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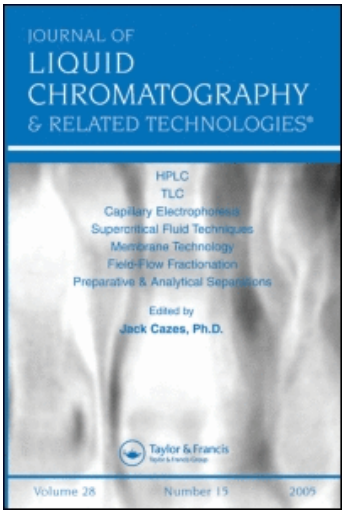

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DETERMINATION BY HPLC OF THE UDP-GLUCURONYL TRANSFERASE ACTIVITY FOR 7-HYDROXYCOUMARIN IN RABBIT TISSUE SAMPLES

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ABSTRACT

HPLC was used to determine the uridine diphosphate (UDP) glucuronyl transferase activity in rabbit tissues. The crude tissue samples prepared were from the liver, kidney, bladder, large intestine, lung, spleen, heart and fat. A metabolic reaction mixture was prepared that included the rabbit tissue sample, 7-hydroxycoumarin and UDP-glucuronic acid (UDPGA). The method was used to determine the level of production of 7-hydroxycoumarin-glucuronide from 7-hydroxycoumarin. Seperation of the analyte was carried out under gradient elution by reverse phase chromatography on a C_{18} column, with UV detection at 320 nm. It was possible to determine 7-hydroxycoumarin-glucuronide (7-OHCG) produced over time as well as the decrease in 7-hydroxycoumarin concentration as the reaction progressed. The rate of the reaction was calculated from a plot of the concentration of 7–OHCG produced versus time for

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each organ, and the rate calculated from the slope of the linear part of the curve. The liver showed the highest activity with a rate of production of 7-OHCG of 2.3 nmol per min per milligram of protein. The kidney showed an activity of 0.22 nmol of 7-OHCG produced per minute per milligram of protein, with the bladder and large intestine showing activities of 0.14 nmol and 7.8 pmol of 7-OHCG produced per minute per milligram of protein. The method used required minimal sample clean up and was reliable and accurate.

INTRODUCTION

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Of all the organs in the body the liver has been established to be quantitatively and qualitatively the most important site for glucuronidation.[1] It has also been shown that glucuronidation occurs predominantly in the periportal reigon of the liver.[2,3] Other extra hepatic tissues also have the ability to glucuronidate compounds, although with a more restricted substrate specificity and capacity than hepatic tissue (Dutton 1980). In rat kidney UDPGT activity towards (-)-morphine and testosterone is absent, while transferase activities towards phenols and bilirubin are present.[4] In rats the gastro-intestinal tract exhibits glucuronidation capacity for phenols.[5] Other organs such as lungs,[6] bronchus,[7] and skin,[8] have also demonstrated activity to simple phenolic xenobiotics. Phenol UDPGT activity at very low levels has been shown in many rat tissues including spleen, thymus, brain and heart.[4]

Coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin are all members of the benzo-pyranones. Coumarin and 7-hydroxycoumarin are found in nature and both have been used as trial drugs in cancer treatment.[10] 7-Hydroxycoumarin is metabolised by UDP-glucuronyl transferase to the glucuronide (Fig 1.). The glucuronide is principally excreted in the urine.[11]

The clinical role of 7-hydroxycoumarin-glucuronide is unclear. It can be detected in the plasma after coumarin administration.[12] Due to the active transport system proposed to exist for glucuronides,[4] it has been suggested that 7-hydroxycoumarin-glucuronide is transported into the cells where glucuronidases present within the cell reconvert the glucuronide to the 7-hydroxycoumarin. After it has exerted its pharmacological action it might then be reglucuronidated before excretion.[13] Analysis of 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin in metabolism studies, and in urine, plasma and serum samples almost always involves the deconjugation of 7-hydroxycoumarin-glucuronide to free 7-hydroxycoumarin, followed by

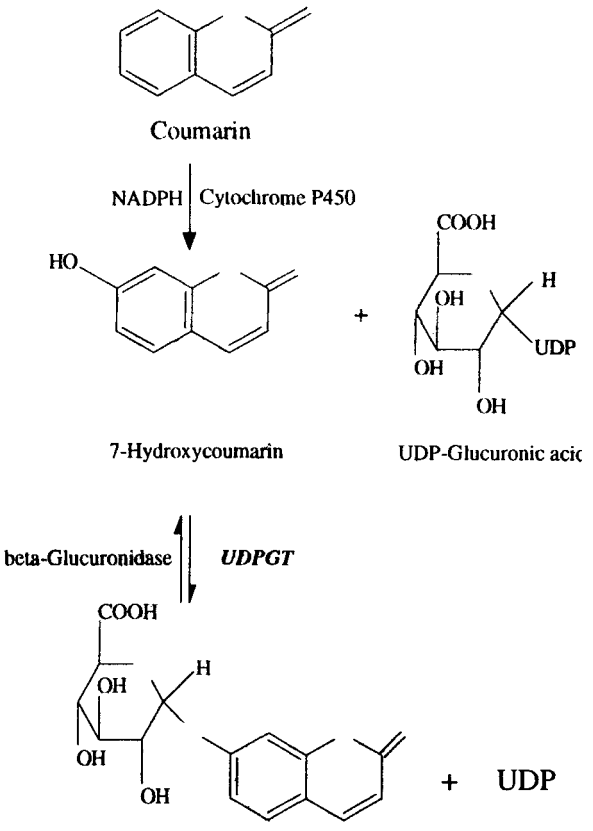

Figure 1. Reaction scheme for the in vivo metabolism of coumarin and 7-hydroxycoumarin.

extensive clean up procedures.[14,15] Killard *et al.*[16] reported on the direct determination of the glucuronide form of 7-hydroxycoumarin as well as the free 7-hydroxycoumarin without deconjugation with β-glucuronidase.

Crude tissue was prepared and used for studying the activity of UDP-glucuronyl transferase on 7-hydroxycoumarin in eight different rabbit tissues. The method applied here was adapted from Killard *et al.*[16] and applied to the separation and determination of 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and the internal standard, 4-hydroxycoumarin with minimal sample clean up.

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EXPERIMENTAL

Chemicals

7-hydroxycoumarin and uridine 5'-diphosphate glucuronic acid (UDPGA) were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid and acetic acid were purchased from BDH Chemicals Ltd., Poole, Dorset, England. 4-Hydroxy-coumarin was purchased from Aldrich Chemicals Ltd., Gillingham, England. 7-hydroxycoumarin-glucuronide was kindly donated by Schaper and Brummer, Salzgitter, Germany. HPLC grade methanol was purchased from Labscan, Dublin, Ireland. 1.1 mL screw cap vials were obtained from Labquip, Dublin, Ireland. Ultra pure water was used for serial dilutions and mobile phase.

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HPLC

The HPLC apparatus consisted of a System Gold solvent module 126, detector module 166, and autosampler, module 507. All components were controlled by System Gold Software™, (Beckmen Instruments Ltd., California, USA). Separation was carried out on a Microbonda Pak C_{18} column (HPLC Technology, Cheshire, UK). with gradient elution of the compound of interest. The solvents employed were A, water-methanol-acetic acid (950:50:2, v/v) and B, 100% methanol. The eluent was monitored at 320 nm. Sample (20µl) was injected onto the column. The 1 mL min^{-1} gradient was as follows: 0-5 min 100% A → 50% A : 50% B ; 5-14 min 50% A : 50% B ; 14-15 min 50% A : 50% B → 100% A.

Preparation of Samples

Organs were obtained from a New Zealand White Rabbit and stored at -20°C until required. A sample of each organ was weighed and homogenised in 50mM Tris-HCL, pH 7.4. The protein concentration in each organ was determined by Bicinchoninic acid (BCA) assay (Pierce, Illinois, USA). A reaction mixture was set up and incubated at 37°C. Samples were taken every 15 mins from time 0 to 120 minutes. The reaction was terminated by addition of 200µl of reaction mixture to 40µl of 20% (w/v) trichloroacetic acid. This mixture was centrifuged prior to analysis.

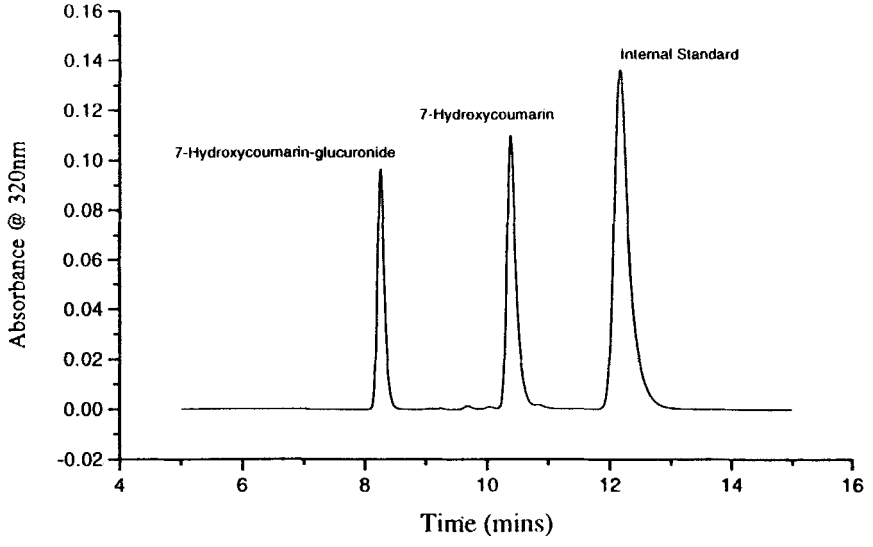

Figure 2. Chromatogram showing separation achieved for 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and the Internal Standard under experimental conditions.

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Preparation of Standards

A series of standards of each analyte were prepared in ultra pure water. A 1 mg/mL stock solution of 7-hydroxycoumarin was prepared in 10% methanol / 90% water and the 1 mg/mL stock solution of 7-hydroxycoumarin-glucuronide was prepared in ultra pure water. The internal standard used was 4-hydroxycoumarin and was prepared in methanol. The standards used for determining concentration of analytes and also precision and accuracy of method were 0, 1, 5, 10, 20, 50, 80, 100 and 200 μg/mL.

RESULTS AND DISCUSSION

HPLC Separation

The purpose of this *in vitro* study was to gain more information on intra organ differences in the metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide in rabbit. The focus of this study was the determination of the rate of production of 7-OHCG by each organ.

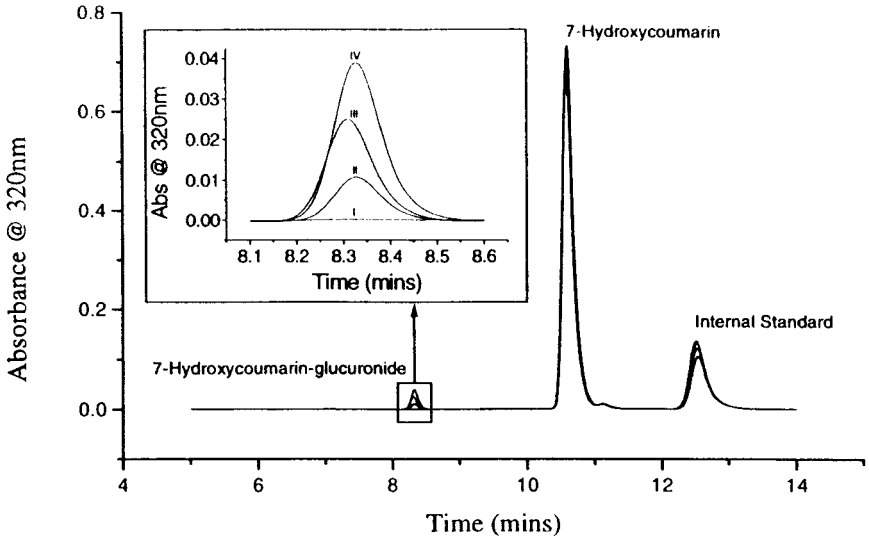

Figure 3. Overlay of four chromatograms of kidney reaction mixture showing analysis for 7-hydroxycoumarin-glucuronide at time 0 (I), time 30 (II), time 60 (III), and time 90min (IV). Reaction mixture analysed as outlined in experimental.

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An HPLC method was adapted from that of Killard *et al.*[16] The parameters were changed by analysing a series of standards under different conditions until the optimum separation of analytes was achieved. Figure 2 shows the resolution obtained with the glucuronide eluting at 8.3, 7-hydroxycoumarin at 10.4, and internal standard (4-hydroxycoumarin) at 12.2 minutes, respectively.

The choice of solvents used for the separation were selected because they had been successfully used previously by Egan and O'Kennedy[14] and Sharifi *et al.*[17] The time between sample injection was 9 minutes as this was the time taken for the absorbance to return to zero after each analysis.

Glucuronidation of 7-hydroxycoumarin by UDPGT

There are very few methods that allow the direct determination of 7-hydroxycoumarin-glucuronide without deconjugation,[16,17] however, with method applied here the metabolite of 7-hydroxycoumarin was monitored over time and its increase could be seen clearly (Fig. 3). It was also possible to monitor the decrease in 7-hydroxycoumarin concentration (results not shown).

Table 1

Activities of Different Organs in Amount of 7-OHCG Producted in pmol per Minute per Milligram of Protein

Tissue	Concentration of 7-OHCG Produced per Min per Milligram of Protein (pmol)
Liver	2300
Kidney	220
Bladder	140
Large Intestine	7.8
Lung	0.0
Spleen	0.0
Heart	0.0
Fat	0.0

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Table 2

Mean Concentrations of 7-hydroxycoumarin-glucuronide ± St. Dev.) Produced by Rabbit Kidney UDPGT and the % Relative Standard Deviation Over Time (n=3)

Time (min)	Mean 7-hydroxycoumarin glucuronide Concentration (μM)	% Relative Standard Deviation
0	0	0
15	2.4 ± 0.09	3.7
30	5.3 ± 0.2	4.0
45	10.3 ± 0.4	4.3
60	12.6 ± 0.6	4.9
75	17.3 ± 0.48	2.7
90	20.7 ± 1.53	7.4

The determination of 7-hydroxycoumarin or its metabolite allow a calculation of the metabolic rate of the reaction for the tissue in question. Eight organs; liver, kidney, large intestine, bladder, lung, spleen, heart and fat were selected for the study. UDPGT activity was not observed in all tissues. Table 1 shows the UDPGT activity in the various organs. The

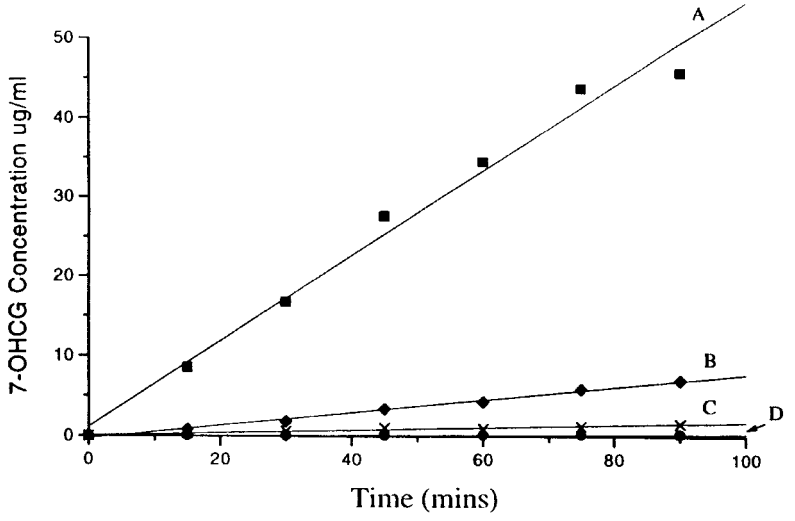

Figure 4. Graph showing rate of production of 7-hydroxycoumarin-glucuronide in four of the tissue samples, liver (A), kidney (B), bladder (C), and large intestine (D).

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highest activity was observed in the liver, with the kidney and bladder showing less activity. Very low activity was observed in the large intestine. The spleen and lung showed trace concentrations of 7-hydroxycoumarin-glucuronide after 90 mins (results not shown), with the fat and heart tissue showing no detectable activity.

A plot of the mean 7-hydroxycoumarin-glucuronide concentration produced (n=3) versus time (Table 2) was used to calculate the activity of UDPGT in the tissue . The rate of reaction was calculated from the linear part of the curve (Fig. 4). It can be seen that the liver and kidney produced appreciable quantities of 7-hydroxycoumarin-glucuronide with relative activities of 2.3 nmol and 0.22 nmol of 7-hydroxycoumarin-glucuronide produced per minute per milligram of protein respectively.

The bladder showed an activity of 0.14 nmol of 7-OHCG produced per minute per milligram of protein, with a very low level of activity in the large intestine of 7.8 pmol of 7-OHCG produced per minute per milligram of protein.

CONCLUSION

UDPGT activity is present in many organs of the one species, here in the rabbit it has been demonstrated to be present in the liver, kidney and to a lesser extent bladder and large intestine. Trace amounts of the glucuronide were found in the spleen and lung, with no detectable activity in the heart and fat tissue. The method used is reliable and accurate and requires minimal sample clean up.

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