected HMQC spectrum in which 1-bond ^{13}C coupled proton resonances have been removed (c) ^{1}H detected spectrum obtained using a $^{3}J_{CH}$ HMQC–COSY pulse sequence.

occurring in benzyl-glutathione and it was hoped that their chemical shift values would identify the degradation reaction products.

One-dimensional spectra obtained from an undegraded bile sample using a HMQC–COSY pulse sequence are contained in Fig. 7 (spectrum A). The pulse sequence used to acquire these spectra was then modified so that the resonances from 1-bond ^{13}C-coupled protons were also excluded (spectrum B). These signals normally appear as large, dominant resonances in HMQC spectra (spectrum A). COSY transferred resonances could be more easily identified in spectra if these resonances were removed. Spectrum C was acquired using a further adapted pulse sequence; $^{3}J_{CH}$–HMQC–COSY (Fig. 1), that produces magnetization transfer from long-range coupled protons to their homonclear coupled partners, in this case from the g_5 protons to the g_2 protons of benzyl-glutathione.

A two-dimensional $^{3}J_{CH}$–HMQC–COSY spectrum obtained from bile samples before degradation is contained in Fig. 8. Although 1-bond coupled proton resonances have not been completely removed from these spectra they do not interfere with other areas of the spectra. This spectrum is equivalent to a normal ^{1}H–^{1}H COSY spectrum with the exception that resonances from protons that do not possess 'long-range' ^{13}C coupling or are coupled to a proton with this coupling have been removed from acquisition. We acquired two-dimensional $^{3}J_{CH}$-HMQC–COSY spectra from a degraded bile sample. From the chemical shift of particular crosspeaks in these spectra we were able to identify benzyl-cysteine as a possible product of degradation reactions. All other crosspeaks produced in this spectrum overlapped those produced by benzyl-glutathione itself. These similarities in chemical shift values show clearly that degradation reaction products are very similar in structure to benzyl-glutathione.

Conclusion

We have found that the structural identification of the excretory products of cefoperazone and benzyl chloride in whole bile could be achieved using relatively simple NMR tech-

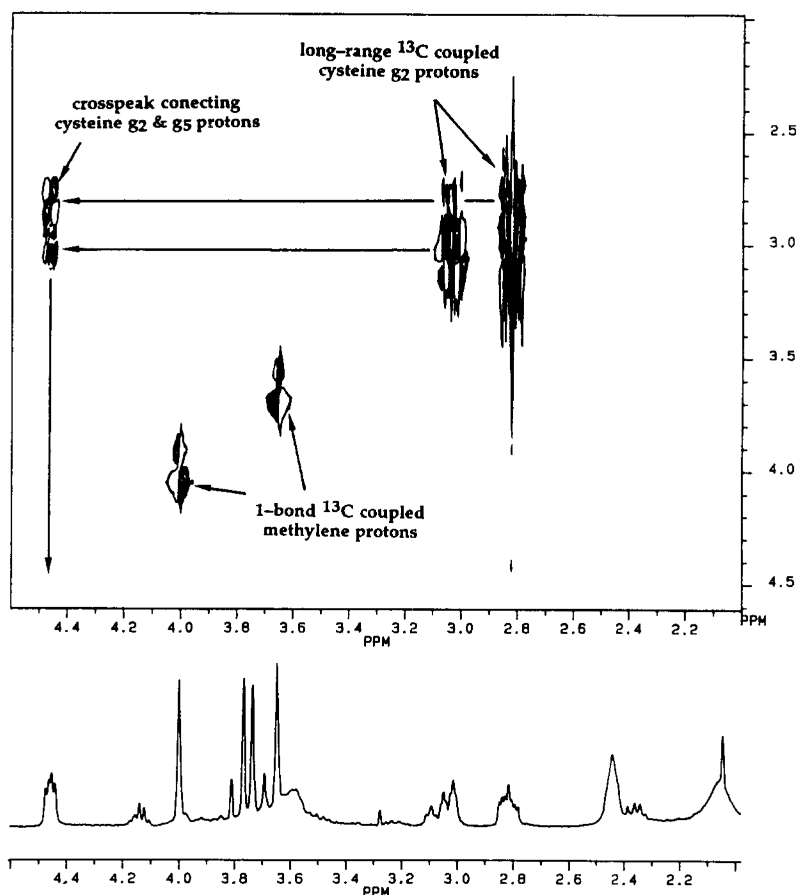


Figure 8
Contour plot of a 400 MHz ^{1}H detected two-dimensional $^{3}J_{CH}$ HMQC–COSY spectrum obtained from a whole bile sample containing undegraded $[CH_2]$-^{13}C labelled benzyl glutathione. This spectrum is equivalent to a ^{1}H–^{1}H COSY spectrum with the exception that resonances of protons without a ^{13}C coupling have been excluded from acquisition.

niques. This analysis was made easier by the fact that both compounds were excreted into bile at a relatively high concentration in comparison to the other sample components. Our accompanying analysis of the biliary metabolites of 4-cyano dimethyı aniline [30] addresses the problems associated with the analysis of xenobiotic metabolites at lower concentrations.

A major difficulty associated with the analysis of bile largely arises because spectra contain resonances from a large number of compounds. It can often be difficult to discriminate resonances produced by a single bile component. Our use of ^{13}C isotopic labelling has demonstrated how $^{1}H/^{13}C$ heteronuclear NMR experiments can be applied to the structural analysis of xenobiotic metabolites and used to avoid crowded, uninformative spectra.

^{13}C labelling studies are most informative when a label is located close to the area of interest in a molecular structure. This was not the case during the above study of benzyl-glutathione and its degradation. This fact prevented us from determining the precise nature of these reactions. It is nevertheless clear that these reactions are not just those of the β-lyase pathway as our NMR results show that apart from benzyl-cysteine at least three other breakdown products are formed which are very similar in structure to benzyl-glutathione. We feel that these reactions may simply involve a mixture of transpeptidation reactions occurring to the amino acid constituents of glutathione rather than the ordered sequence of reactions seen in the β-lyase pathway.

NMR has the potential to examine molecules within intact cells. The NMR techniques described above could be applied to *in vitro* cell systems in which ^{13}C labelled compounds undergo biotransformation. It remains to be seen whether under these circumstances NMR could reveal the mechanisms by which

particular xenobiotic metabolites are formed *in vivo*.

Acknowledgements — David Ryan would like to thank the SERC and Shell Research Ltd for a CASE studentship and Dr C.-W. Chung for constructing the pulse sequences used in this paper.

References

[1] V.J. Feil, in *Xenobiotic Conjugation Chemistry* (G.D. Paulson, J. Caldwell, D.H. Hutson and J.J. Men, Eds) ACS Symposium Series 299. ACS, Washington (1989).

[2] H.W. Dorough and J.D. Webb, in *Xenobiotic Conjugation Chemistry* (G.D. Paulson, J. Caldwell, D.H. Hutson and J.J. Men, Eds) ACS Symposium Series 299. ACS, Washington (1989).

[3] S. Cohen, *J. Bio. Chem.* **258**, 14294–14308 (1983).

[4] R.E. London, *Prog. NMR Spectro.* **20**, 337–383 (1988).

[5] M.C. Malet-Martino and R. Martino, *Xenobiotica* **19**, 583–607 (1989).

[6] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectro.* **21**, 449–501 (1989).

[7] K.P.R. Gartland, S.M. Sanins and J.K. Nicholson, *NMR Biomed.* **3**, 166–172 (1990).

[8] T.D. Spurway, P.J. Philips, I.D. Wilson and A. Warrander, *J. Pharm. Biomed. Anal.* **8**, 969–973 (1990).

[9] K.P.R. Gartland, F.W. Bonner, J.A. Timbrell and J.K. Nicholson, *Arch. Toxicol.* **63**, 97–106 (1989).

[10] P. Huetter, K. Albert, E. Bayer, K.P. Zeller and F. Hartmann, *Biochem. Pharmacol.* **36**, 2729–2733 (1987).

[11] M.C. Malet-Marino, J. Bernadou, R. Martino and J.P. Armand, *Drug Metab. Disp.* **16**, 78–84 (1988).

[12] C.G. Curtis, G.M. Powell and S.L. Stone, *J. Physiol.* **213**, 14–15 (1970).

[13] B.D. Ross, *Perfusion Techniques in Biochemistry*. Clarendon Press, Oxford (1972).

[14] R.M.C. Dawson, in *Data for Biochemical Research* (R.M.C. Dawson, W.H. Elliot and K.M. Jones, Eds), pp. 507. Oxford University Press, Oxford.

[15] J.K.M. Sanders and B.K. Hunter, *Modern NMR spectroscopy* 2nd edn. Oxford University Press, Oxford (1993).

[16] D. Neuhaus, J. Keeler and R. Freeman, *J. Magn. Res.* **61**, 553–568 (1985).

[17] U. Wollburn and D. Leibfritz, *Mag. Res. Chem.* **29**, 238–243 (1991).

[18] N. Ballatori, R. Jacob and J.L. Boyer, *J. Bio. Chem.* **261**, 7860–7865 (1986).

[19] W.A. Abbott and A. Meister, *Proc. Natl Acad. Sci. USA* **83**, 1246–1250 (1986).

[20] D.L. Rabenstein and D.A. Keire, in *Glutathione — Biochemical & Medical Aspects* (D. Avramovic, R. Poulson and D. Dolphin, Eds), p. 67. Wiley, New York (1989).

[21] S. Naylor, R.P. Mason, J.K.M. Sanders, D.H. Williams and G. Moneti, *Biochem. J.* **249**, 573–579 (1988).

[22] C.D. Klassen, M.D. Amdur and J. Doull, in *Casarett and Doull's Toxicology — the Basic Science of Poisons*, 3rd edn, p. 108. Collier Macmillan, London (1986).

[23] P.G. Smith and L.Z. Benet, *Drug Metab. Dispos.* **14**, 503–505 (1986).

[24] D.R. Dohn, M.J. Graziono and J.E. Casida, *Biochem. Pharmacol.* **37**, 3485–3495 (1988).

[25] L. Muller, *J. Am. Chem. Soc.* **101**, 4481 (1979).

[26] J. Cavanah and J. Keeler, *J. Mag. Res.* **77**, 356–362 (1988).

[27] L. Braunschweiller and R.R. Ernst, *J. Mag. Res.* **53**, 521–528 (1983).

[28] A. Bax and R. Freeman, *J. Mag. Res.* **44**, 542–561 (1981).

[29] R.C. Crouch, J.P. Shocklor and G.E. Martin, *Tetrahedron Lett.* **31**, 5273–5276 (1990).

[30] D.A. Ryan, J.K.M. Sanders, G.C. Curtis and H. Hughes, *J. Pharm. Biomed. Anal.* (in press).

[Received for review 1 June 1994; revised manuscript received 12 October 1994]