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# Analysis of the glucuronidation of 7-hydroxycoumarin by HPLC

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**Abstract**

The *in-vitro* metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide was investigated in bovine liver homogenate. A metabolic reaction mixture was prepared that included a crude preparation of uridine diphosphate (UDP) glucuronyl transferase, 7-hydroxycoumarin and UDP-glucuronic acid. A HPLC method was developed to separate coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and an internal standard, 4-hydroxycoumarin. Samples were separated by reverse-phase HPLC, on a C18 column, with a 1 ml $min^{-1}$ gradient elution with UV detection at 320 nm. The limit of quantification of the method, for 7-hydroxycoumarin-glucuronide, was 1.47 $\mu$M, and the linear range was from 0–295.7 $\mu$M. Concentrations of 7-hydroxycoumarin-glucuronide produced were calculated from a plot of 7-hydroxycoumarin-glucuronide concentration versus the mean absorbance ratio ($n = 4$) (7-hydroxycoumarin-glucuronide absorbance/4-hydroxycoumarin absorbance). It was possible to monitor the decrease in the 7-hydroxycoumarin content as it was metabolised as well as the increase in 7-hydroxycoumarin-glucuronide as it was produced enzymatically. The identity of the compound produced was confirmed by photodiode array spectral analysis. A plot of time versus 7-hydroxycoumarin-glucuronide produced indicates that the metabolism is linear for the first 90 min and reached a plateau at 150 min. The rate of reaction in the first 90 min was $2.96 \pm 0.06$ (RSD 1.7%, $n = 3$) nmol of 7-hydroxycoumarin-glucuronide produced per minute per milligram of protein. After 150 min $0.34 \pm 0.005$ mM (RSD 1.4%) 7-hydroxycoumarin-glucuronide was produced, from 0.77 mM 7-hydroxycoumarin introduced into the reaction mixture and $58.0\% \pm 5.3\%$ (or $0.44 \pm 0.02$ mM) of the 7-hydroxycoumarin remained. These results show that it is possible to monitor the production of the phase II metabolite of coumarin with minimal sample clean-up and without the need for deconjugation of the glucuronide moiety. The method was very reliable and applicable for the direct determination of 7-hydroxycoumarin-glucuronide in an *in-vitro* metabolic assay.

*Keywords:* HPLC; 7-Hydroxycoumarin; 7-Hydroxycoumarin-glucuronide; Uridine diphosphate-glucuronyl transferase

## 1. Introduction

The liver has been identified as the most important site for glucuronidation. Glucuronidation of xenobiotics and endogenous substances is a major detoxification pathway. Compounds are made more water soluble, facilitating excretion in the urine [1,2]. The coumarins are a large group of naturally occurring flavanoids with a backbone structure of 1,2-benzopyran-2-one. Coumarin has been shown to be an ideal "probe dryg" for both

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been shown to be an ideal "probe dryg" for both *in-vitro* and *in-vivo* studies of cytochrome P450 enzyme systems [3]. The ideal "probe drug" is typically enzyme-specific, not inhibitory to other enzymes present, easily available, sampling is not difficult or inconvenient for the volunteer, "polyfunctional" (one can detect environmental/host influences), and the kinetics/metabolism formation is determined by metabolism and not by blood flow or protein binding. Coumarin, its mode of action, its analysis, and its pharmacological applications in humans have been reviewed by O'Kennedy and Thornes [4].

In humans, coumarin is metabolised in the liver, initially to 7-hydroxycoumarin and subsequently to 7-hydroxycoumarin-glucuronide [5]. The majority of coumarin administered to humans is excreted in the 7-hydroxycoumarin-glucuronide form [5]. As metabolites of coumarin, the determination of the metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide is critical in assessing both phase I and phase II metabolism enzyme systems. Coumarin is a prodrug for 7-hydroxycoumarin as it is believed that 7-hydroxycoumarin is the active form of the drug rather than coumarin itself. Thus, 7-hydroxycoumarin and coumarin are currently being investigated as potential anti-cancer agents [6]. Coumarin has also been used in the treatment of brucellosis and high protein oedemas [7].

The clinical role of 7-hydroxycoumarin-glucuronide is unclear. It can be monitored circulating in plasma after the administration of coumarin [5]. Casley-Smith and Casley-Smith [8] have suggested that due to the active transport shown to exist for glucuronides, it is possible that 7-hydroxycoumarin-glucuronide is transported into the cells. Glucuronides present in the cell reconvert the glucuronide to the 7-hydroxycoumarin. After it has exerted its pharmacological action, it might then be reglucuronidated before excretion [8]. The metabolism of coumarin to 7-hydroxycoumarin is species dependent [9–12], and even within species there is great variability in coumarin metabolism [13,14]. This is due to different levels of the phase I cytochrome P-4502A6 present in liver. However, little research has concentrated on investigation of the particular glucuronyl transferase that metabolites 7-hydroxycoumarin to its more hydrophilic metabolite.

Analysis of 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin in metabolism studies and in urine, plasma and serum samples, almost always requires the deconjugation of 7-hydroxycoumarin-glucuronide to free 7-hydroxycoumarin followed by extensive clean-up procedures [15,16]. HPLC [15], spectrofluorimetry [13], and capillary electrophoresis [16] have all been used for the determination of total 7-hydroxycoumarin in urine after *in-vivo* metabolism, and as a coumarin metabolite [10,17] in *in-vitro* metabolism studies. Bogan et al. [18] and Sharifi et al. [19] reported on the direct determination of the glucuronide form of 7-hydroxycoumarin as well as the free 7-hydroxycoumarin without deconjugation with $\beta$-glucuronidase. The ideal method would have a minimal clean-up procedure that does not interfere with the compounds of interest.

Whole bovine liver homogenate was prepared and used for studying the metabolism of 7-hydroxycoumarin to 7-hydroxcoumarin-glucuronide. A method was developed for the separation and determination of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and internal standard, 4-hydroxycoumarin, and was applied to the determination of the phase II metabolite of coumarin in an *in-vitro* assay with minimal sample clean-up.

## 2. Materials and methods

### 2.1. Chemicals

Uridine diphosphate (UDP)-glucuronic acid (UDPGA), 7-hydroxycoumarin, 4-hydroxycoumarin, magnesium chloride, and saccharic acid, 1,4-lactone, were purchased from Sigma (St. Louis, MO). 7-hydroxycoumarin-glucuronide and coumarin were kindly donated by Schaper and Brummer (Salzgitter, Germany). The methanol and acetic acid used for the mobile phase preparation were purchased from Labscan (Dublin, Ireland) and were of HPLC-grade. Solvent A was

50/950/2 (v/v/v) methanol/water/acetic acid mixture, and solvent B was 100% methanol. The solvents were prepared, mixed, and sonicated for 20 min. Analar-grade trichloroacetic acid and Tris–HCl were obtained from BDH Chemicals (Poole, UK).

### 2.2. Liver sample preparation

A bovine liver was obtained and stored at −80°C until required. The crude protein solution was prepared according to the method of Tegtmeier [20]. The protein solution (4 mg $ml^{-1}$) was stored in 50 mM Tris–HCl buffer pH 7.4 at −80°C until required. The protein concentration was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL, USA).

### 2.3. Incubation mixture and preparation of standards

The concentrations of the components of the metabolic incubation solution were: crude liver homogenate (1 mg $ml^{-1}$), 7-hydroxycoumarin (0.77 mM), UDPGA (1.25 mM), magnesium chloride (6.25 mM), Tris–HCl (125 mM, pH 7.4), saccharic acid 1,4-lactone (6.25 mM), water, and absolute ethanol. The final volume was 4 ml. 200 μl of the reaction mixture was removed at specific time intervals and added to 40 μl of a 20% (w/v) solution of trichloracetic acid, mixed, and centrifuged at 13 000 rev $min^{-1}$ for 5 min. The reaction was carried out at 37°C in an open 10 ml glass tube (Medical Supply Co., Dublin, Ireland) with mixing before sampling. The reaction was initiated by the addition of the enzyme solution and samples were removed at specific time intervals.

A series of 7-hydroxycoumarin-glucuronide (7-OHCG) (0–2.96 mM) standards was prepared in water from a 1 mg $ml^{-1}$ (2.96 mM) stock solution. A standard curve was constructed from standards prepared as follows: 20 μl 7-OHCG standard, 40 μl enzyme solution, 40 μl water, 100 μl Tris–HCl, and 40 μl trichloracetic acid were added together and centrifuged as above. The total volume was exactly the same as that of the sample removed from the metabolic solution. 190 μl of the sample or standard supernatant was added to 10 μl of a 1 mg $ml^{-1}$ solution of the internal standard in a 1.1 ml sample vial for analysis by HPLC.

### 2.4. HPLC analysis

The HPLC system consisted of a System Gold (Beckman Instruments Ltd., Fullerton, CA, USA) solvent module 126, detector module 166, photodiode array detector 168, and autosampler module 507. All of the components were controlled by System Gold Software. Separation was carried out on a Phenomenex (Macclesfield, UK) Bondclone 10 C18 column with gradient elution of the compounds of interest. The eluent was monitored at 320 nm. 20 μl of sample was injected onto the column. The 1 ml $min^{-1}$ gradient was as follows: 0–14 min: Solvent A (100%) → Solvent A (50%)–Solvent B (50%); 14–22 min: Solvent A (50%) → Solvent B (50%); 22–23 min: Solvent A (50%) → Solvent B (50%) → Solvent A (100%); 23–32 min: Solvent A (100%).

## 3. Results and discussion

### 3.1. HPLC separation development

A new HPLC method for the separation of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin (Fig. 1) was developed. This was applied to the determination of 7-hydroxycoumarin-glucuronide, following its metabolism from 7-hydroxycoumarin by UDP-glucuronyl transferase (UDPGT) with the substrate UDPGA. In the isocratic method of Egan and O'Kennedy [15] 7-hydroxycoumarin-glucuronide, which is very hydrophilic, was not retained on the column and elutes in the void volume. A gradient elution was developed to retard the 7-hydroxycoumarin-glucuronide on the column. Some of the previous methods for the analysis of coumarins [15,19] used a water/methanol/acetic acid mobile phase. Thus, these solvents were assessed for their potential to separate the above compounds.

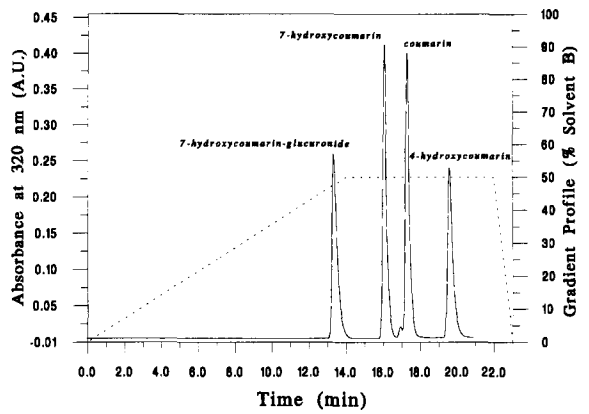


Fig. 1. Reverse-phase chromatographic separation of 7-hydroxycoumarin-glucuronide (13.3 min), 7-hydroxycoumarin (16.0 min), coumarin (17.3 min), and the internal standard 4-hydroxycoumarin (19.6 min) prepared in water. Chromatogram of time (min) versus absorbance at 320 nm (absorbance units). The gradient profile, represented as a precentage of solvent B (100% methanol) versus time (min) is overlaid. The samples were analysed as described in Section 2.

The separation was optimised for the determination of 7-hydroxycoumarin-glucuronide, as well as the reactant 7-hydroxycoumarin, and an internal standard 4-hydroxycoumarin (Fig. 2) in a metabolic assay. The gradient elution (Fig. 1) allowed the successful retardation of 7-hydroxycoumarin-glucuronide on the column. It was well

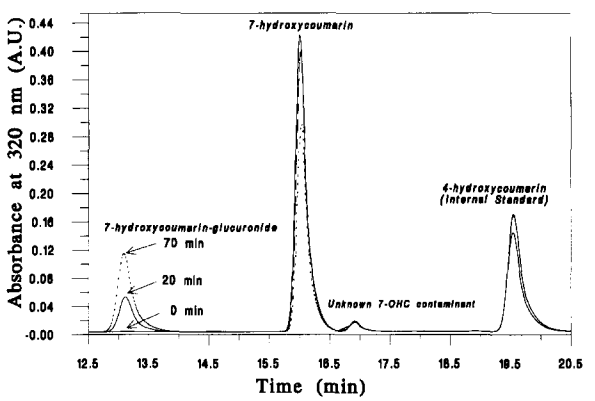


Fig. 2. Overlay of three chromatograms, time (min) versus absorbance at 320 nm (absorbance units) from 12.5 min to 20.5 min, of samples taken at 0 min, 20 min, and 70 min. The chromatograms show the decrease in 7-hydroxycoumarin content, and the increase in 7-hydroxycoumarin-glucuronide content over time. Samples were analysed as described in Section 2.

Table 1
Mean peak absorbance ratios ( ± SD) for 7-hydroxycoumarin-glucuronide standards prepared as described in Section 2 ($n = 4$) calculated from peak absorbance 7-hydroxycoumarin-glucuronide/peak absorbance 4-hydroxycoumarin

| 7-Hydroxycoumarin-glucuronide concentration ($\mu$M) | Mean peak absorbance ratio ($n = 4$) | %RSD |
|---|---|---|
| 0 | 0 | 0 |
| 1.47 | $0.006 \pm 2 \times 10^{-4}$ | 1.3 |
| 2.96 | $0.010 \pm 2 \times 10^{-4}$ | 3.0 |
| 5.92 | $0.019 \pm 1.5 \times 10^{-3}$ | 8.3 |
| 14.79 | $0.048 \pm 1.5 \times 10^{-3}$ | 3.2 |
| 29.58 | $0.094 \pm 3.0 \times 10^{-3}$ | 3.2 |
| 59.15 | $0.185 \pm 6.9 \times 10^{-3}$ | 3.7 |
| 147.89 | $0.458 \pm 9.9 \times 10^{-3}$ | 2.2 |
| 236.62 | $0.727 \pm 40.7 \times 10^{-3}$ | 5.6 |
| 295.77 | $0.876 \pm 59.0 \times 10^{-3}$ | 6.7 |

resolved from the other coumarins present, and from any of the components of the reaction mixture. As the concentration of methanol increased the compounds eluted in the order 7-hydroxycoumarin-glucuronide, 7-hydroxycoumarin, coumarin and finally 4-hydroxycoumarin (Fig. 1). 4-Hydroxycoumrain was chosen as internal standard as it was well resolved from the other compounds and has an absorbance at 320 nm. Previous methods for the determination of 7-hydroxycoumarin-glucuronide [15,16] required its deconjugation to 7-hydroxycoumarin. This HPLC method did not require any deconjugation or extraction. There was minimal sample clean-up. The addition of trichloroacetic acid acts to precipitate the protein out of solution, thus stopping the reaction, and also as a sample clean-up to prevent any fouling of the HPLC system.

The method had a limit of quantitation, for 7-hydroxycoumarin-glucuronide, of 0.5 $\mu$g ml$^{-1}$ (1.47 $\mu$M). The linear range was from 0–100 $\mu$g ml$^{-1}$ (0–295.7 $\mu$M). To prevent the endogenous $\beta$-glucuronidase, present in the liver homogenate, causing the deconjugation of 7-hydroxycoumarin-glucuronide, the standards were prepared in protein denatured with trichloracetic acid. Table 1 gives the mean peak absorbance ratios for the 7-hydroxycoumarin-glucuronide standards and

their percentage RSDs ($n = 4$). The percentage RSDs are all below 6%.

### 3.2. Glucuronidation of 7-hydroxycoumarin by UDPGT

There are very few methods that allow the direct determination of 7-hydroxycoumarin-glucuronide without deconjugation [18,19]. The metabolite of 7-hydroxycoumarin was monitored over time and its increase could be seen clearly (Fig. 2). An added advantage of the method was that it was not possible to monitor the decrease in the 7-hydroxycoumarin concentration as it was consumed. The determination of 7-hydroxycoumarin or its metabolite allow a calculation of the metabolic rate of the reaction.

A plot of the mean 7-hydroxycoumarin-glucuronide concentration produced ($n = 3$) versus time (Table 2) gave a linear plot for the first 90 min. After 90 min the metabolic rate decreased and the level of 7-hydroxycoumarin-glucuronide no longer increased linearly. It reached a plateau at about 150 min (Fig. 3). The rate of reaction for the first 90 min was $2.96 \pm 0.06$ $\mu$M ($n = 3$) 7-hydroxycoumarin-glucuronide produced per min per milligram of protein. $0.34 \pm 0.005$ mM ($n = 3$) 7-hydroxycoumarin-glucuronide was produced from 0.77 mM 7-hydroxycoumarin added to the reaction mixture. The 7-hydroxycoumarin content decreased over time (Fig. 2) and, at 150 min, $58.0\% \pm 5.3\%$ ($0.44 \pm 0.02$ mM) ($n = 3$) of the initial amount added remained (calculated from the decrease in peak absorbance ratios for 7-hydroxycoumarin absorbance/4-hydroxycoumarin absorbance). In the absence of 7-hydroxycoumarin, enzyme, or UDPGA, no 7-hydroxycoumarin-glucuronide was produced.

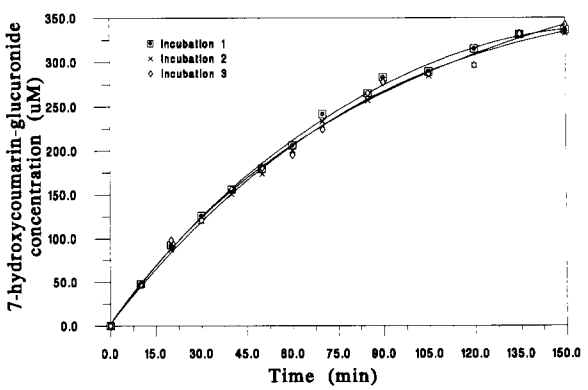


Fig. 3. Metabolism of 7-hydroxycoumarin by UDPGT with the cofactor UDPGA. A plot of the 7-hydroxycoumarin-glucuronide concentration ($\mu$M) (produced enzymatically) versus time (min) is shown.

Table 2
Mean concentrations of 7-hydroxycoumarin-glucuronide ($\pm$SD) produced by bovine liver UDPGT and the % RSD over time ($n = 3$)

| Time (min) | Mean 7-hydroxycoumarin glucuronide concentration ($\mu$M) $\pm$SD | %RSD |
|---|---|---|
| 0 | 0 | 0 |
| 10 | $47.1 \pm 0.7$ | 1.6 |
| 20 | $96.2 \pm 5.5$ | 5.9 |
| 30 | $122.4 \pm 3.1$ | 2.5 |
| 40 | $153.8 \pm 3.1$ | 2.0 |
| 50 | $177.8 \pm 3.9$ | 2.2 |
| 60 | $200.5 \pm 5.5$ | 2.7 |
| 70 | $233.1 \pm 12.4$ | 5.3 |
| 85 | $262.3 \pm 4.7$ | 1.7 |
| 90 | $280.4 \pm 3.8$ | 1.4 |
| 105 | $287.2 \pm 3.0$ | 1.1 |
| 120 | $302.6 \pm 10.6$ | 3.5 |
| 135 | $331.3 \pm 0.6$ | 2.0 |
| 150 | $336.7 \pm 5.0$ | 1.5 |

A similar metabolic assay study was carried out with capillary electrophoresis (CE) as the mode of separation with UV detection at 320 nm [18]. There was no statistical difference between the CE method and the HPLC method when the results were compared. The rate of reaction from the CE study was $3.1 \pm 0.13$ $\mu$M ($n = 3$) 7-hydroxycoumarin produced per min per milligram of protein. Samples were removed from the reaction mixture and analysed immediately by CE without any sample preparation. The application of 30 kV stopped the reaction by separating the individual components of the reaction. However, it was not possible to monitor the decrease in 7-hydroxycoumarin content by CE, due to interference from mangesium chloride.

## 4. Conclusion

7-hydroxycoumarin is metabolised by UDPGT to 7-hydroxycoumarin-glucuronide. Both compounds can be separated and determined by HPLC. The method developed requires minimal sample preparation and is reliable and accurate. Errors due to incomplete deconjugation or loss during extraction are removed. The method is ideal for studying the *in-vitro* metabolism of 7-hydroxycoumarin and *in-vivo* metabolism of coumarin as all the major coumarin metabolites in humans can be separated and determined.

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