Biochemical Pharmacology, Vol. 44, No. 2, pp. 231–241, 1992.
Printed in Great Britain.

0006-2952/92 $5.00+0.00
Pergamon Press Ltd

STUDIES ON THE BIOCHEMICAL EFFECTS OF THE ALDOSE REDUCTASE INHIBITOR 2,7-DIFLUOROSPIROFLUORENE-9,5′-IMIDAZOLIDINE-2′,4′-DIONE (AL 1576, HOE 843)

DETECTION OF D-GLUCARIC AND D-GLUCURONIC ACID EXCRETION BY HIGH RESOLUTION ^{1}H AND ^{13}C NMR SPECTROSCOPY

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(*Received* 23 *March* 1992; *accepted* 1 *May* 1992)

Abstract—The effects of two aldose reductase inhibitors on the biochemical composition of rat urine were investigated using high resolution ^{1}H and ^{13}C NMR spectroscopy. We report the elevated excretion of D-glucaric acid (DGA) and D-glucuronic acid (GCA) following treatment with 2,7-difluorospirofluorene-9,5′-imidazolidine-2′,4′-dione (Imirestat, IM, Al 1576, HOE 843) at 50 mg/kg/day for 1 month, but not with 3-4-bromo-2-fluorobenzyl-4-oxo-3-phthalazine-1-ylacetic acid (Ponalrestat, Statil), dosed at 50 mg/kg/day for 2 weeks. Sugar aciduria was also detected following treatment with the cytochrome P450 inducer phenobarbitone (PB) at 45 mg/kg/day for 1 month, although the qualitative and quantitative pattern of excretion of sugar acids differed greatly between the IM and PB treatment groups. The levels of GCA excreted are elevated 11-fold by IM treatment from 19.0 to 210.0 μmol/24 hr, but only 2.5-fold by PB, from 9.7 to 23.9 μmol/24 hr. DGA was not detectable in control urine, although levels did increase by 30% during the study from 7.5 to 10.9 μmol/24 hr, between day 8 and day 29, with IM treatment, and by 60% from 1.7 to 4.9 μmol/24 hr following PB administration for the same time period. This predominant elevation of DGA and GCA caused by IM treatment far exceeds previous records. In contrast, PB treatment resulted in an increase in intensity of a number of partially resolved sugar resonances, but at a much lower level than resulted from IM treatment. A raised level of DGA and GCA is usually associated with hepatic P450 induction; however, we report here profound DGA and GCA uria as a result of the inhibition of the aldehyde reductase, hexonate dehydrogenase (EC 1.1.1.19, EC 1.1.1.20). This mechanism is not closely linked to P450 induction, corroborating the current view that elevated excretion of DGA is not a reliable indicator of hepatic enzyme induction. This study further demonstrates the use of high resolution NMR spectroscopy in the detection of a novel biochemical effect which may go unnoticed during routine clinical chemistry tests.

Many of the complications associated with diabetes such as blindness, nephropathy and peripheral neuropathy have been associated with sorbitol accumulation [1]. The conversion of glucose to sorbitol by aldose reductase in hyperglycaemic tissue becomes a problem as sorbitol is poorly transported across biological membranes. The subsequent osmotic changes in the lens, kidney, and nervous tissue are typically associated with the development of the above conditions [2]. The experimental drug, Imirestat (IM‡,2,7-difluorospirofluorene-9-5′-imidazolidine-2′,4′-dione, Al 1576, HOE 843), was designed to act as an aldose reductase inhibitor (ARI), and so alleviate these problems.

Aldose reductase (alditol:$NADP^{+}$ oxidoreductase, EC 1.1.1.21) is part of a group of several aldehyde reductases, which have been isolated from tissues such as liver, kidney, skeletal muscle and brain [3]. It is closely related to, but distinct from, another aldehyde reductase known as aldehyde reductase (hexonate dehydrogenase, L-gulonate:$NADP^{+}$ oxidoreductase, EC 1.1.1.19, EC 1.1.1.20). They have been classified as ALR1 (aldehyde reductase), and as ALR2 (aldose reductase) [4] and have broad overlapping substrate specificites.

The biological and potential toxicological effects of ARIs have been incompletely studied and are poorly understood. Given the recent developments in high resolution NMR spectroscopy of biological fluids as a probe of toxicological events [5–9] we have investigated the effects of IM on the urine composition of rats using ^{1}H and ^{13}C NMR spectroscopy. In an earlier investigation, a 2-year toxicity study, D-glucaric acid (DGA) and D-glucuronic acid (GCA) were detected in rat urine after 6 months of treatment with IM by ^{1}H NMR and ^{13}C NMR (Gilbert and Troke, unpublished data).

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‡ Abbreviations: IM, Imirestat; ARI, aldose reductase inhibitor; DGA, D-glucaric acid; FID, free induction decay; GCA, D-glucuronic acid; IM, Imirestat: PB, phenobarbitone; TCA, trichloroacetic acid.

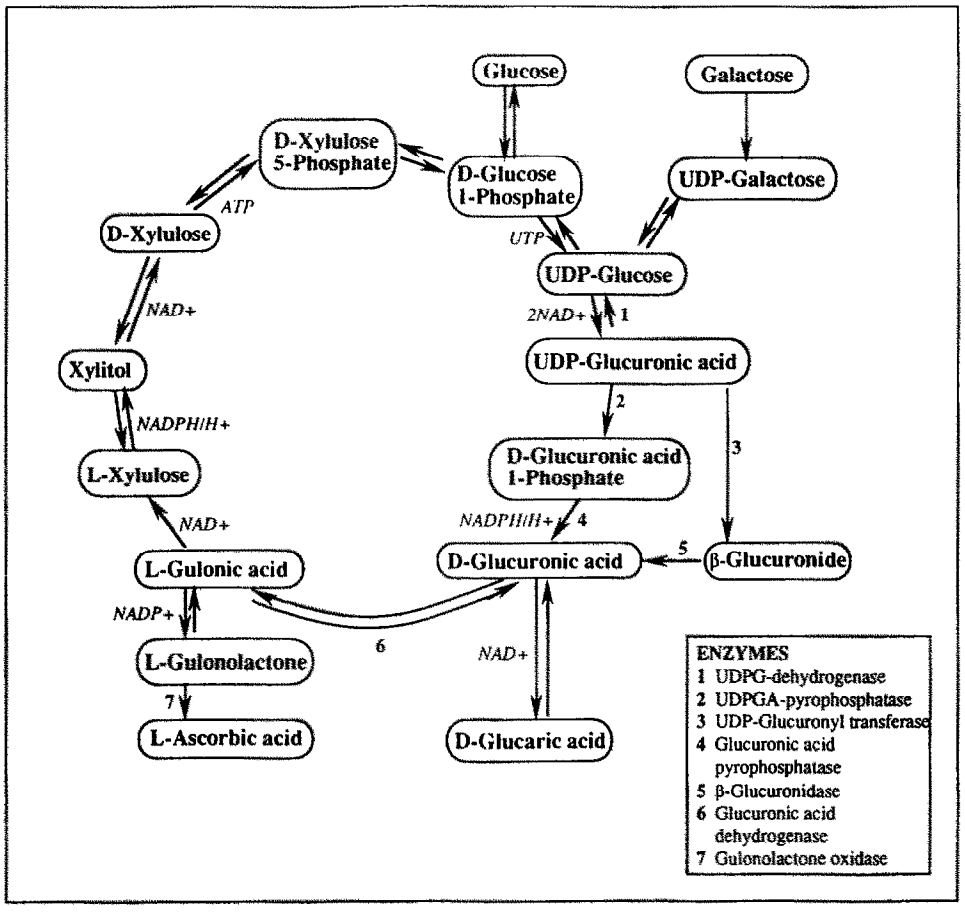

Fig. 1. D-Glucuronic acid pathway.

DGA is an end product of the glucuronic acid pathway (Fig. 1). It was first detected in the urine as an inhibitor of β-glucuronidase [10], and has been associated with hepatic microsomal enzyme induction, i.e. cytochrome P450 [11, 12]. Many chemicals are known to induce certain P450 isoenzymes and elevate DGA excretion in man. A wide variety of chemicals cause this effect in man [13], for example phenobarbitone (PB) [14], which induces P450IIB, ethanol [15], which induces P450IIE, and 2,3,7,8-tetrachloridibenzo-*p*-dioxin [16], an inducer of P450IA. PB also elevates DGA excretion in the rat [17], but 2,3,7,8-tetrachlorodibenzo-*p*-dioxin does not, despite the fact that it induces P450IA [18] illustrating the complexities associated with DGA excretion and P450 enzyme induction. Whilst these two phenomena have long been associated the causal mechanism, if one exists, remains unknown. Conventionally, DGA is detected by a labour-intensive enzymatic method [10, 19]; however, the alternative measurement of a spectrum of GCA pathway metabolites also exists using GLC [17] or high-resolution GC [20]. It was of interest to us to be able to detect elevation of sugar acids such as DGA by NMR, this technique allowing simultaneous detection not only of the sugar acid(s) of interest, but also providing information about other low molecular mass compounds in the urine.

The present NMR study was conducted over a 1-month period to measure and collate the time-course of ARI-induced sugar aciduria using IM and another ARI Ponalrestat (3-4-bromo-2-fluorobenzyl-4-oxo-3-phthalazine-1-ylacetic acid, Statil) with a classical inducer of hepatic drug-metabolizing enzymes, PB. PB was the inducer of choice as DGA excretion is associated primarily with this type of P450 inducer rather than the 3-methylcholanthrene-type inducer [21]. The principal aim of this work was to investigate the relationships (if any) between enzyme induction and sugar aciduria due to ARIs, in order to elucidate the mechanism, as this ultimately affects the utility of sugar aciduria as a marker of P450 induction.

MATERIALS AND METHODS

Materials and dosing. IM was obtained from Alcon (Fort Worth, TX, U.S.A.), Ponalrestat was obtained from ICI (Macclesfield, U.K.) and PB was from Evans Medical Ltd (U.K.). IM was administered daily by the p.o. route at 50 mg/kg, as a suspension in a 2% starch mucilage (N = 3) for 1 month. PB

was administered similarly at 45 mg/kg (N = 3). Control animals received 0.9% saline solution (N = 3). Ponalrestat was dissolved in sodium carbonate (250 mM) and dosed for two weeks by the p.o. route at 50 mg/kg (N = 3).

Experimental animals. Female Wistar rats (139–145 g, mean weight 140 g, source Bantin and Kingman, Hull, U.K.), were housed in glass Metabowls and were provided with food and water *ad lib.* throughout the experiment. Urine was collected for the periods 0–6 hr and 6–24 hr for the first 7 days of the experiment, after which collection was every 24 hr for 22 days. At the end of the study the rats were killed by decapitation, and sera were collected. The livers were excised from the animals to prepare hepatic microsomes and a TCA extract from a section of the liver.

^{1}H *NMR spectroscopy of urine.* For each urine sample collected, routine ^{1}H NMR spectra were recorded on a Bruker WM250 spectrometer operating at 250 MHz ^{1}H resonance frequency, at 25°. Urine samples, 0.5 mL, were freeze-dried and reconstituted in 0.7 mL of D_2O (lock signal), containing sodium-tetradeuterio-(trimethylsily) propionate to give a final concentration of 207 μM. This provided a quantitation standard and an internal chemical shift reference (δ = 0 ppm). For each sample, 128 free induction decays (FIDs) were collected into 16,384 data points (acquisition time 2.048 sec). The intense water signal was suppressed by a secondary irradiation field (gated-off during acquisition) at the water resonance frequency. To aid resonance assignment ^{1}H NMR spectra were collected on selected samples at 500 and 600 MHz. For 500 MHz experiments ^{1}H NMR spectra of urine were measured on a JEOL GSX 500 spectrometer. Urine samples, 1.0 mL, were freeze-dried and reconstituted in 0.7 mL D_2O (lock signal). For each sample 64 FIDs were collected into 32,768 data points (acquisition time 2.73 sec). 600 MHz ^{1}H NMR spectra were measured on a Varian VXR 600S spectrometer. Urine samples, 2 mL, were freeze-dried and reconstituted in 0.7 mL D_2O (lock signal). For each sample 64 FIDs were collected into 35,968 data points (acquisition time 2.997 sec).

^{1}H *NMR spectroscopy of blood sera.* Sera were collected by centrifugation (3000 rpm × 10 min) of blood which had been allowed to clot. Sera samples, 0.65 mL, were freeze-dried and reconstituted in 0.65 mL D_2O (lock signal). Carr Purcell Meiboom Gill (CPMG) spin-echo ^{1}H 500 MHz NMR spectra [22] were collected for each sample, with 128 FIDs being collected into 32,768 data points (acquisition time 2.559 sec).

^{1}H *NMR spectroscopy of liver extracts.* Frozen liver sections, 1.5 g, were ground to a powder in liquid nitrogen, and then homogenized in 4 mL NaCl (0.9%). Following centrifugation (3000 rpm × 15 min) protein was precipitated from the supernatant by the addition of TCA solution (6%), 2 mL of TCA solution per 1 mL of supernatant. Centrifugation as before produced the TCA extract which was freeze-dried. Each sample was reconstituted in 0.7 mL D_2O (lock signal). 500 MHz ^{1}H NMR spectra were recorded with 128 FIDs being collected into 32,768 data points (acquisition time 2.559 sec).

^{13}C *NMR spectroscopy of urine.* ^{13}C{^{1}H} NMR spectra were recorded on a Bruker WM250 spectrometer operating at 62.8 MHz ^{13}C resonance frequency, at 25°, using broadband ^{1}H decoupling. Urine samples from the IM and PB groups, 2 mL, were freeze-dried and reconstituted in 0.7 mL D_2O (lock signal). Data were acquired using the DEPT ($3\pi/4$) pulse sequence [22]. For each sample, 30,814 FIDs, were collected into 32,768 data points (acquisition time 0.819 sec).

Quantitation of GCA and DGA excretion levels. GCA excretion levels were quantitated, using sodium-tetradeuterio-(trimethylsilyl) propionate as an internal standard, by peak height measurement of the α and β anomeric proton signals. DGA excretion levels were quantitated by comparison of ^{13}C peak height ratios of DGA to GCA with a standard spectrum of DGA and GCA.

Measurement of hepatic cytochrome P450 levels. Each liver was perfused through the portal vein with ice-cold saline, 0.9%. Microsomes were prepared by differential high speed centrifugation [23]. Each microsomal pellet was resuspended on the basis of 4 mL/g fresh weight in a 100 mM Tris–HCl, 20% glycerol, pH 7.4 buffer. Protein was determined by the Lowry method [24], and cytochrome P450 was measured as the reduced carbon monoxide adduct [25].

RESULTS

Typical 500 MHz ^{1}H NMR spectra of urine collected during this study are shown in Fig. 2. The control (a), PB (b) and IM (d) samples were collected on day 28 of dosing, and the Ponalrestat sample (c) was collected on day 7 of dosing. The standard spectra of the sugar acids DGA (e) and GCA (f) are also shown. The control spectrum (a) shows many signals typically seen in this chemical shift range of the spectrum of rat urine, e.g. citrate (2.51–2.70 ppm, AB spin system), methylamine (2.61 ppm, singlet), dimethylamine (2.73 ppm, singlet), dimethylglycine (2.93 ppm, singlet), creatinine (3.04 and 4.05 ppm, singlets), trimethylamine-*N*-oxide (3.27 ppm, singlet), taurine (3.43, triplet), glycine (3.57 ppm, singlet), hippurate (3.97 ppm, singlet as sample was lyophilised and reconstituted in D_2O), allantoin (5.39 ppm, singlet) and lactate (4.13 ppm, quartet).

In comparing the NMR spectra from an IM-treated animal (d) with a control animal (a) several differences are immediately apparent. Firstly, the signals due to the α (5.25 ppm) and β (4.63 ppm) anomeric (H_1) protons of GCA are clearly visible, together with an increase in overall intensity and complexity in the region 3.3–3.8 ppm. The presence of GCA was confirmed by addition of the standard compound to the urine and remeasurement of the spectrum. ^{1}H NMR spectra measured at 600 MHz show the GCA resonances more clearly due to improved dispersion of the chemical shifts (Fig. 3b). The expanded region of the spectrum (c) shows that the GCA β-anomeric proton signal (4.67 ppm) is resolved from the glucose β-anomeric proton signal (4.69 ppm). ^{1}H NMR spectra measured at 600 MHz indicate that the glucose excreted in the urine during

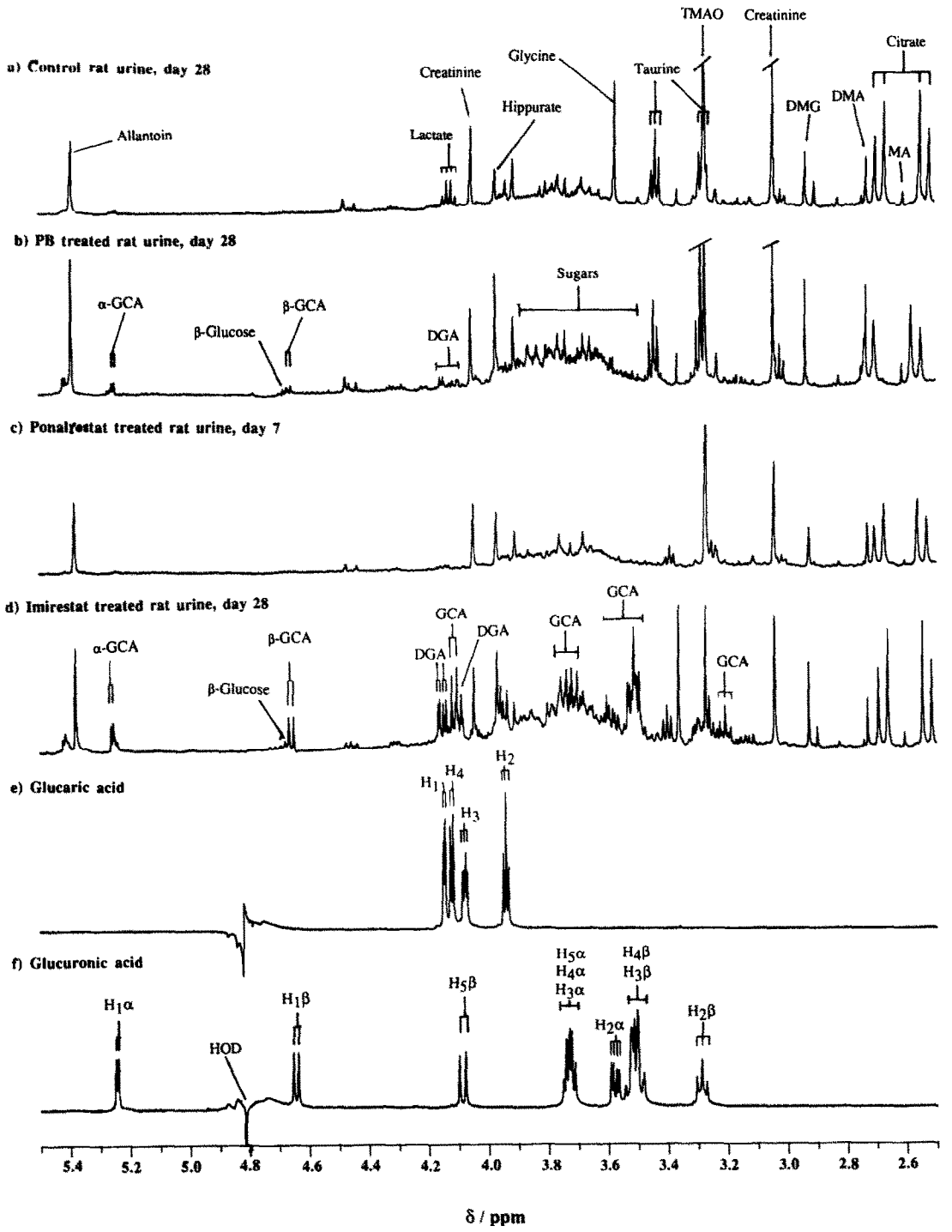

Fig. 2. ^{1}H NMR spectra (500 MHz) of (a) control (b) PB-treated (45 mg/kg p.o.), (c) Ponalrestat-treated (50 mg/kg p.o.) and (d) IM-treated (50 mg/kg p.o.) rat urine; (e) D-glucaric acid and (f) D-glucuronic acid. MA, methylamine; DMA, dimethylamine; DMG, dimethylglycine; TMAO, trimethylamine-*N*-oxide.

treatment was negligible and could be discounted in quantitation measurements, as the level of excretion did not appear to change throughout the experimental period. The signals due to DGA are also indicated on the 500 MHz ^{1}H NMR spectrum (Fig. 2e) following assignment of the resonances on addition of the standard compound to urine. Comparison with the spectrum in Fig. 3 again shows that the

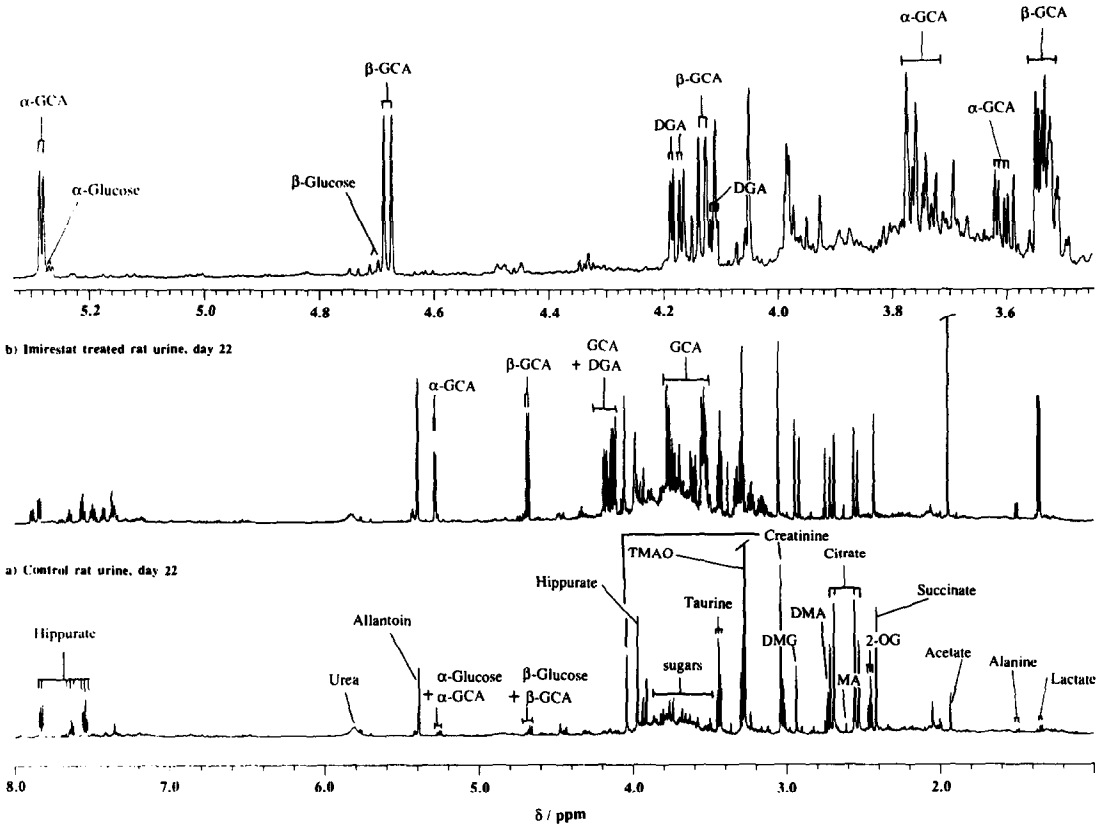

Fig. 3. ^{1}H NMR spectrum (600 MHz) of (a) control and (b) IM-treated rat urine (50 mg/kg p.o.) with an expansion (δ = 3.4–5.2) of spectrum (b) above.

resonances are much better resolved at the higher field strength. In contrast, Ponalrestat treatment appears to have very little effect on the excretion of sugars and/or polyols, in fact this spectrum shows no major differences when compared with the control rat urine spectrum (Fig. 2a).

The changes in rat urine following PB administration are more subtle than those seen in IM-treated rats when observed by ^{1}H NMR. The 28-day urine spectrum (Fig. 2b) shows a slight increase in GCA, as indicated, and also a possible increase in DGA. However, in addition to this the general "area under the curve" between the regions 3.4 and 3.8 ppm is increased, which probably indicates that a number of different sugars and/or polyols are being excreted at a slightly increased level rather than two sugars being excreted at a very high level. A clearer picture of the excretion profile of the PB treated rat is obtained from the ^{13}C NMR spectrum (Fig. 4).

The benefit of ^{13}C over ^{1}H NMR spectroscopy is the increased signal dispersion due to the greater chemical shift range of ^{13}C. This is particularly useful for the assignment of sugar resonances. Although the signal to noise ratio differs between the spectra of the ^{13}C data it is still possible to compare the resonance patterns and then relative intensities of the signals. The control spectrum (Fig. 4a) from an untreated rat shows that the resonances from GCA can be observed (*) but little else is detectable in this region of the spectrum. The spectrum from IM-treated rat urine (Fig. 4c) confirms that almost all of the elevated resonances assigned in the proton spectrum are due to DGA (●) and GCA (*). In contrast it can be seen in the PB-treated rat urine spectrum that there are many other minor resonances present, which require further NMR work at higher field to assign.

The time-course of sugar acid elevation due to IM and PB treatment can be followed by ^{1}H NMR as shown in Figs 5 and 6. Having determined which signals were due to which sugars at high field, quantitation of the routinely collected spectra was possible. The levels of urinary GCA following these treatments were quantitated from the 250 MHz ^{1}H NMR spectra (Fig. 7). The quantitative results support qualitative observations from the spectra, i.e. that excretion of GCA is elevated rapidly following IM treatment (11-fold). In contrast PB caused a smaller, but significant elevation of urinary GCA (2.5-fold). DGA levels were quantitated from

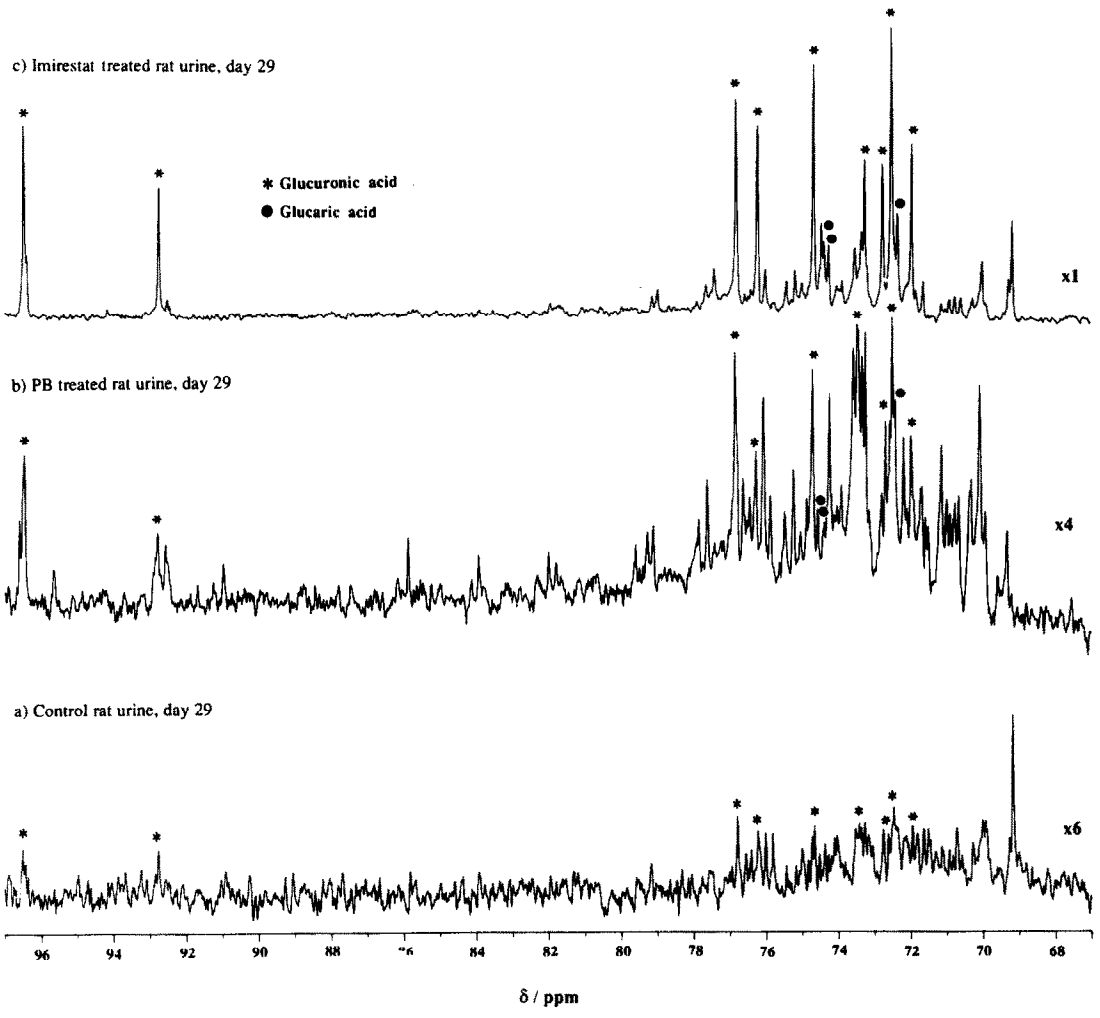

Fig. 4. ^{13}C NMR spectra (62.8 MHz) of (a) control, (b) PB-treated (50 mg/kg p.o.) and (c) IM-treated (50 mg/kg p.o.) rat urine.

^{13}C NMR spectra, where peak superposition does not pose a quantitation problem. It can be seen from the data (Fig. 7) that a 30% elevation of DGA is observed with IM treatment and a 60% elevation of DGA is seen with PB.

Little difference in the biochemical composition of the sera was observed between the treated and control groups (results not shown), indicating that the observed effect is not due to overflow of DGA and GCA from the blood. Additionally, ^{1}H NMR analysis of TCA extracts of the liver (results not shown) showed no elevation of the sugar acids in the liver, indicating that the sugar acids are not stored in the liver but are excreted as soon as they are present in excess of normal physiological levels.

The hepatic P450 levels measured at the end of the study (Table 1) show that IM does not act as a typical enzyme inducer like PB, as significantly lower levels of P450 are detected in the IM group compared with the PB group. If a sugar acid:P450 ratio is considered (Table 1) there is a 15-fold difference between the PB and IM groups. This illustrates that the increase in sugar acid levels in the IM group is not accompanied by the concomitant increase in P450 levels which would be expected if the effect were due to P450 induction. This comparison of the biochemical effects of IM and PB by NMR shows that as the profiles of sugar acid excretion differ greatly between the two compounds, then the effects observed in this study are probably caused by differing mechanisms.

DISCUSSION

We report here a novel biochemical effect of a test drug which would probably have gone unnoticed during the series of routine biochemical measurements which are normally performed as part of a drug development programme. Early studies [11, 12] investigating the relationship between hepatic enzyme induction and sugar aciduria only measured levels of DGA excretion. The use of GC

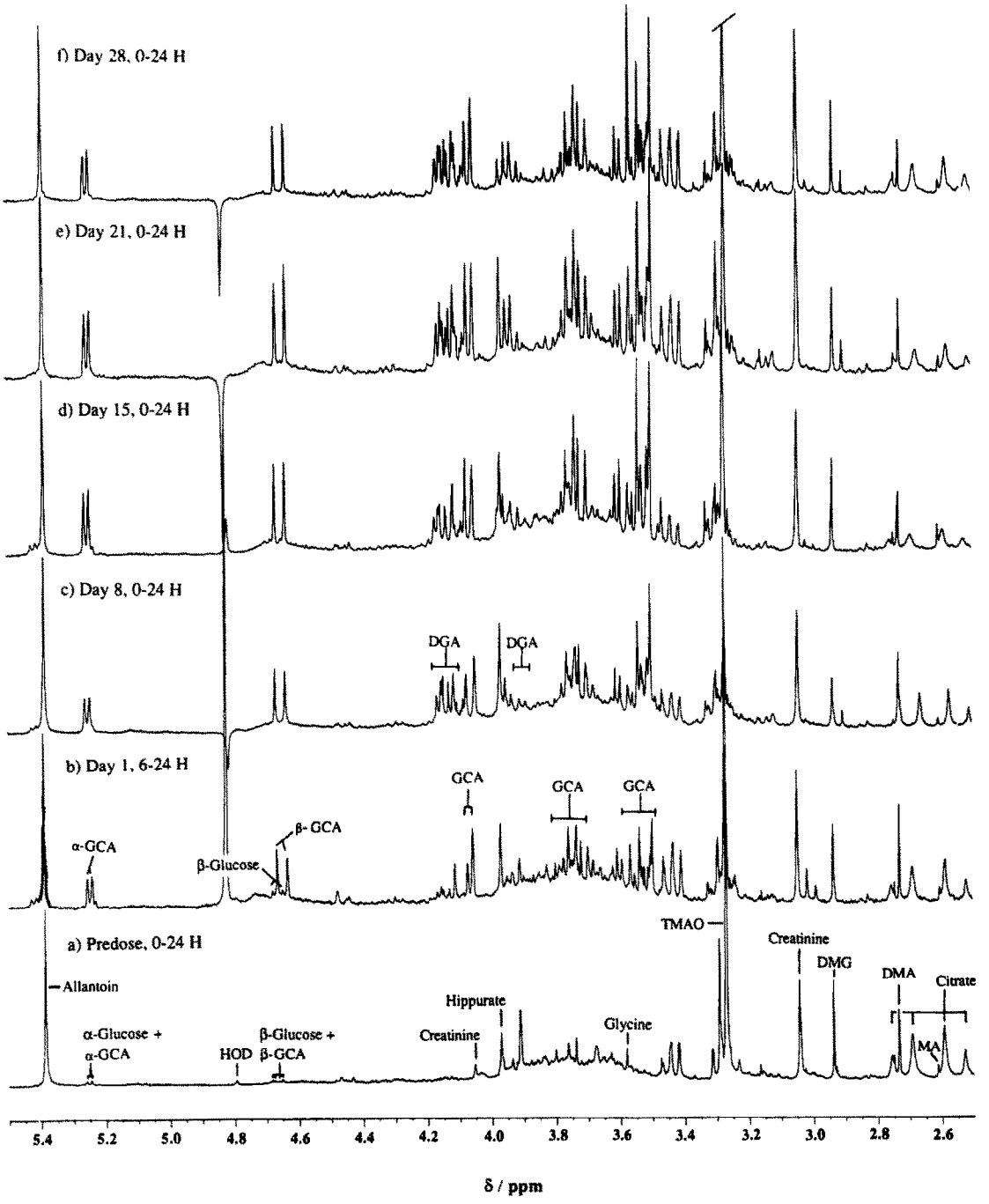

Fig. 5. ^{1}H NMR spectral time-course (250 MHz) of rat urine following dosing with IM (50 mg/kg p.o.).

enabled additional metabolites of the glucuronic acid pathway (GCA, gulonic acid, ascorbic acid, and xylitol) to be detected simultaneously [17, 26, 27]. Indeed, Lake *et al.* [17] suggested that it is more informative to observe the levels of more than one metabolite and thus time-course excretion patterns, which this chromatographic technique allowed. At that time NMR was not a technique commonly employed to analyse biofluids. However, the great developments in the field of high resolution NMR spectroscopy of biofluids since that time [5–9] now make NMR the ideal tool to screen for novel metabolic effects during toxicity testing, as prior knowledge of sample composition and chemical derivatization are not prerequisites to analysis by this method.

The levels of GCA and DGA determined in this study by NMR compare well with control values

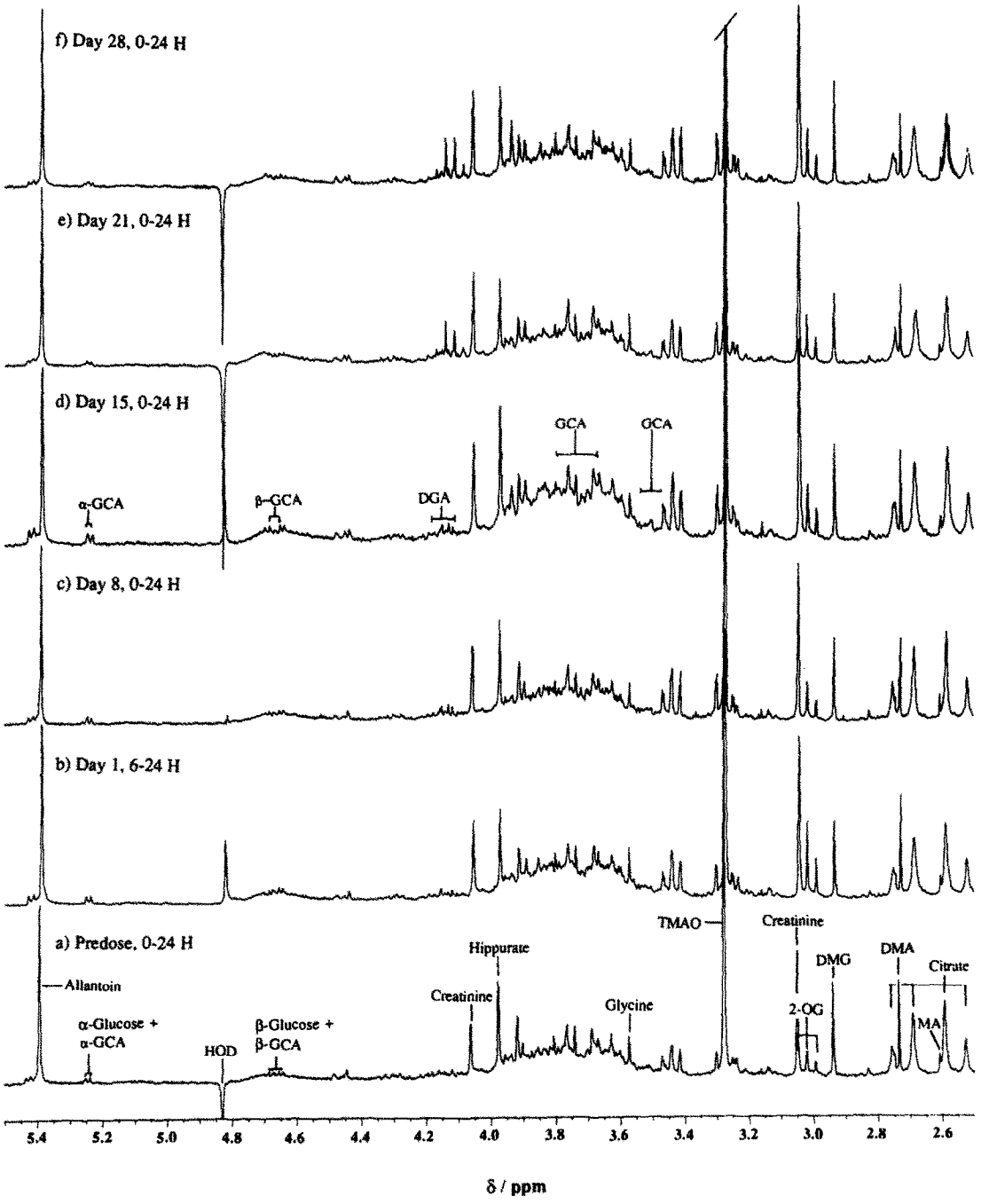

Fig. 6. ^{1}H NMR spectral time-course (250 MHz) of rat urine following dosing with PB (45 mg/kg p.o.).

determined by other analytical methods. We report here 9.7 μmol GCA/24 hr for the PB predose control group, and 19.0 μmol GCA/24 hr for the IM predose control group. Lake *et al.* [17] reported 1.0–5.2 μmol GCA/24 hr for male Sprague–Dawley, and 15.5 μmol GCA/24 hr, for male Wistar rats (Lake *et al.* [26]). Warrander and Waring [27] reported 8.5 μmol GCA/24 hr for female Wistar rats. The elevation of DGA and GCA following PB treatment also compares well with Lake *et al.* [26]. However, in contrast IM treatment elevated GCA levels vastly and raised DGA levels considerably.

This comparison of the urinary excretion profiles following IM and PB treatment shows that IM treatment elevated two sugar acids only, whereas PB treatment elevated an additional number of minor polyol and/or sugar-like metabolites with incompletely resolved NMR signals. The large and

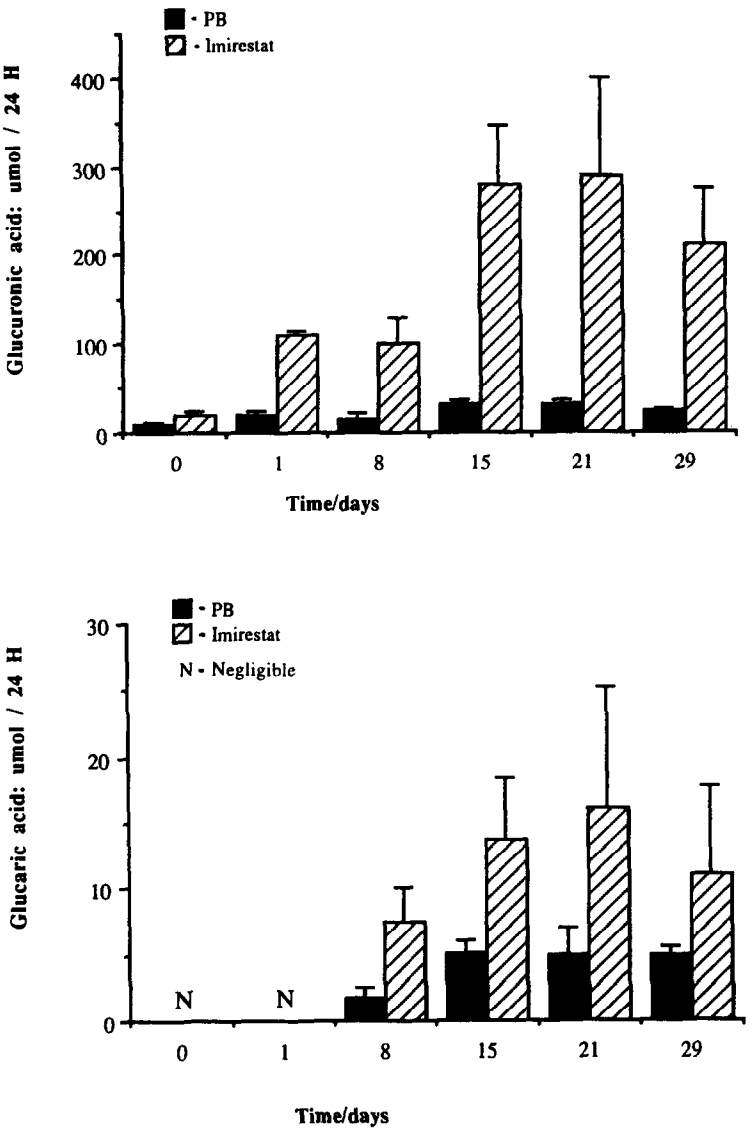

Fig. 7. The effects of IM and PB on the levels of excretion of glucuronic acid and glucaric acid.

Table 1. The effect of IM and PB treatment on levels of hepatic cytochrome P450

Parameter	Treatment group		
	Control	HOE 843	PB
Hepatic Cytochrome P450 (nmol/mg protein)	0.41 ± 0.02	0.63 ± 0.02*	1.08 ± 0.09*
GCA:P450 ratio (day 29)	33†	332 ± 132	22 ± 2
DGA:P450 ratio (day 29)	ND	18 ± 11	5 ± 1

Values are means ± SE, N = 3.
* $P < 0.01$.
† Single animal only.
ND, not detectable.

probably unprecedented elevations of DGA and GCA caused by IM treatment indicate that IM does not act in the same way as the typical enzyme inducer PB. As neither GCA nor DGA is elevated in the serum or liver extract by either IM or PB treatment, this suggests that on reaching a threshold level, GCA is excreted directly into the urine due to its high polarity, as metabolic recycling of the molecule is not possible. The only metabolic route for GCA is conversion to the end product DGA, which is similarly excreted.

We propose that the sugar aciduria observed following IM treatment is due to a secondary pharmacological effect of the ARI. Recent work [28] has shown that several other developed ARIs exhibit non-specific inhibition of ALR1. Sato and Kador [28] studied the selectivity of inhibition, by ARIs, of ALR2 over ALR1. Of the 10 ARIs tested, selectivity for aldose reductase ranged from only twice as selective (IM) to 38 times more selective (Quercitrin and Alrestatin). Ponalrestat was even more highly selective for ALR2 by a factor greater than 100. This high specificity for ALR2 coupled with the absence of sugar aciduria following Ponalrestat treatment substantiates the hypothesis that the effects observed with IM treatment are due almost entirely to inhibition of ALR1 rather than the effect of hepatic enzyme induction. Although this theory accounts for the effects of IM, it does not however explain the more subtle effects of PB treatment. It is known that PB also inhibits ALR1 [29]; however, the results presented here show that this inhibitory action is certainly not as marked as that of IM, and indicate that one or more biochemical events may be responsible for the effects observed.

The ^{13}C NMR spectrum of PB-treated rat urine shows approximately 50 resonance signals in the region 68–100 ppm that are elevated above control levels, which could be equivalent to at least 10 small carbohydrates such as sugars, sugar acids and polyols. This study also reveals that the region of the ^{1}H NMR spectrum of urine containing resonances that are typically assigned to glucose or a "sugar envelope" is much more complex than previously assumed, as each piece of information about a compound is masked by chemical noise arising from a multiplicity of overlapping signals from other molecules present in the sample. The problem with analysing this region (δ 3.5–4.0) of the spectrum lies in the close chemical and structural similarity of many sugars and polyols, thus many of the resonances arising from sugar and polyol molecules all appear in the same region of the spectrum. Assignment of many resonances in this region is ongoing, by use of advanced 2D NMR methods. These results additionally implicate that PB treatment may give rise to novel biochemical effects, previously unidentified by other analysis techniques.

It may be postulated that the action of ARIs affects the balance of such glucose-utilizing pathways as glycolysis and the pentose phosphate pathway. This seems unlikely as the polyol and glucuronic acid pathways are only minor routes of glucose catabolism compared with glycolysis and the pentose phosphate pathway.

The findings of this study demonstrate that the use of high resolution NMR spectroscopy as an analytical tool for body fluid analysis is of benefit in the detection of novel effects which may go undetected if routine clinical, biochemical and toxicological procedures are employed in toxicity testing during a drug development programme.

Acknowledgements—We wish to thank the SERC (VH) and Hoechst UK for financial support, MRC and SERC for provision of central NMR facilities, ICI Pharma for the supply of Ponalrestat, and Dr R Turcan and staff at Hoechst Pharmaceutical Research Labs, Dept. of Drug Metabolism for their assistance with this work.

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