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Short Communication

Isolation and characterization of the *S*-glucuronide of 4-nitrothiophenol formed by microsomal glucuronyl transferase

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Introduction

Over the last three decades, occasional reports have indicated that *S*-glucuronides are metabolites of thiols and dithioic acids [1]. Recently, an *S*-glucuronidated compound, formed in rats after administration of malotilate, was isolated by chromatography from bile, and NMR and mass spectrometry were used to establish that it was a mono-*S*-glucuronide of a dithiol metabolite of the drug [1]. Subsequently the apparent kinetic parameters of its formation using rat liver microsomes were investigated by HPLC [2].

It has been assumed that *S*-glucuronides are formed from acceptor thiols by the catalytic action of microsomal UDP-glucuronate glucuronyltransferases (EC 2.4.1.17). Dutton and Illing [3] showed that in the presence of a rat liver microsomal fraction and the donor substrate UDP-glucuronate, simple thiols were converted into products with the characteristics of glucuronides and that the acceptor thiols were regenerated by treating the products with β-glucuronidases. However, these products were not isolated or characterized. Because of their charged nature and inherent instability, glucuronides are difficult to isolate but recent advances in chromatography now allow their isolation without extensive degradation [4].

This paper describes the microsomal transferase-catalysed formation of the β-*S*-glucuronide of 4-nitrothiophenol, its isolation and purification by HPLC, and its characterization by NMR spectroscopy.

Experimental

Materials

Ammonium UDP-glucuronate (UDP-GlcUA), UMP, uridine and 4-nitrophenyl glucuronide were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, UK). 4-Nitrophenol (spectrophotometric grade) was from BDH Ltd (Poole, Dorset, UK). A crude preparation of 4-nitrothiophenol was obtained from Aldrich Chemical Co. Ltd (Gillingham, Dorset, UK) and acetonitrile for HPLC was from Rathburn Chemicals Ltd (Walkerburn, Peebleshire, UK). 4-Nitrothiophenol was purified according to a published method [5].

Methods

Male guinea pigs (Hartley strain, 250–400 g) were from David Hall, (Newchurch, Burton-on-Trent, Staffs, UK). They were starved overnight, killed and liver microsomal fractions were prepared and washed thoroughly with 154 mM KCl [6]. The microsomal membranes were resuspended in 154 mM KCl at a concentration of about 20 mg of protein ml^{-1} and were used immediately or were stored frozen at −20°C for subsequent use. Microsomal protein was determined using a biuret reagent [7]. Rates of disappearance of 4-nitrophenol (0.32 mM) and 4-nitrothiophenol

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(0.32 mM) catalysed by microsomal UDP-glucuronyltransferases were measured at 37°C in reaction mixtures containing 3.2 mM UDP-GlcUA [8]. When the thiophenol was used, reaction mixtures were flushed with and kept under N_2. Rates of disappearance of these compounds were negligible in the absence of UDP-GlcUA (see also ref. 6).

To separate the products of the reaction with 4-nitrothiophenol, reaction mixtures under N_2 contained the concentrations of substrates given above, 65 mM Tris–HCl buffer (pH 8.0) and 1 mg of microsomal protein in total volumes of 0.8 ml. After 1 h of shaking at 37°C, the mixtures were cooled on ice and the microsomal protein was removed by centrifugation. Portions (20 μl) of the supernatant solutions were analysed by HPLC using a column (150 × 4.6 mm) packed with a mixed phase material 5 μm Spherisorb S5 ODS/NH_2 (Phase Separations, Queensferry, UK), eluting with acetonitrile–0.1 M KH_2PO_4 (pH 4.8) (10:190, v/v) at 1 ml min^{-1}.

To isolate the material suspected of being 4-nitrothiophenyl glucuronide, a reaction mixture of 20 ml total volume and identical composition was employed. After the 1 h reaction period and removal of microsomal protein, the supernatant solution was freeze-dried. The residue was dissolved in H_2O (3 ml) and the solution was chromatographed in portions as described previously via a 1 ml injection loop. The fractions of eluate containing the product were combined and freeze-dried and the residue was extracted with three portions (2 ml) of ethanol. The solvent was removed with a stream of N_2 and the residue was dissolved in methanol (5 ml). The methanolic solution was then rechromatographed; fractions of eluate containing the product were combined, freeze-dried and extracted with ethanol as before.

The ^{1}H-NMR spectra of 4-nitrophenyl glucuronide and the isolated reaction product were determined using solutions in deuterated pyridine with tetramethylsilane as reference standard and a Brüker WH-360 NMR spectrometer.

Results and Discussion

Confirming the report by Dutton [9], measurements of the rates of disappearance of 4-nitrophenol and 4-nitrothiophenol showed that with microsomal fractions in the presence of UDP-GlcUA (3.2 mM) the thiophenol was converted much more slowly. In a typical experiment, initial rates of conversion of the phenol and the thiophenol were 65.2 and 9.9 nmol min^{-1} per mg of microsomal protein, respectively.

Similar studies (unpublished results) with this enzyme system and the thiocarbamide drug, 6-*n*-propyl-2-thiouracil suggest that this compound was not an acceptor molecule, although strong circumstantial chemical and pharmacological evidence has been published that an *S*-glucuronide of the drug is formed under these conditions [10].

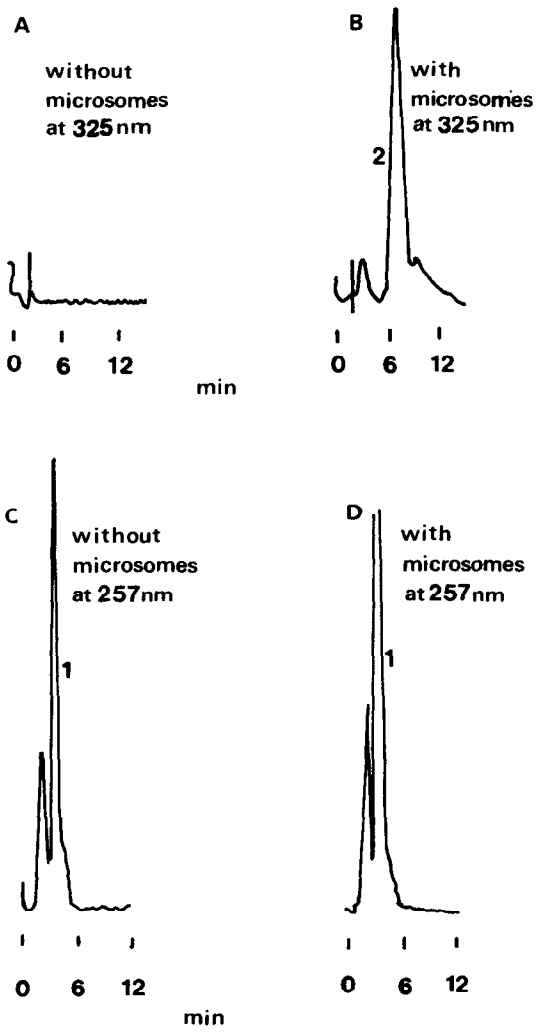


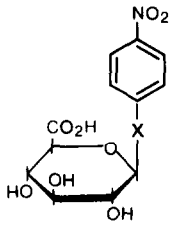
Figure 1
Typical chromatograms of reaction mixtures containing 4-nitrothiophenol, UDP-glucuronic acid and microsomal membranes. 4-Nitrothiophenol (0.32 mM) and UDP-GlcUA (3.2 mM) without addition of microsomal membranes (A, 325 nm; C, 257 nm). Complete reaction mixture (B, 325 nm; D, 257 nm). See Experimental for details. Peak 1 is UDP-GlcUA, peak 2 is the putative *S*-glucuronide.

Although the acetonitrile–phosphate buffer HPLC mobile phase produced a good separation (t_R = 7 min) of the putative *S*-glucuronide (Fig. 1), 4-nitrothiophenol was strongly retained on the mixed phase column. Moreover UDP-GlcUA (the donor molecule; t_R = 3 min), and UMP (t_R = 3 min) and uridine (t_R = 3 min; possible degradation products formed in these reaction mixtures [11]) did not absorb at 325 nm, the wavelength of detection for the *S*-glucuronide, but at 257 nm. 4-Nitrothiophenol has UV absorption maxima at 321 and 409 nm in methanol, whilst the isolated product had one maximum at 326 nm, which was near the wavelength of detection for some experiments.

Comparison of the 360 MHz NMR spectrum of the product with the spectrum of 4-nitrophenyl-β-glucuronide confirmed that the product was the *S*-glucuronide of 4-nitrothiophenol (Table 1) since the characteristic doublet signals at 8.02 ppm and 7.84 ppm can be assigned to the aromatic protons in the nitrothiophenol moiety. Moreover, whilst it was possible to assign some of the other signals to the glucuronyl group the most characteristic signal in the NMR spectra of *O*- [12] and *S*-glucuronides [1] is in the region 5–6 ppm caused by the protons on the anomeric Cl-carbons. In this study these signals appeared as doublets at 5.92 ppm for 4-nitrophenyl-β-glucuronide and at 5.62 ppm for the isolated product.

The HPLC system developed with the mixed phase column permitted the measurement and isolation of the *S*-glucuronide, and therefore would be suitable for more detailed kinetic studies of its transferase-catalysed formation.

Table 1
NMR spectral data for 4-nitrophenyl-β-glucuronide and the isolated reaction product



Carbon position	X = O (ppm)	X = S (ppm)
C1 (1H)	5.92 (d)	5.62 (d)
C2, C3 (2H)	4.47 (m)	
C4 (3H)	4.74 (t)	4.54 (t)
C5 (1H)	4.93 (d)	4.65 (d)
C2′ (2H)	7.34 (d)	7.84 (d)
C3′ (2H)	8.15 (d)	8.02 (d)

d = doublet; m = multiplet; t = triplet.

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References

[1] M. Nakaoka, H. Hakusui and T. Takegoshi, *Xenobiotica* **19**, 209–216 (1989).
[2] M. Nakaoka and H. Hakusui, *J. Chromatogr.* **526**, 254–259 (1990).
[3] G.J. Dutton and H.P.A. Illing, *Biochem. J.* **129**, 539–550 (1972).
[4] J. Tomasic, in *Drug Metabolite Isolation and Determination* (E. Reid and J.P. Leppard, Eds), pp. 149–160. Plenum Press, London (1983).
[5] C.P. Price and G.W. Stacey, *J. Am. Chem. Soc.* **68**, 498–500 (1946).
[6] A.B. Graham and G.C. Wood, *Biochim. Biophys. Acta* **311**, 45–50 (1973).
[7] E. Layne, *Meth. Enzymol.* **3**, 447–454 (1957).
[8] D.T. Pechey, A.B. Graham and G.C. Wood, *Biochem. J.* **175**, 115–124 (1977).
[9] G.J. Dutton, in *Glucuronidation of Drugs and Other Compounds* (G.J. Dutton, Ed.), pp. 88–91. CRC Press, Boca Raton, FL (1980).
[10] L.R. Lindsay, A. Vaughn, K. Kelly and H.Y. Aboul-Enein, *Biochem. Pharmacol.* **26**, 833–840 (1977).
[11] B.I. Knight and G.G. Skellern, *J. Chromatogr.* **192**, 247–249 (1980).
[12] M.P. Harrison, in *Drug Metabolite Isolation and Determination* (E. Reid and J.P. Leppard, Eds), pp. 191–196. Plenum Press, London (1983).

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