Biochemical Pharmacology, Vol. 43, No. 5, pp. 971–978, 1992.
Printed in Great Britain.

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

MEASUREMENT OF GLUCURONIDATION BY ISOLATED RAT LIVER CELLS USING [^{14}C]FRUCTOSE

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(*Received* 8 *August* 1991; *accepted* 25 *November* 1991)

Abstract—We have developed a simple and sensitive method for the study of the relative rates of glucuronidation of compounds, in isolated liver cells, based on the incorporation of ^{14}C from fructose into glucuronide conjugates. Liver cells from fasted rats are used to minimize any reduction of the specific activity by glycogenolysis. Although rates of glucuronidation are lower in isolated liver cells from fasted rats than in those from fed rats, because of a reduction in the concentration of UDP-glucuronic acid, it is possible to compare the rates of glucuronidation of different compounds. Radiolabelled glucuronides are separated from [^{14}C]fructose and [^{14}C]glucose, produced by the liver cells, by normal-phase HPLC on a polar amino-cyano column. The specific activity of the glucuronide was found to be approximately 50% of that of the [^{14}C]fructose. Absolute amounts of glucuronide can be determined by measuring the specific activity of the [^{14}C]glucose, also produced by liver cells from fructose, which reflects that of the glucose-6-phosphate and hence the UDP-glucuronic acid used for glucuronidation, although for the measurement of relative rates this would not be necessary. We have used this method to examine the kinetics of the glucuronidation of *N*-acetyl-*p*-aminophenol (acetaminophen), 4-nitrophenol and 1-naphthol in isolated rat liver cells. The method should be applicable to the study of the rates of glucuronidation of a range of aglycones and, unlike other methods, does not require glucuronide standards or radiolabelled aglycone.

Glucuronidation by the UDP-glucuronosyltransferases (EC 2.4.1.17) is the most common conjugation reaction in mammals for the biotransformation of various xenobiotic and endogenous substances such as steroids, bile salts, catecholamines and lipids [1, 2]. The liver parenchymal cell is a major site of glucuronidation; glucuronides are more water soluble than the unconjugated substrates and are rapidly eliminated in urine and bile.

Glucuronidation is often studied in liver microsomes using UDP[^{14}C]glucuronic acid as donor [3, 4]. These studies, however, are of limited value in predicting rates of metabolism *in vivo*; studies in isolated liver cells are more useful [5]. It is not possible to use UDP[^{14}C]glucuronic acid in studies in whole cells because it is non-permeant. The study of the relative rates of glucuronidation of a range of substrates in isolated liver cells requires a rapid and sensitive assay for processing a large number of samples. Several methods have been described that are applicable to the study of glucuronidation in isolated liver cells but these are specific for particular substrates [6–19]. These methods involve chromatographic separation of products with various detection systems or solvent extraction. They all need standards or radiolabelled substrates for quantification, so that a new assay has to be developed for each novel substrate.

The UDP-glucuronic acid for glucuronidation in liver cells is normally derived from both glycogenolysis and gluconeogenesis [20]. We predicted that, in liver cells isolated from fasted rats in which the glycogen had been depleted, radiolabel from gluconeogenic substrates would be incorporated into UDP-glucuronic acid and glucuronides, with the specific activity of the glucuronide being that of the radiolabelled substrate (Scheme 1). It is known that rates of glucuronidation are lower in liver cells from fasted rats than those from fed rats [9, 21–23], probably due to a decrease in the concentration of UDP-glucuronic acid in the cells of the fasted rats. However, it is valid to compare the rates of glucuronidation of a range of compounds using cells from fasted rats.

We have developed a general method, based on the incorporation of ^{14}C from fructose into glucuronides and their quantitation using normal phase HPLC to study the kinetics of glucuronidation in isolated rat liver cells; this new method has been applied to the glucuronidation of *N*-acetyl-*p*-aminophenol (acetaminophen), 4-nitrophenol and 1-naphthol.

MATERIALS AND METHODS

Animals. Male Wistar rats (200–250 g), fasted for 18–22 hr, were used throughout.

Chemicals. D-[U-^{14}C]fructose and 1-[1-^{14}C]naphthol were from Amersham International (Aylesbury, U.K.). Pico-fluor 40 scintillation fluid was from Canberra Packard Ltd. (Pangbourne, U.K.). Firefly lantern extract (FLE-50), 6-phosphogluconate dehydrogenase (6PGDH†) (EC 1.1.1.44 from *Leuconostoc mesenteroides*) and collagenase (Type

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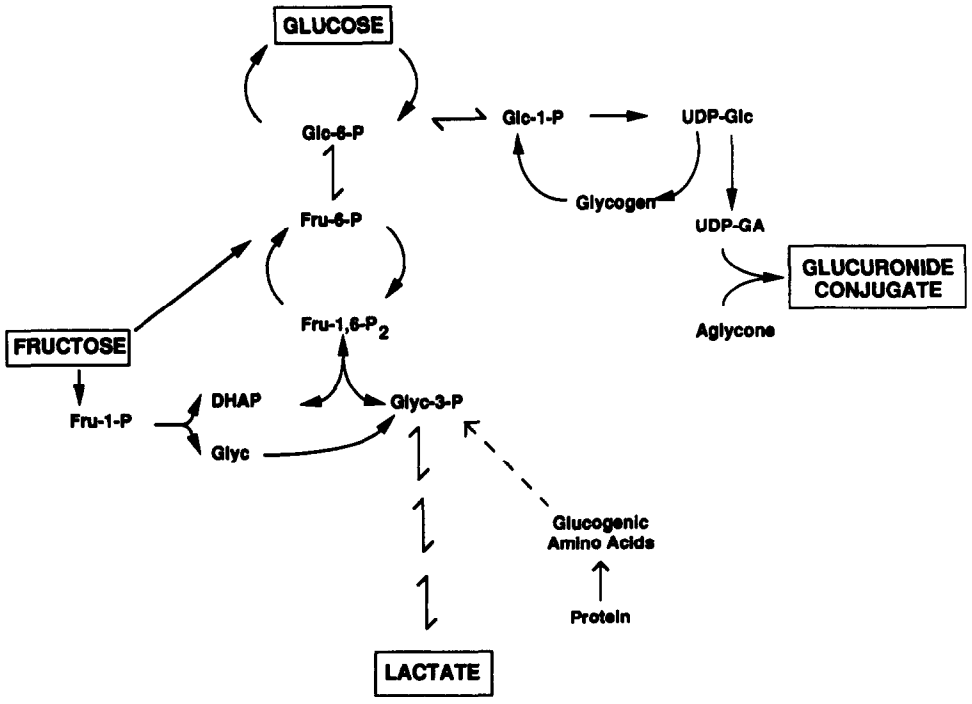
† Abbreviations: PAC, polar amino-cyano; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.



Scheme 1. Metabolic pathway showing the incorporation of fructose into hepatic UDP-glucose pools and glucuronic acid conjugates. Glc, glucose; P, phosphate; Fru, fructose; Glyc, glyceraldehyde; DHAP, dihydroxyacetone phosphate; GA, glucuronic acid.

IV) were from the Sigma Chemical Co. (Poole, U.K.). Hexokinase (EC 2.7.1.1 from yeast) and G6PDH (EC 1.1.1.49 from *L. mesenteroides*) were from Boehringer Mannheim U.K. (Lewes, U.K.). All other chemicals were of analytical grade or the highest grade commercially available and were obtained from Sigma or BDH Chemicals (Poole, U.K.).

Preparation and incubation of liver cells. Liver cells were isolated essentially according to the two-step procedure of Seglen [24]. The Krebs–Henseleit buffer [25] used for the cell preparation and incubation consisted of 118 mM NaCl, 4.73 mM KCl, 1.18 mM $MgSO_4.7H_2O$, 1.18 mM KH_2PO_4 and 25 mM $NaHCO_3$; pH 7.4 when gassed with O_2:CO_2 (19:1). The liver was perfused with Ca^{2+}-free buffer containing 0.1 mM ethylene glycol bis-(aminoethylether)tetra-acetate followed by buffer containing 1.25 mM Ca^{2+} and collagenase (0.5 mg/mL). The cell suspension released after rupture of the liver capsule was filtered through nylon gauze and centrifuged at 50 *g* to sediment parenchymal cells. The supernatant was removed by aspiration and the pellet washed twice with buffer. The final pellet was weighed and resuspended to give approximately 1 mg dry weight/mL (1 mg dry weight/mL = 3.8×10^5 cells/mL [26]) in buffer containing 1.25 mM Ca^{2+}. Dry weights were later determined by centrifugation (150 *g* for 5 min) of portions of cell suspension in preweighed 2 mL glass vials, aspiration of the supernatants, and drying of the pellets overnight at 120° before reweighing.

Substrates dissolved in dimethyl sulphoxide were added to 1-mL portions of cell suspension (final concentration of dimethyl sulphoxide 1% v/v), together with 200 μM [^{14}C]fructose (0.5 μCi/mL) in 20 mL polyethylene scintillation vials. Vials were capped and gassed with O_2:CO_2 (19:1) at room temperature. Glucuronidation did not occur at a measurable rate during the period at room temperature. Vials were transferred to a reciprocating water bath (Mickle Engineering, Gomshall, U.K.) at 37° and shaken at 100 cycles/min. Incubation was for 20–90 min and was terminated by centrifugation of 1 mL portions through 250 μL of silicone oil (Dow Corning 550:dinonylphthalate, 2:1 v/v) at 12,000 *g* for 30 sec to separate cells from medium. Supernatants were heated in a boiling water bath for 5 min before centrifugation (12,000 *g* for 1 min) to remove denatured protein, and stored at −20° for subsequent determination of glucuronide by HPLC.

Cell viability was assessed by measurement of ATP content by bioluminescence using firefly luciferin–luciferase [27] and a LKB 1251 luminometer in the integration mode. For this, cell incubations (1 mL) were terminated by addition of 0.1 mL of 20% (v/v) $HClO_4$ and were stored at −20° before analysis. ATP content was calculated by reference to known standards. The normal ATP content of isolated rat liver cells was 8–10 nmol/mg dry weight. The effect of added substrates on the ATP content of the cells was routinely checked.

HPLC. A System Gold high performance liquid chromatograph (Beckman, High Wycombe, U.K.)

was used throughout, comprising a model 126 binary solvent delivery module, model 167 scanning UV detector and model 506 autosampler. The outflow from the UV detector was collected with a Frac-100 fraction collector (Pharmacia). Fractions (0.5 min) were collected directly into scintillation vials (Mini 'Poly-Q' vials, Beckman) for determination of radioactivity.

Normal phase PAC HPLC of glucuronides. Portions (0.1 mL) of supernatants were analysed on a 4.5 mm × 25 cm Partisil 5PAC (polar amino-cyano) column equipped with a guard column packed with Co:Pell PAC (both from Whatman, Maidstone, U.K.) at a flow rate of 1 mL/min. The mobile phase consisted of a linear gradient from 100% acetonitrile to 67% 0.01 M tetrabutylammonium hydrogen sulphate in H_2O over 20 min. Sixty seven per cent tetrabutylammonium hydrogen sulphate was maintained for 5 min and the system returned to 100% acetonitrile over the subsequent 10 min. The column was washed for a further 10 min with acetonitrile before injecting the next sample. Fractions (0.5 min) were collected for determination of radioactivity. In order to identify the glucuronide peaks, some samples were hydrolysed by mixing 200 μL portions with 40 μL 0.5 M sodium phosphate buffer, pH 7, and 1000 U β-glucuronidase (*Escherichia coli*, Sigma). After 2 hr at 37°, samples were heated in a boiling water bath for 5 min before centrifugation to remove denatured protein. Portions (0.1 mL) were analysed by the HPLC method. Glucuronide peaks were identified by their disappearance following this β-glucuronidase treatment. The retention times for 4-nitrophenol- and 1-naphthol-glucuronides was 9.75 min and for acetaminophen-glucuronide 11.25 min. The added [^{14}C]fructose and [^{14}C]glucose, produced in the liver cell incubations, eluted together at 13.5 min. The sensitivity was approximately 60 pmol/100 μL injection, equivalent to 0.6 μM in the incubations, with the specific activity of fructose we used. Unlabelled 4-nitrophenol glucuronide and 1-naphthol glucuronide (Sigma) were located by monitoring their extinction at 300 nm.

Reverse phase HPLC of glucuronides. Analyses were performed on a 4.6 mm × 25 cm Ultra Techsphere 5 ODS column (HPLC Technology, Macclesfield, U.K.) equipped with a guard column packed with Co:Pell ODS (Whatman), at a flow rate of 1 mL/min with a 100 μl injection volume.

Acetaminophen, acetaminophen glucuronide and acetaminophen sulphate were separated by a modification of the method of Adriaenssens and Prescot [12] with mobile phase A (0.1 M sodium phosphate, pH 4.5: isopropanol: 98% (v/v) formic acid, 100:1.7:0.1) and quantified by peak area using the extinction at 254 nm and acetaminophen as external standard. The concentrations of acetaminophen metabolites were calculated as 'acetaminophen equivalents' since their extinction coefficients are similar to each other [14]. The retention times for acetaminophen, acetaminophen glucuronide and acetaminophen sulphate were 12.7, 5.8 and 8.8 min, respectively.

Determination of the specific activity of [^{14}C]-*glucose.* Glucose was measured spectrophotometrically by the hexokinase/G6PDH method [28],

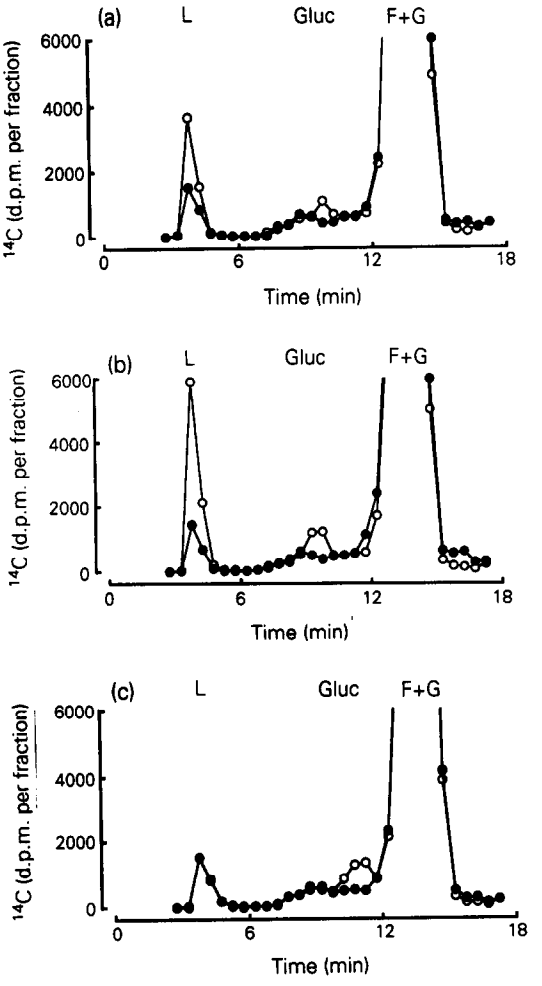


Fig. 1. Separation of [^{14}C]glucuronides by HPLC on a PAC column. Isolated rat liver cells were incubated with aglycone and [^{14}C]fructose. Samples of incubation medium were treated with (●) or without (○) β-glucuronidase, as described in the text, and 100 μL were run on PAC HPLC. Incubation conditions used were (a) 50 μM 4-nitrophenol and 0.78 mg dry weight/mL cells for 90 min, (b) 50 μM 1-naphthol and 1.1 mg dry weight/mL cells for 60 min and (c) 5 mM acetaminophen with 1.1 mg dry weight/mL cells for 40 min. Representative elution profiles are shown. L, lactate; F + G, fructose and glucose; Gluc, glucuronide.

modified by further reaction with 6PGDH, which results in the formation of ribulose 5-phosphate with the release of CO_2.

Samples of boiled supernatants from the cell incubations were centrifuged (12,000 *g* for 1 min) to remove denatured protein. A sample of 0.1 mL was added to 0.9 mL of 100 mM Tris buffer, pH 7.5, containing 1 mM magnesium acetate, 1.7 mM NAD^+ and 1.1 mM ATP. The reaction was carried out at 37° in disposable cuvettes. The change in extinction at 340 nm was measured following the addition of 1 U HK/1U G6PDH and again following the addition of 0.1 U 6PGDH. The time to reach the end point was determined by continuous extinction

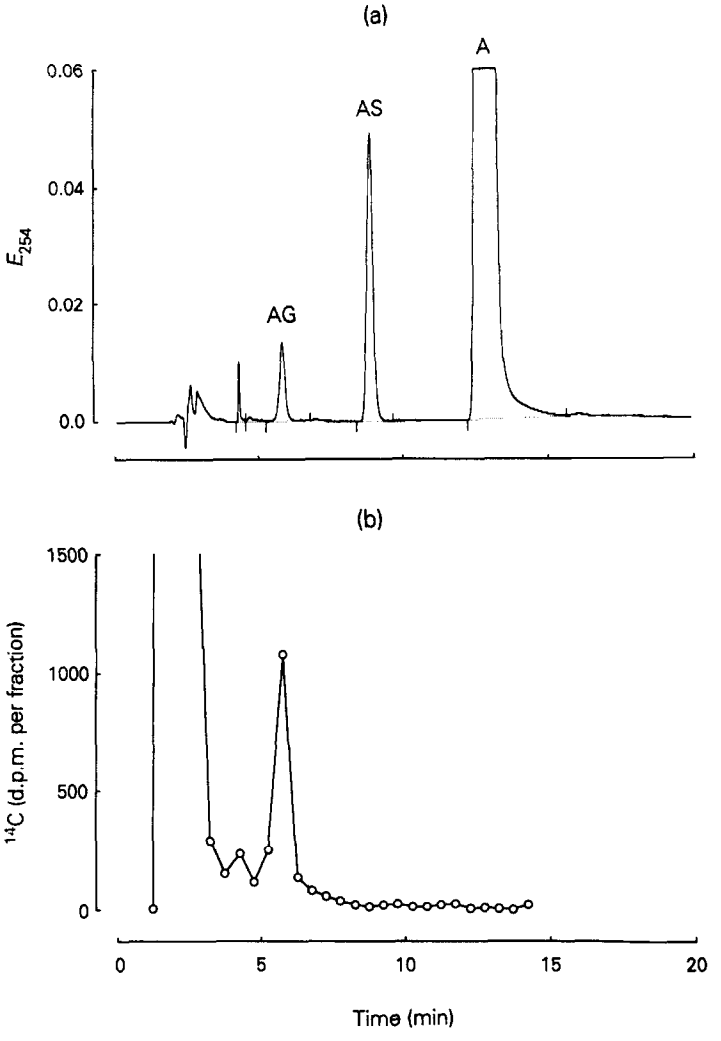


Fig. 2. Elution profile for acetaminophen and its metabolites by reverse phase (C_{18}) HPLC. A representative profile is shown for a 100-μL sample from a 40-min cell incubation with 1 mM acetaminophen and 1.1 mg dry weight of cells/mL. (a) E_{254}, (b) radioactivity determined in 0.5-min fractions. AG, acetaminophen glucuronide; AS, acetaminophen sulphate and A, acetaminophen.

measurement of standards and samples. The glucose concentration was calculated using an extinction coefficient for NADH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$. There was no change in extinction from fructose. Duplicate samples for [^{14}C]glucose determination were incubated in the same way in 20 mL glass scintillation vials closed with rubber stoppers from which were suspended disposable centre wells. Enzyme additions were made by syringe injection and at the end of the incubation 0.1 mL 20% (v/v) $HClO_4$ was injected to drive off $^{14}CO_2$. 2-Phenethylamine/methanol (0.2 mL, 1:1, v/v) was injected into the centre well, which contained a piece of folded filter paper. Vials were then shaken periodically at room temperature for 1 hr for absorption of $^{14}CO_2$. The wells were removed and added directly to vials containing 5 mL Pico-fluor 40 scintillation fluid. The radioactivity in the original glucose was calculated as being six times that in the single carbon released as $^{14}CO_2$. This method for determining the [^{14}C] glucose specific activity was reproducible and rapid, it being possible to assay 20–40 samples in <2 hr.

Computer fitting of data. For glucuronidation of acetaminophen the kinetic parameters were determined by computer fitting of results from individual experiments to the Michaelis–Menten equation by non-linear least squares regression.

RESULTS

Identification of [^{14}C]*glucuronides*

The radiolabelled glucuronides of 4-nitrophenol, 1-naphthol and acetaminophen were separated from [^{14}C]fructose precursor by gradient HPLC on a PAC column (Fig. 1). The retention times were 9.8, 9.5 and 11.2 min, respectively. Acetaminophen glucuronide eluted near to the fructose peak, but we were unable to improve the separation

Table 1. Determination of acetaminophen glucuronide by various methods

	C_{18} HPLC		PAC HPLC	
E_{254}	From [^{14}C]fructose specific activity	From [^{14}C]glucose specific activity	From [^{14}C]fructose specific activity	From [^{14}C]glucose specific activity
4.25 ± 0.98	2.10 ± 0.55	3.66 ± 0.25	1.64 ± 0.41	2.87 ± 0.14

Values are expressed in nmol/mg dry weight of cells.

Samples from cell incubations with acetaminophen (2 mM for 40 min) were separated by C_{18} or PAC HPLC. The amount of glucuronide was calculated from the extinction (E_{254}), the specific activity of the fructose and the specific activity of the glucose (C_{18} method) or the specific activity of the fructose or glucose (PAC method). Results are means ± range of two experiments.

by modification of the HPLC conditions. The glucuronide peaks were identified by their disappearance following treatment of samples with β-glucuronidase. Authentic unlabelled 4-nitrophenyl-glucuronide and 1-naphthyl-glucuronide had retention times of 9.9 and 9.6 min, respectively. Acetaminophen glucuronide was not available. Any [^{14}C]glucose formed by the liver cells eluted with [^{14}C]fructose at 13.8 min. A radioactive peak was also seen with the same retention time as lactate (3.8 min).

Quantification of glucuronides using the specific activity of [^{14}C]*fructose*

In order to check the quantification of the glucuronide produced in cell incubations using the specific activity of the [^{14}C]fructose, some samples from incubations with acetaminophen were also separated by an isocratic reverse phase HPLC method on a C_{18} column, with both UV detection and determination of radioactivity. We were unable to do this using the PAC HPLC with UV detection due to high background noise. Figure 2 shows the elution profile for acetaminophen, acetaminophen sulphate and acetaminophen glucuronide. The retention times were 12.7, 8.8 and 5.8 min, respectively. The glucuronide peak was identified by its disappearance following β-glucuronidase treatment. The sulphate peak was identified by running samples from cells incubated with [^{35}S]SO_4^{2-} as described previously [29]. With samples from cells incubated with 2 mM acetaminophen for 40 min the amount of glucuronide calculated from the specific activity of the fructose was only 49% of that calculated by extinction.

As it would be technically difficult to measure the specific activity of the UDP-glucuronic acid in the cells, we developed a method to measure the specific activity of the [^{14}C]glucose, also produced by the liver cells from fructose (Scheme 1). Since the phosphoglucomutase step (G6P–G1P) is thought to be at equilibrium (because the phosphoglucomutase activity [30] is far higher than the fluxes through G6P/G1P) the specific activity of the G6P, the precursor for glucose, and the G1P, the precursor for UDP-glucuronic acid, should remain the same under a wide variety of conditions.

We found the specific activity of the [^{14}C]glucose was 63 ± 6% (mean ± SEM, N = 5) of the fructose specific activity. We have also measured 1-naphthol glucuronidation with either [^{14}C]fructose or [^{14}C]1-naphthol and separation by PAC HPLC. When the glucuronide from [^{14}C]fructose was calculated using the specific activity of the [^{14}C]glucose there was no significant difference from that calculated from the [^{14}C]naphthol specific activity (5.03 ± 0.83 and 7.13 ± 2.09 nmol/mg dry weight of cells with 100 μM 1-naphthol and 60 min incubation, mean ± range of two experiments). Table 1 shows a comparison between the amounts of glucuronide, separated by C_{18} or PAC HPLC and calculated from the specific activity of the fructose, the specific activity of the glucose or by extinction. Quantifying the amount of glucuronide from the extinction and from the radioactivity using the specific activity of the [^{14}C]-glucose gave the same result when samples were run on C_{18} HPLC. The amount of glucuronide calculated using either the specific activity of the [^{14}C]glucose or that of the [^{14}C]fructose with PAC HPLC was 78% of that calculated in the same way with

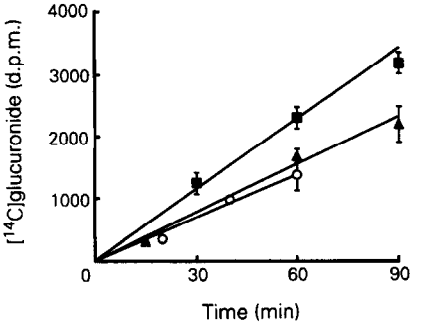


Fig. 3. The time-course of [^{14}C]glucuronide production by isolated rat liver cells incubated with 50 μM 4-nitrophenol (▲), 50 μM 1-naphthol (■) or 5 mM acetaminophen (○). For 4-nitrophenol and 1-naphthol 1.2 mg dry weight of cells/mL was used and 33 μL of supernatant were run on PAC HPLC. For acetaminophen 1.1 mg dry weight of cells/mL was used and 100 μL of supernatant were run on PAC HPLC. Results are means ± SD of triplicate determinations in a representative experiment (4-nitrophenol and 1-naphthol) or means ± range of duplicate determinations in a representative experiment (acetaminophen).

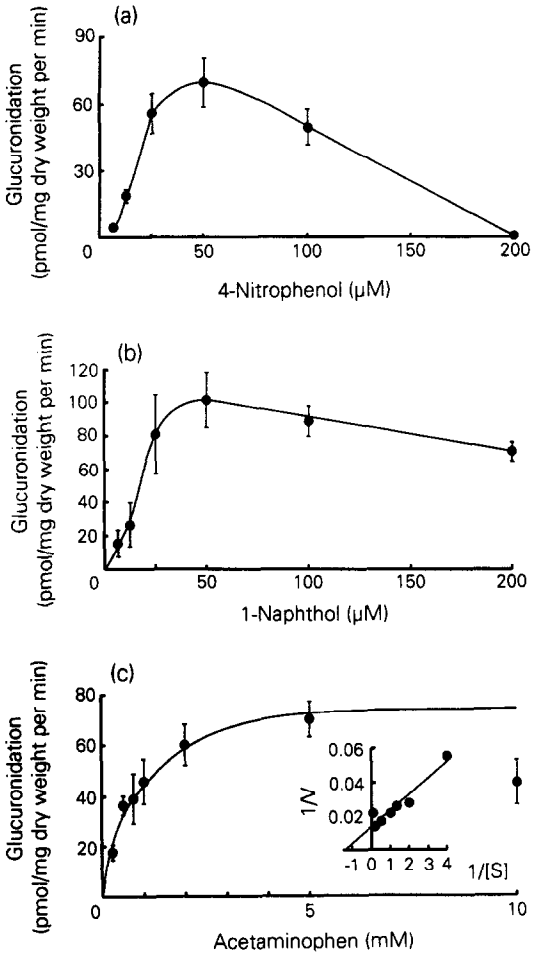


Fig. 4. The concentration dependence of glucuronidation measured by the PAC HPLC method. The conditions used were (a) 0.78–1.25 mg dry weight of cells/mL and 60 min incubation, (b) 0.63–1.16 mg dry weight of cells/mL and 60 min incubation and (c) 1.1 mg dry weight/mL cells and 40 min incubation. The inset shows $1/v$ against 1/[S] and the line corresponds to the kinetic parameters obtained as described in the text. The results are means ± SEM, N = 3. The absolute rates were calculated taking the specific activity of the glucuronide to be that of the glucose produced from fructose in the same experiments.

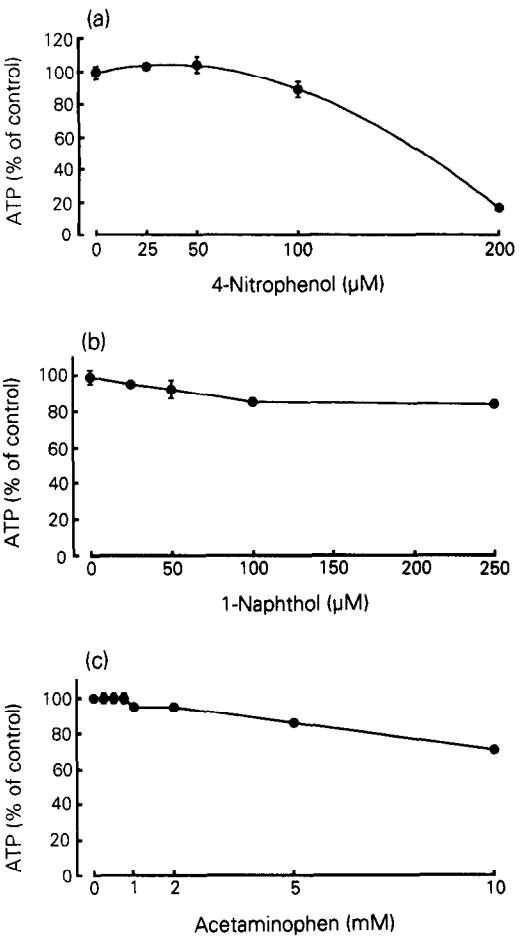


Fig. 5. The effect of the compounds on the ATP content of isolated liver cells. The results are means ± SD of triplicate determinations from a representative experiment (a and b) or the mean ± range of duplicate determinations from a representative experiment (c).

C_{18} HPLC. This is probably due to a slight underestimation in determining the radioactivity of the glucuronide peak from the PAC column, as it runs close to the fructose/glucose peak.

Consequently, we have used the specific activity of the [^{14}C]glucose to calculate the amount of glucuronide in the kinetic experiments.

Kinetic studies of glucuronidation

Incubation of isolated rat liver cells with 4-nitrophenol, 1-naphthol and acetaminophen resulted in the time-dependent formation of the corresponding glucuronide, measured in the incubation medium. By using a suitable amount of cells and incubation time, linear rates of glucuronide formation were achieved (Fig. 3).

The concentration dependence of glucuronidation of the three substrates is shown in Fig. 4. With all three aglycones apparent substrate inhibition was seen. This is, however, due to the toxicity of the compounds as the concentrations inhibiting glucuronidation also caused a decrease in ATP content (Fig. 5) With acetaminophen up to 5 mM (at which concentration there was only a small effect on ATP content), glucuronidation was as predicted by the Michaelis–Menten equation. The kinetic parameters derived from the data in Fig. 4 are shown in Table 2.

DISCUSSION

There is a need for simple and robust assays for rates of drug metabolism in cell preparations *in vitro*. Such assays should not be dependent on the availability of radiolabelled drugs or unlabelled

Table 2. Kinetic parameters for the glucuronidation of 4-nitrophenol, 1-naphthol and acetaminophen determined from the data in Fig. 4 compared with those for the sulphation determined in isolated liver cells from fed rats

	Glucuronidation		Sulphation*	
	V_{max} or maximum rate (pmol/mg dry weight of cells/min)	K_m or EC_{50} (μM)	V_{max} or maximum rate (pmol/mg dry weight of cells/min)	K_m or EC_{50} (μM)
4-Nitrophenol	70 ± 11	18 ± 1	233 ± 19	1.8 ± 0.3
1-Naphthol	105 ± 18	19 ± 2	137 ± 33	0.31 ± 0.05
Acetaminophen	83 ± 8	850 ± 140	302 ± 38	38 ± 3

For glucuronidation of 4-nitrophenol and 1-naphthol, the maximum rate and EC_{50} were determined graphically for individual experiments. For acetaminophen glucuronidation kinetic parameters were determined by computer fitting of results from individual experiments to the Michaelis–Menten equation by non-linear least squares regression.

* Sulphate conjugation data are from Ref. 29 and were measured by incorporation of ^{35}S from [^{35}S]sulphate and a barium precipitation procedure. Results are means ± SEM from three or four experiments.

metabolite standards because these are either not readily available or prepared or are of low specific activity. The method described in this paper is a practically simple procedure which can be applied to large numbers of independent samples simultaneously. It offers a potentially high sensitivity because of the relatively high specific activities possible with the incorporation of ^{14}C from fructose. The lowest rate of glucuronidation which can be measured by this method, with the fructose specific activity used in the present study, was calculated to be 10 pmol/mg dry weight/min. As we propose to use the method to study compounds that are rapidly glucuronidated this sensitivity should be adequate. If greater sensitivity is needed this can probably be achieved by increasing the specific activity of the fructose, the incubation time and the amount of sample injected onto the HPLC. The method would not be useful for compounds that are metabolized slowly. The method should be applicable to the study of a range of aglycones as a similar PAC HPLC method has been used to separate a number of other radiolabelled glucuronides from liver microsome incubations with UDP[^{14}C]glucuronic acid [4].

The method described uses liver cells isolated from fasted rats to reduce any dilution of the radiolabel by glycogenolysis. It is known that rates of glucuronidation are lower in cells from fasted rats than in those from fed rats, probably due to a decrease in the concentration of UDP-glucuronic acid in the cells. However, it is valid to compare rates of glucuronidation of compounds with our method. It would probably not be suitable to use our method for measuring the effects of factors such as species of animal, age, sex and nutritional status on rates of glucuronidation.

Many studies have been performed with subcellular fractions but as the rate of metabolism is affected by a number of factors such as transport of substrates to the active site of the enzymes involved, the availability of cofactors and the presence of competing endogenous substrates, studies in isolated liver cells should be of more value in predicting routes and rates of metabolism *in vivo* [5]. Moreover, this method should also be useful for studying the metabolism of compounds which require phase I metabolism before conjugation (phase II) occurs [5, 18], since the intact liver cells should contain the cofactors and enzymes required for both phases of metabolism; we have not assessed this in the present study.

The liver is the major site of both sulphation and glucuronidation and a number of substrates are both sulphated and glucuronidated. Some studies show that sulphation is more important than glucuronidation only at low substrate concentrations, both *in vivo* [15] and *in vitro* [9, 13, 15, 16, 19], but thorough kinetic studies need to be undertaken to confirm if this is the case for all substrates. The K_m or EC_{50} values for glucuronidation we have determined are significantly higher than those for sulphation that we have determined in isolated liver cells from fed rats (Table 2). The K_m for acetaminophen glucuronidation was similar to that obtained in our laboratory using isolated liver cells from fed rats (1.29 ± 0.06 mM, mean ± SEM, N = 3) [31]. The rates of glucuronidation we have obtained with the method are lower than those reported in liver cells from fasted rats [21, 32], but this may be due to the use of a different strain of rat or the use of rats in which the glucuronyl transferase is induced. The rates of 1-naphthol glucuronidation we obtained were similar to those of Schwarz [9], measured in 48-hr fasted rats.

Fructose is also known to decrease rates of glucuronidation at high concentrations (10 mM and above) [11, 20]. At this concentration it is known to decrease ATP concentrations in liver *in vivo* due to its rapid utilization in the phosphorylation of fructose to fructose-1-phosphate [33]. ATP is necessary for the rephosphorylation of UDP to UTP which is required for the synthesis of UDP-glucuronic acid. In our studies we have used only 200 μM fructose which we have shown to have no effect on ATP levels in the liver cells.

The assay we have developed should be of considerable use in determining whether novel compounds are glucuronidated and measuring the relative rates of glucuronidation of a range of aglycones.

REFERENCES

1. Burchell B and Coughtrie MWH, UDP-glucuronyltransferases. *Pharmacol Ther* **43**: 261–289, 1989.
2. Dutton GJ, *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, FL, 1980.
3. Bansal SK and Gessner T, A unified method for the assay of uridine diphosphoglucuronyltransferase activities toward various aglycones using uridine diphospho[U-^{14}C]glucuronic acid. *Anal Biochem* **109**: 321–329, 1980.
4. Coughtrie MWH, Burchell B and Bend JR, A general assay for UDPglucuronyltransferase activity using polar amino-cyano stationary phase HPLC and UDP[U-^{14}C]-glucuronic acid. *Anal Biochem* **159**: 198–205, 1986.
5. Billings RE, McMahon RE, Ashmore J and Wagle SR, The metabolism of drugs in isolated rat hepatocytes. *Drug Metab Dispos* **5**: 518–526, 1977.
6. Levvy GA, The measurement of glucuronide synthesis by tissue preparations. *Biochem J* **44**: 295–299, 1949.
7. Diamond G and Quebbemann AJ, Rapid separation of *p*-nitrophenol and its glucuronide and sulfate conjugates by reverse-phase high-performance liquid chromatography. *J Chromatogr* **177**: 368–371, 1979.
8. Karakaya A and Carter DE, High-performance liquid chromatography of glucuronide and sulphate conjugates using ion-pair chromatography. *J Chromatogr* **195**: 431–434, 1980.
9. Schwarz LR, Modulation of sulfation and glucuronidation of 1-naphthol in isolated rat liver cells. *Arch Toxicol* **44**: 137–145, 1980.
10. Ullrich D and Bock KW, Glucuronide formation of various drugs in liver microsomes and in isolated hepatocytes from phenobarbital- and 3-methylcholanthrene-treated rats. *Biochem Pharmacol* **33**: 97–101, 1984.
11. Eacho PI, Sweeny D and Weiner M, Effects of glucose and fructose on conjugation of *p*-nitrophenol in hepatocytes of normal and streptozotocin-diabetic rats. *Biochem Pharmacol* **30**: 2616–2619, 1981.
12. Adriaenssens PI and Prescot LF, High performance liquid chromatographic estimation of paracetamol metabolites in plasma. *Br J Pharmacol* **6**: 87–88, 1978.
13. Suolinna E and Mäntylä E, Glucuronide and sulphate conjugation in isolated liver cells from control and phenobarbital- or PCP-treated rats. *Biochem Pharmacol* **29**: 2963–2968, 1980.
14. Howie D, Adriaenssens PI and Prescot LF,L Paracetamol metabolism following overdosage: application of high performance liquid chromatography. *J Pharm Pharmacol* **29**: 235–237, 1977.
15. Koster H, Halsema I, Scholtens E, Knippers M and Mulder GJ, Dose-dependent shifts in the sulfation and glucuronidation of phenolic compounds in the rat *in vivo* and in isolated hepatocytes. *Biochem Pharmacol* **30**: 2569–2575, 1981.
16. Andersson B, Berggren M and Moldéus P, Conjugation of various drugs in isolated hepatocytes. *Drug Metab Dispos* **6**: 611–616, 1978.
17. Mulder GJ and Hagedorn AH, UDP glucuronyltransferase and phenolsulfotransferase *in vivo* and *in vitro*. *Biochem Pharmacol* **23**: 2101–2109, 1974.
18. Pang KS, Kong P, Terrell JA nd Billings RE, Metabolism of acetaminophen and phenacetin by isolated rat hepatocytes. *Drug Metab Dispos* **13**: 42–50, 1985.
19. Mizuma T, Hayashi M and Awazu S, Factors influencing drug sulfate and glucuronic acid conjugation rates in isolated hepatocytes: significance of preincubation time. *Biochem Pharmacol* **34**: 2573–2575, 1985.
20. Bánhegyi B, Garzó T, Antoni F and Mandl J, Glycogenolysis—and not gluconeogenesis—is the source of UDP-glucuronic acid for glucuronidation. *Biochim Biophys Acta* **967**: 429–435, 1988.
21. Aw TK and Jones DP, Control of glucuronidation during hypoxia. *Biochem J* **219**: 707–712, 1984.
22. Price VF, Miller MG and Jollow DJ, Mechanisms of fasting-induced potentiation of acetaminophen hepatotoxicity in the rat. *Biochem Pharmacol* **36**: 427–433, 1987.
23. Price VF and Jollow DJ, Effect of glucose and gluconeogenic substrates on fasting-induced suppression of acetaminophen glucuronidation in the rat. *Biochem Pharmacol* **38**: 289–297, 1989.
24. Seglen PO, Preparation of isolated rat liver cells. In: *Methods in Cell Biology* (Ed. Prescott DM), pp. 29–83. Academic Press, New York, 1969.
25. Krebs HA and Henseleit K, Untersuchungen über die Harnstoffbildung im Tierkörper. *Biol Chem Hoppe Seyler* **210**: 33–66, 1932.
26. Siess EA, Brocks DG and Wieland OH. Subcellular distribution of adenine nucleotides and of metabolites of tricarboxylate cycle and gluconeogenesis in hepatocytes. In: *Metabolic Compartmentation* (Ed. Sies H), pp. 235–257. Academic Press, London, 1982.
27. Stanley PE and Williams SG, Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal Biochem* **29**: 381–392, 1969.
28. Bondar RJL and Mead DC, Evaluation of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* in the hexokinase method for determining glucose in serum. *Clin Chem* **20**: 586–590, 1974.
29. Dawson J, Knowles RG and Pogson CI, Quantitative studies of sulphate conjugation by isolated rat liver cells using [^{35}S]sulphate. *Biochem Pharmacol* **42**: 45–49, 1991.
30. Joshi JG, Hooper J, Kuwaki T, Sakurada T, Swanson JR and Handler P, Phosphoglucomutase, V. Multiple forms of phosphoglucomutase. *PNAS* **57**: 1482–1489, 1967.
31. McPhail ME, Dawson J, Pogson CI and Burchell B, A comparison of *N*-acetyl-*p*-aminophenol metabolism in liver snips and isolated liver cells from male Wistar rats. *Biochem Soc Trans* **18**: 1216, 1990.
32. Shipley LA and Weiner M, Effects of adenosine on glucuronidation and uridine diphosphate glucuronic acid (UDPGA) synthesis in isolated rat hepatocytes. *Biochem Pharmacol* **36**: 2993–3000, 1987.
33. Burch HB, Max P Jr, Chyu K and Lowry OH, Metabolic intermediates in liver of rats given large amounts of fructose or dihydroxyacetone. *Biochem Biophys Res Commun* **34**: 619–626, 1969.