Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 945–949, 1990
Printed in Great Britain

0731-7085/90 $3.00 + 0.00
© 1990 Pergamon Press plc

Short Communication

^{1}H-NMR spectroscopy of biofluids and the investigation of xenobiotic-induced changes in liver biochemistry*

S. SEQUEIRA,‡ P.W. SO,‡ J.R. EVERETT,§ C.R. ELCOMBE,|| A.S. KELVIN** and J.K. NICHOLSON†‡

‡*Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H OPP, UK*
§*SmithKline Beecham, Brockham Park, Surrey RH3 7AJ, UK*
||*ICI, CTL, Alderley Park, Cheshire SK10 4TG, UK*
***SmithKline Beecham, Honeypot Lane, Stock, Essex CM4 9PE, UK*

Keywords: *Galactosamine (GAL); β-naphthoflavone (BNF); ^{1}H-NMR; biochemical toxicology; hepatotoxicity.*

Introduction

Alterations in body fluid composition after toxic insult generally reflect abnormal cellular biochemical changes induced by the toxin. Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) can simultaneously detect both changes in levels of endogenous and xenobiotic metabolites in body fluids and therefore, is potentially an important exploratory tool for the biochemical toxicologist [1]. We have used ^{1}H-NMR to investigate the effects of two model xenobiotics, i.e. galactosamine (GAL) and β-naphthoflavone (BNF) in the rat, these compounds producing marked alterations in liver structure and function. Administration of GAL induces a highly-reproducible, dose-dependent hepatotoxicity [2] bearing a striking pathological resemblance to viral hepatitis [3]. Its metabolism predominates in the liver and involves the depletion of uracil nucleotides by the formation of uracil nucleotide hexosamines [2, 4]. BNF is a well known inducer of hepatic enzymes, including cytochrome *p* 448 [5] and is potentially hepatotoxic. Both compounds are of considerable toxicological interest but have poorly understood biochemical effects or mechanisms of toxicity. The NMR studies reported here were designed to give new insights into the effects of the compounds on hepatic intermediary biochemistry and on biofluid composition.

Methods and Materials

Animal treatment and sample collection

Galactosamine. Fifteen male Sprague Dawley rats (190–250 g) were dosed with 400 mg kg^{-1} GAL·HCl in 0.9% saline vehicle intraperitoneally (i.p.). Urines samples were collected over 2 days, 0–8, 8–24 and 24–48 h after dosing. The experiment was repeated with a 7-day observation period and the urine samples collected 0–7, 7–24, 24–48, 48–72, 72–96 and 96–168 h after dosing. A further three rats were used as controls and given an equivalent dose of the saline vehicle. Control urine was collected for 24 h prior to dosing for all rats. All rats were allowed free access to food and water. The urine samples were prepared for NMR analysis by addition of 120 μl of ^2H_2O and 80 μl of 2.5 mg ml^{-1} sodium-3-(trimethylsilyl)-[^{2}H]$_4$-propionate (TSP) in ^2H_2O. On termination, blood was removed from the inferior vena cava or dorsal aorta and plasma separated. Both plasma and liver extracts were freeze-dried and redissolved in ^2H_2O.

* Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.
† Author to whom correspondence should be addressed.

β-Naphthoflavone. Six male Sprague Dawley rats (200–250 g) were housed in individual metabolism cages and allowed to acclimatize for 24 h. Three of the rats were injected (i.p.) with BNF (in corn oil 100 mg kg^{-1}) once daily for 3 days prior to termination. The other three animals (controls) received corn oil alone according to the same schedule. Urine samples were collected on ice for 24 h prior to dosing and 8 and 24 h after each dose.

NMR measurements

NMR spectra were recorded either on JEOL GSX500 or Bruker WH400 spectrometers operating at 500 and 400 MHz proton resonance frequencies, respectively. For the single pulse experiments performed on urine, 64 or 128 free induction decays (FIDS) were collected using 45° pulses, and a delay of 4 s between successive pulse cycles to ensure full T_1 relaxation. The intense signal resulting from water was suppressed by application of a gated secondary irradiation field at its resonance frequency. Chemical shifts were referenced to internal TSP ($\delta = 0$ ppm). For blood plasma, spin–echo methods [6] were employed to edit out the broad envelope of signals resulting from the relatively large quantities of proteins as these would mask the signals from the highly mobile low molecular weight metabolites. The Hahn-spin echo pulse sequence: D–($90°_x$–τ–$180°_y$–τ–collect FID) eliminates broad resonances from macromolecules via T_2 relaxation during the delay period 2 τ. The remaining resonances, mainly from the mobile low molecular weight species, are phase-modulated according to their coupling multiplicities and coupling constants. With the value of 60 ms, singlets and triplets are phased upright and doublets are phase-inverted where coupling constants are 7–8 Hz.

Results and Discussion

NMR spectra of biological fluids give characteristic patterns of resonances from endogenous compounds that can be related to and aid the understanding of, disease or toxicological processes [1]. ^{1}H NMR spectra showing changes in urinary metabolite profiles following GAL treatment over part of the long time-course of the experiment is shown in Fig. 1. NMR spectroscopy revealed that GAL caused an increase in urinary acetate, lactate, dimethylglycine and taurine concentrations 7–24 h after treatment, and these metabolite concentrations continued to increase for the remainder of the observation period (Fig. 1). Hypertaurinuria and the decrease in mobile plasma triglycerides (Fig. 2) appeared to be associated with acute hepatic dysfunction reported in previous studies [1, 7]. Increases were also noted for formate and propanoate 24–48 h after dosing. Urinary concentrations of Kreb's cycle intermediates (i.e. citrate, α-ketoglutarate and succinate) appeared to decrease 24–48 h after dosing before increasing again from 72 to 96 h onwards following GAL administration. Urinary hippurate, phenylalanine, creatine and creatine followed a similar time-course (Fig. 1). The changes in urinary metabolites following GAL treatment is summarized in Table 1. The most striking biochemical change detectable by ^{1}H NMR was an increase in the concentrations of urinary and plasma betaine (Figs 1 and 2). Also noted was the increase in hepatic betaine (data not shown) and the late increase in urinary betaine (Fig. 1). It has been suggested that betaine acts as a methyl donor when normal methylating pathways are impaired, e.g. by ethanol [8]. Betaine is demethylated to form dimethylglycine by the transfer of the methyl group of betaine to homocysteine by betaine–homocysteine methyltransferase (Fig. 3). This methylation step is one of two mechanisms by which methionine is conserved in the rat liver [8]. The other pathway involves the use of 5*N*-methyltetrahydrofolate as the methyl donor, an important metabolite of the folate pathway. The novel NMR data presented here suggest that GAL induces a severe disturbance in the hepatic methylation pathways which may contribute to the onset of the hepatotoxic episode. Hence a previously unknown and important consequence (mechanism) of GAL toxicity may involve the disruption of the folate pathway.

BNF also causes mild hypertaurinuria (at 24 h, Fig. 4), suggesting a degree of hepatotoxicity consistent with the observation of fatty liver at post-mortem and the decrease in mobile triglycerides observed in the plasma (data not shown). Further, ^{1}H-NMR analysis also revealed a substantial decrease in the urinary excretion of citrate, α-ketoglutarate and succinate (Fig. 4) indicating strong inhibition of renal carbonic anhydrase (RCA). Interestingly, this inhibition was not maintained on the third dosing day (Figs 4f and 4g)

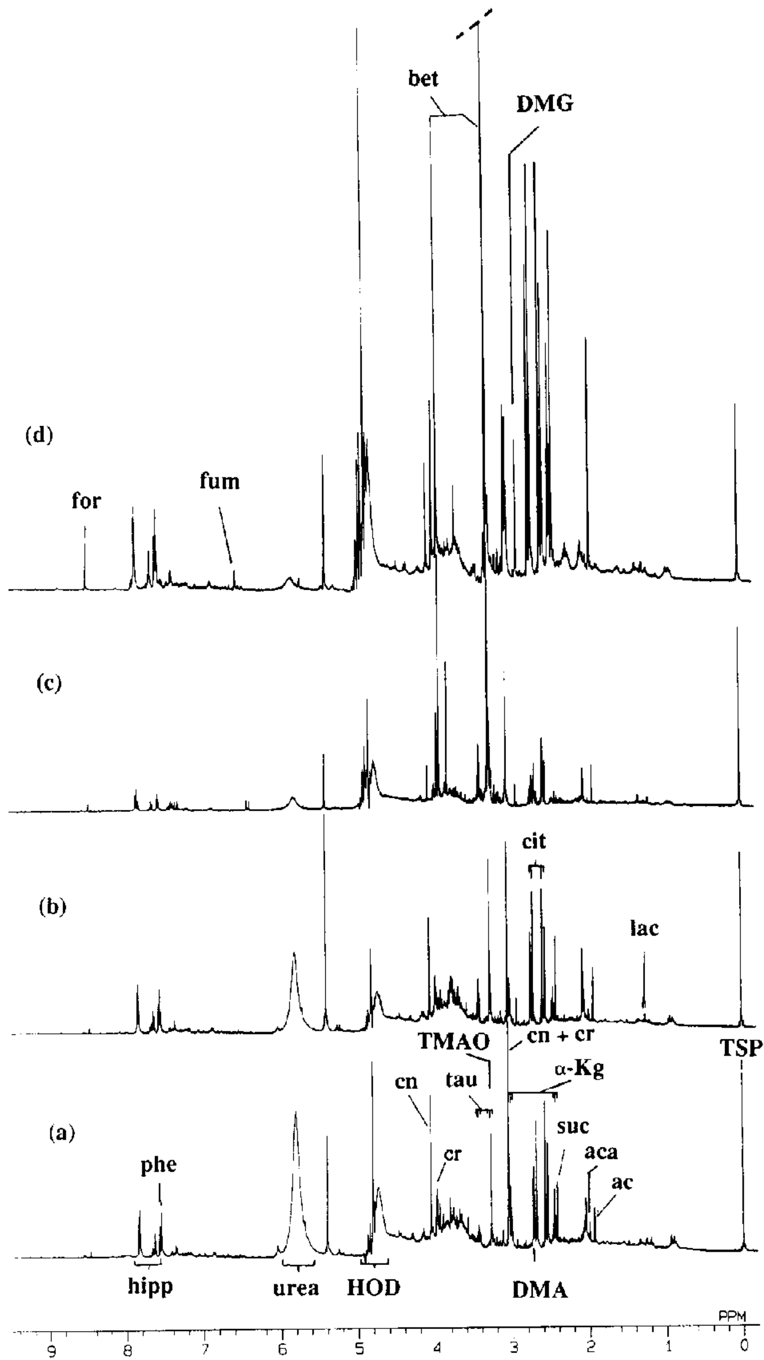

Figure 1
Spectra 500 MHz of urinary profiles taken at (a) 24 h predose, (b) 7–24 h, (c) 48–72 h and (d) 96–168 h following GAL administration over an observation period of 7 days. (ac, acetate; aca, acetamide; suc, succinate; α-Kg, α-ketoglutarate; cit, citrate; cr, creatine; cn, creatinine; TMAO, trimethylamine oxide; tau, taurine; hipp, hippurate; phe, phenylalanine; for, formate; fum, fumarate; bet, betaine; DMG, dimethylglycine; lac, lactate).

possibly indicating the induction of a new RCA isoform or a change in BNF metabolism. When RCA activity is low, intracellular pH of tubular epithelium is lowered, i.e. renal tubular acidosis (RTA) occurs [9]. RTA causes an increase in the flux of Kreb's cycle intermediates across the mitochondrial membrane into the matrix and stimulation of mitochon-

drial aconitase. This increases the concentration gradient between the tubular lumen and cytosolic compartment, hence tubular reabsorption of Kreb's cycle acids is enhanced and urinary concentrations of these metabolites reduced. These effects are similar to those observed following acetazolamide (a powerful RCA inhibitor) treatment [9].

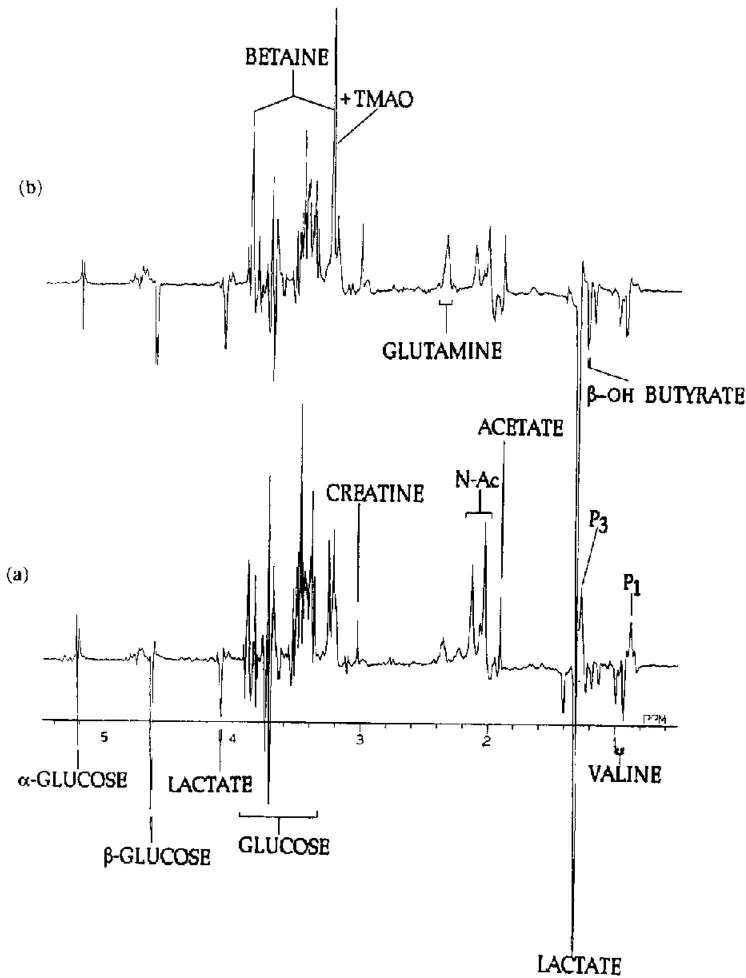

Figure 2
Spectra 500 MHz of plasma (freeze-dried) taken on termination of 48 h (a) following administration of 0.9% saline, and (b) following a single i.p. dose of 400 mg kg^{-1} GAL·HCl. (val, valine; glu, glucose; gln, glutamine; Hb, hydroxybutyrate; P_1 and P_3, methyl and methylene protons of triglycerides; *N*-Ac, *N*-acetylglycoproteins; other abbreviations are as before).

Table 1
Changes in metabolite concentrations following GAL administration (time-course experiment) are summarized below: 0, no increase; +, increase; and −, decrease in metabolite concentrations (number of symbols indicates magnitude of change, 0 = no change)

Metabolite	Time interval (h)					
	0–7	7–24	24–48	48–72	72–96	96–168
Acetate	0	+	++	+	+	++
Lactate	0	+	0	+	+	+
Dimethylglycine	0	+	+	+	+	++
Betaine	0	0	+	++	+++	+++
Taurine	0	0	++	+	+	0
Formate	0	0	0	+	++	+++
Citrate	0	0	−	−−−	−−	0
α-Ketoglutarate	0	−	−−−	−−	0	0
Succinate	+	−	−−	−−−	0	0

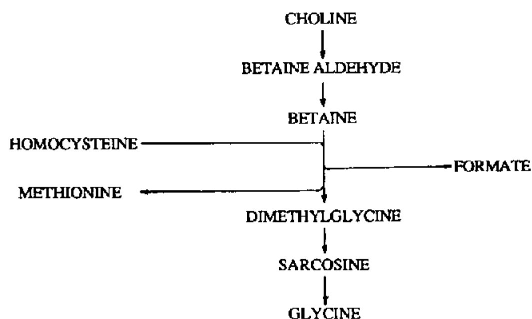

Figure 3
Metabolism of betaine in the rat liver.

Conclusion

¹H-NMR spectroscopic analysis of biofluids and cell extracts provides a powerful analytical tool for exploring the metabolic properties of xenobiotics. It has been shown that GAL causes betainaemia, hepatic betainosis and betainuria, suggesting some perturbation of the folate pool. NMR revealed that BNF is a strong RCA inhibitor as well as having mild hepatotoxic effects. This work exemplifies the general ability of NMR in the study of the biochemical effects of poorly understood and potentially hepatotoxic xenobiotics.

References

[1] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectrosc.* **21**, 449–501 (1989).
[2] K. Decker and D. Decker, *Rev. Physiol. Biochem.* **71**, 77–106 (1974).
[3] A. Medline, F. Schaffner and H. Popper, *Expt. Molec. Path.* **12**, 201–211 (1970).
[4] F. Maley, M.L. Tarentino, J.F. McGarrahan and R. Delgiacco, *Biochem. J.* **107**, 637–644 (1968).
[5] B.G. Lake and R.C. Longland, *Biochem. Pharmacol.* **27**, 2357–2361 (1986).
[6] J.K. Nicholson, M.J. Buckingham and P.J. Sadler, *Biochem. J.* **211**, 605–616 (1983).
[7] S.M. Sanins, J.A. Timbrell, C. Elcombe and J.K. Nicholson, *Method. Surveys Biochem. Anal.* **18**, 375–380 (1988).
[8] A.J. Barak and D.J. Tuma, *Life Sci.* **32**, 771–774 (1982).
[9] J.K. Nicholson and K.P.R. Gartland, *Method. Surveys Biochem. Anal.* **17**, 397–408 (1986).

[*Received for review 30 April 1990; revised manuscript received 27 June 1990*]

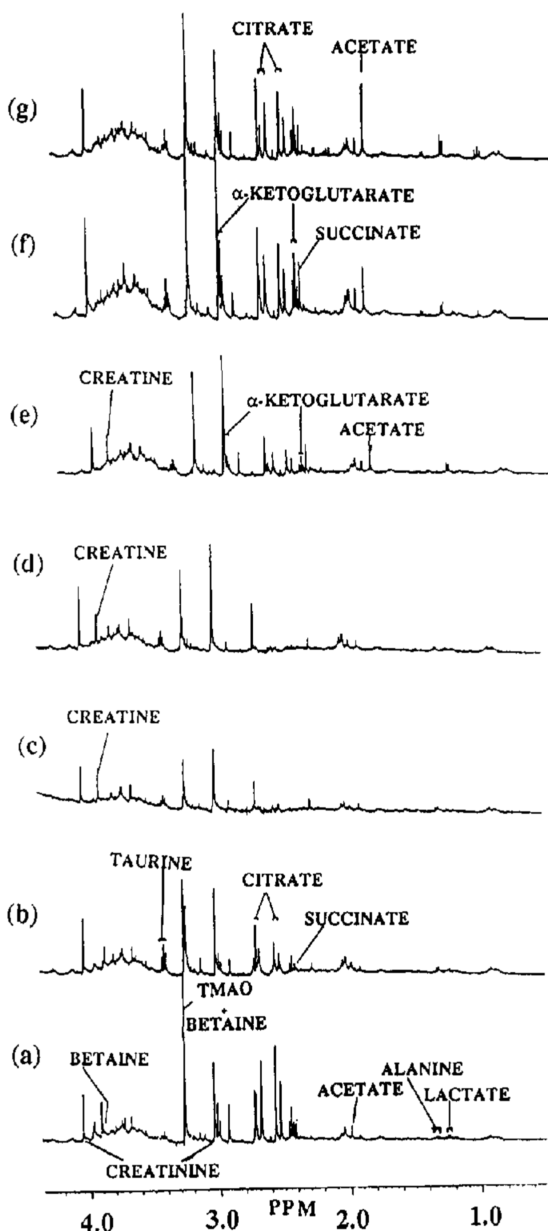

Figure 4
Spectra (400 MHz) of urine collected at (a) 24 h predose; (b) dose 1, 0–8 h; (c) dose 1, 8–24 h; (d) dose 2, 0–8 h; (e) dose 2, 8–24 h; (f) dose 3, 0–8 h; and (g) dose 3, 8–24 h following treatment with BNF (100 mg kg^{-1}).