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In vitro glucuronidation of 7-hydroxycoumarin and determination of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide by capillary electrophoresis

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Abstract

A method was developed for the determination of the in vitro production of 7-hydroxycoumarin glucuronide and β-glucuronidase activity in rabbit tissue homogenates (liver, kidney, heart, lung, spleen, large intestine and fat). Separation of 7-hydroxycoumarin (7-HC) and 7-hydroxycoumarin glucuronide (7-HCG) by capillary electrophoresis was carried out on a 27 cm untreated fused silica capillary using a 100 m*M* phosphate buffer, pH 7.0 at 17.5 kV, with detection at 320 nm. 7-HC and 7-HCG were separated within 4 min and could be determined in the same run. Rabbit tissues, containing uridine diphosphate (UDP) glucuronyl transferase (UDPGT), were homogenised in Tris–HCl, pH 7.4, and used for the production of 7-HCG by the reaction of 7-HC and UDP-glucuronic acid (UDPGA). The conversion of 7-HC to 7-HCG is catalysed by UDPGT and the reverse reaction by β-glucuronidase. A sample of the reaction mixture was removed and added to acetonitrile, centrifuged and analysed by capillary electrophoresis. For the reverse reaction (β-glucuronidase reaction), the rabbit tissue samples were homogenised in 100 m*M* acetate buffer, pH 4.3. To this 7-HCG was added and its metabolism to 7-HC and the decrease in 7-HCG content was determined after stopping the reaction with a β-glucuronidase inhibitor, protein precipitation and centrifugation. β-Glucuronidase activity was observed in all tissue types, but not all tissues displayed UDPGT activity. The highest UDPGT activity was detected in the liver, followed by the kidney. The limit of detection was 1 μg/ml for 7-HC, and 2 μg/ml for the glucuronide, with a linear detection range for both analytes from 0–100 μg/ml.

Keywords: Hydroxycoumarin; Glucuronides; Hydroxycoumarin glucuronide; Enzymes; Uridine diphosphate glucuronyl transferase; Glucuronidase

1. Introduction

The uridine diphosphate glucuronyl transferases (UDPGTs) are a family of closely related membrane bound enzymes that are responsible for the transfer of the glucuronyl group from UDP-glucuronic acid (UDPGA) to many endogenous and exogenous molecules having functional groups of oxygen, nitrogen, sulphur and carbon. The liver is shown to be the most important organ in the body for glucuronidation both quantitatively and qualitatively [1]. For most drugs glucuronidation occurs predominantly in the periportal region of the liver [2,3]. However, Conway et al. have shown that glucuronidation of 7-hydroxycoumarin (7-HC) is 3-fold greater in the pericental areas compared to the periportal areas of the liver [4]. Generally, 7-HC is the principal phase I metabolite of coumarin in humans [5]. Coumarin and 7-HC have both been used as trial drugs in cancer treatment [6]. The majority of coumarin and 7-HC

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administered is excreted as 7-hydroxycoumarin glucuronide (7-HCG), within 24 h, for most individuals.

The exact pharmacological role of 7-HCG, if any, has not yet been established. It was suggested by Casley-Smith [7] that, since active transport is proposed to exist for glucuronides, it is possible that 7-HCG is transported back into the cells. Glucuronidases present within the cell may reconvert the glucuronide to the 7-HC. After it has exerted its pharmacological action it might then be reglucuronidated before excretion.

CE was used for the determination of 7-HC following both in vivo [8,9] and in vitro [10,11] metabolism of coumarin. It was also used for the determination of 7-HC and 7-HCG in plasma and urine and has shown mean intra-assay precision and accuracy of 4.3% for 7-HC and 3.8% for 7-HCG. Mean inter-assay precision and accuracy was 5.7% for 7-HC and 6.7% for 7-HCG [9]. The method of Bogan et al. [9] was applied for the direct determination of both compounds after an in vitro incubation, and for the determination of UDP-glucuronyl transferase activity in whole organ homogenates.

Until now CE has not been applied to the study of β-glucuronidase activity. Previous methods for the study of β-glucuronidase activity have involved complex systems [12]. β-Glucuronidase has also been used for qualitative work on morphine and other drugs [13]. Early 7-HCG studies relied on the use of β-glucuronidase to convert the 7-HCG back to 7-HC where it was determined by various methods [14]. This paper describes the application of the method of Bogan et al. [9] as a new procedure for the assay of β-glucuronidase activity in whole organ homogenates.

2. Experimental

7-HC, uridine 5′-diphosphate glucuronic acid (UDPGA), D-saccharic acid-1,4-lactone, magnesium chloride Tris–HCl and β-glucuronidase were purchased from Sigma (St. Louis, MO, USA). Absolute ethanol was purchased from Merck, (Darmstadt, Germany). Na_2HPO_4 and NaH_2PO_4 were obtained from Riedel-de Haen (Hanover, Germany). The electrolyte buffer consisted of 100 m*M* phosphate buffer (sodium salt), pH 7.0, (adjusted and set by adding the acid form of the sodium salt to the basic form until the desired pH was obtained). All buffers and dilutions were made using ultrapure water.

3. Sample preparation

Organs were obtained from New Zealand White Rabbits and stored at −20°C until required. A 10 g sample of each organ was weighed and homogenised in 10 ml of 50 m*M* Tris–HCl, pH 7.4, for the UDPGT experiment, and homogenised in 10 ml of 100 m*M* acetate buffer, pH 4.3, for the β-glucuronidase experiment. These were diluted to 10 mg/ml (mg wet mass of organ/ml of buffer). This constituted the protein solution to be used in the reaction solution. The protein concentration in each was determined by Bicinchonnic acid (BCA) assay (Pierce, IL, USA), after dialysis of an aliquot of protein solution against 50 volumes of phosphate-buffered saline solution (PBS) overnight at 4°C. A standard curve was constructed from a range of standards, 0–2 mg/ml, bovine serum albumin (BSA), prepared in PBS.

3.1. Reaction solution for assay of UDPGT activity

Table 1 shows the eight components necessary for the reactions, along with the respective volumes. A 1 mg/ml solution of 7-hydroxycoumarin was prepared in ethanol–ultrapure water (10:90). All other solutions were prepared in ultrapure water. Reactions were carried out in 10 ml blood tubes (Medical Supply, Dublin, Ireland). The reaction was initiated by addition of 1 ml of the protein solution, to make a final reaction volume of 4 ml. The reaction was incubated in a dark oven at 37°C, and terminated by adding 50 μl of acetonitrile (AnalaR grade) to 150 μl of reaction mixture. This was then centrifuged at 8000*g* to remove the protein and any other material that might interfere with analysis. The supernatant was then immediately analysed.

3.2. Reaction solution for β-glucuronidase assay

Table 2 outlines the components used. This was

Table 1
Stock and final concentrations of components used in the incubation solution for the study of the UDP-glucuronyl transferase activity in rabbit tissue

Component	Stock solution		
	Concentration	Volume (μl)	Final concentration
7-Hydroxycoumarin	6.17 m*M*	500	0.771 m*M*
Enzyme solution	10 mg/ml	1000	–
D-Saccharic acid-1,4- lactone	50 m*M*	500	6.25 m*M*
UDPGA	50 m*M*	100	1.25 m*M*
$MgCl_2$	1 m*M*	25	$6.25 \cdot 10^{-3}$ m*M*
Absolute ethanol	–	125	–
Tris–HCl (pH 7.4)	1 *M*	500	125 m*M*
Ultrapure water	–	1250	–

prepared to a final volume of 140 μl and the reaction allowed to continue for 30 min at 37°C in a dark oven until it was stopped by the addition of 50 μl of 50 m*M* D-saccharic acid-1,4-lactone. 50 μl of acetonitrile was then added and the solution centrifuged at 8000*g* for 5 min. The supernatant was immediately analysed.

4. Controls

For the β-glucuronidase assay a reaction solution of each of the tissues was prepared with one change, in that 50 μl of D-saccharic acid-1,4-lactone (β-glucuronidase inhibitor) was added to each before the addition of 7-HCG and the reaction monitored after 30 min. In the case of all controls monitored, there was no breakdown to 7-HC from 7-HCG.

A similar control was prepared for the UDPGT assay. A reaction solution was prepared with the protein precipitated out of solution by acetonitrile before addition of the UDPGA with monitoring after 30 min. There was no detectable production of the glucuronide in each of the controls monitored.

5. Standard curve preparation

5.1. UDPGT assay

A range of standards were prepared consisting of 0–200 μg/ml of 7-HC, prepared in ethanol–ultrapure water (10:90), and 7-HCG prepared in ultrapure water. 20 μl of each were spiked into 40 μl of 1 *M* Tris–HCl, pH 7.4, 50 μl of acetonitrile and 40 μl of each protein solution. The resulting mixture was vortexed and centrifuged to remove the protein. Previous CE studies required denaturation of protein solution by boiling to remove any interference due to endogenous β-glucuronidase present [15]. However, in this case it was decided to remove the protein as this avoids any further reaction of any type in the mixture prior to analysis which might otherwise affect the results. 7-HCG levels were calculated from a plot of 7-HCG concentration standards versus absorbance (peak height).

5.2. β-Glucuronidase study

The standard curve for the β-glucuronidase study

Table 2
Stock concentrations and final concentrations of components used in the incubation solution for the β-glucuronidase study in rabbit tissue

Component	Stock solution concentration	Volume (μl)	Final concentration
Enzyme solution	10 mg/ml	40	2.86 mg/ml
7-HCG	6.17 m*M*	40	1.76 m*M*
Acetate buffer, (pH 4.3)	100 m*M*	60	42.86 m*M*

was obtained using solutions that consisted of 40 μl of 7-HC standards, 60 μl of 100 m*M* acetate buffer, pH 4.3, and 100 μl of enzyme solution, (either β-glucuronidase or homogenate). To this the 50 μl of D-saccharic acid-1,4-lactone and 50 μl of acetonitrile were added. The protein was then centrifuged as before. Levels of 7-HC were calculated from a plot of 7-HC concentration versus absorbance (peak height).

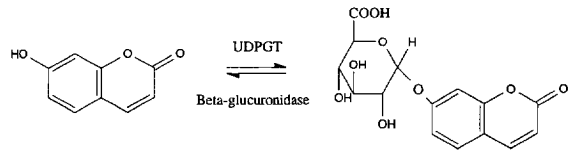

Fig. 1. Structures of 7-HC and 7-HCG and the in vitro reactions studied by capillary electrophoresis. 7-HC is metabolised to 7-HCG by the action of UDPGT and the reverse reaction is catalysed by β-glucuronidase.

6. CE separation

Separation was carried out on a P/ACE System 5500 CE instrument, with detection by a P/ACE UV absorbance detector (Beckman Instruments, Fullerton, CA, USA). All components were controlled by System Gold Software (Beckman Instruments). Separation was carried out on an untreated fused silica capillary, 27 cm×50 μm I.D.×375μm O.D. (19.3 cm capillary to detector distance). Prior to running samples, the capillary was conditioned with 0.1 *M* HCl for 10 min, 0.1 *M* NaOH for 10 min and finally 100 m*M* phosphate buffer, pH 7.0 electrolyte buffer for 10 min. The capillary was conditioned between each run by a 3 min rinse with 100 m*M* phosphate buffer. Samples were applied by 8-s pressurised injection at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). Separation was achieved at 17.5 kV, 25°C, with detection at 320 nm. Typical running current was 150 μA. The same capillary was used without any deleterious effects on performance or reproducibility for the duration of the study.

7. Results and discussion

The majority of coumarin administered in humans is excreted as 7-HCG [8]. It is the principal phase II metabolite and is produced from the main phase I metabolite, 7-HC. Fig. 1 shows the reaction scheme for both UDPGT and β-glucuronidase.

7.1. UDPGT assay

The method used for the determination of 7-HC and (7-HCG) in urine and plasma was applied to the determination of 7-HCG in an in vitro metabolic assay for rabbit liver, kidney, heart, lung, spleen and large intestine.

Separation of 7-HCG from endogenous species present in the metabolic mixture and from 7-HC is achieved in under 4 min. Fig. 2 shows an electropherogram of the reaction mixture as outlined in Section 2 for the kidney. It also shows an overlay of electropherograms of samples taken at time 0, 40 and 80 min. The increase in levels of 7-HCG can be seen. The mean amount ($n=3$) of glucuronide produced was calculated for each organ. Table 3 shows those results calculated for the liver. The results obtained demonstrate the liver to be the major site of glucuronidation for 7-HC, followed by the kidney, and with a small amount of glucuronide produced by

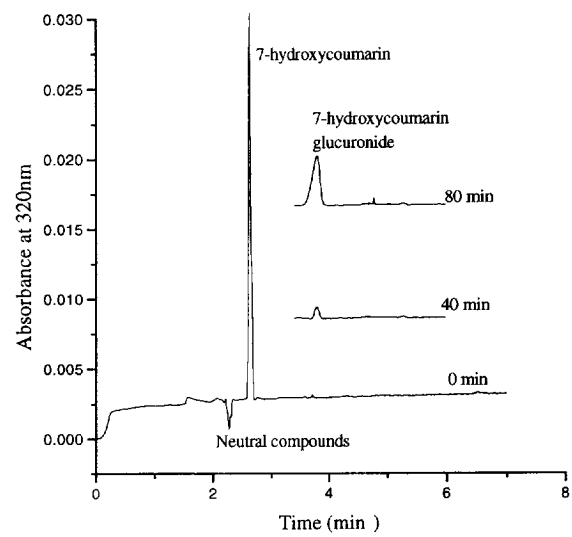

Fig. 2. CE electropherogram of the kidney metabolic solution as outlined in Section 2. It shows an overlay of electropherograms of samples taken at 0, 40 and 80 min overlays shown between 3.4 and 5.9 min.

Table 3
Mean concentrations of 7-HCG±standard deviation (S.D.) produced by rabbit liver UDPGT and the % relative standard deviation (R.S.D.) over time ($n=3$)

Time (min)	Mean 7-HCG concentration (μM) ±S.D.	R.S.D. (%)
0	0	0
20	9.8±0.5	5.0
40	23.66±0.88	3.7
60	37.56±0.83	2.0
80	48.55±0.69	1.4
100	58.11±1.01	1.7
120	63.11±0.76	1.2
140	76.44±1.17	1.5
160	87.11±1.16	1.3

the large intestine (Table 4). The limit of detection for the 7-HCG was 2 μg/ml. Percentage relative standard deviation values for each peak were all ≤5%.

7.2. β-Glucuronidase study

The method of Bogan et al. [8] when applied as a new procedure for the assay of β-glucuronidase activity proved effective for detection of both 7-HC and 7-HCG. Separation of both compounds was achieved in under 4 min and the increase in 7-HC concentration with time can be seen as shown in Fig. 3. The assay was used to monitor β-glucuronidase activity for each organ in terms of conversion of 7-HCG to 7-HC (Fig. 4). β-Glucuronidase activity was also determined in units/g wet mass of organ. The same six organs chosen for the UDPGT assay

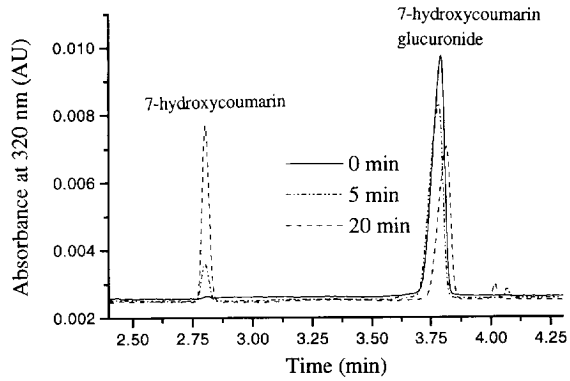

Fig. 3. Portion of electropherogram from liver incubation showing the increase in breakdown of 7-HCG to 7-HC with time.

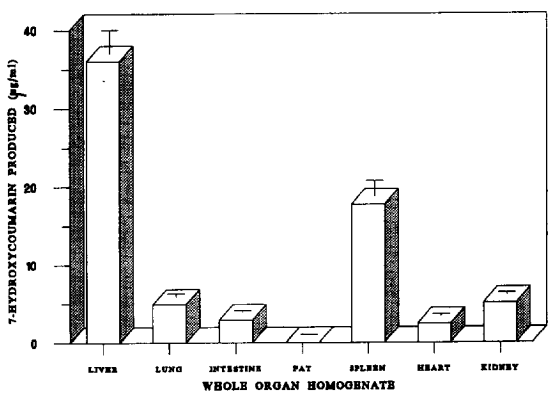

Fig. 4. Plot showing the comparison of endogenous β-glucuronidase activity in each organ i.e. 7-HC produced (μg/ml) in the study of the deconjugation of 7-HCG to 7-HC.

were chosen for this study. As in the case of the UDPGT assay, the liver proved to be the most active site for deconjugation of 7-HCG (Table 5), followed

Table 4
Table showing the activities of the different organs in amount of 7-HCG produced in pmol per minute per milligram of protein

Tissue	Concentration of 7-HCG produced per min per milligram of protein (pmol)
Liver	2100
Kidney	200
Large intestine	7.8
Lung	0.0
Spleen	0.0
Heart	0.0
Fat	0.0

Table 5
β-Glucuronidase activity (units/g wet mass of organ) in whole organ homogenates as determined after analysis by capillary electrophoresis

Organ homogenate	β-Glucuronidase activity units/g wet mass of whole organ
Liver	12 660
Lung	1710
Intestine	1003
Fat	0
Spleen	6200
Heart	800
Kidney	1780

by the spleen with about half the activity of the liver. The kidney and lung displayed low activity relative to the liver, with the heart and intestine showing the lowest activity. The fat, as expected, showed no detectable activity versus 7-HC produced. The activity is expressed in units where 1 unit is that which will liberate 1.0 μg of phenolphthalein from phenolphthalein glucuronide per hour at 37°C at pH 5.0 (30 min assay). The limit of detection for the 7-HC was 1 μg/ml.

8. Conclusion

The CE method was found to be very fast and reliable for the direct determination of 7-HC and 7-HCG as in vitro metabolites of reactions involving UDP-glucuronyl transferase and β-glucuronidase. Minimal sample clean up was necessary which reduces errors due to complex sample preparation (extraction, precipitation, etc.). The method would also facilitate studies of promoters and inhibitors on both enzymes.

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