The use of capillary electrophoresis for studying the in vitro glucuronidation of 7-hydroxycoumarin

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Abstract

An assay, utilizing CE, has been developed for studying the in vitro metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide. A reaction mixture containing a crude preparation of bovine uridine diphosphate (UDP) glucuronyl transferase (UDPGT) and the substrates uridine diphosphate glucuronic acid (UDPGA) and 7-hydroxycoumarin was prepared. Aliquots were removed from the reaction mixture at specific time intervals and analyzed on a 57-cm untreated silica capillary for the presence of 7-hydroxycoumarin-glucuronide. Samples were electrophoresed in a 100 m*M* phosphate/11 m*M* deoxycholic acid (sodium salt)/acetonitrile electrolyte buffer, pH 7.0, at 30 kV, with detection at 320 nm. The method allowed the determination of 7-hydroxycoumarin-glucuronide without sample cleanup, with a limit of detection of 2 µg/mL, and a linear detection range of 0 µg/mL to 100 µg/mL. The 7-hydroxycoumarin-glucuronide concentrations were calculated from calibration curves of standards prepared in enzyme solution. The initial rate of production of 7-hydroxycoumarin-glucuronide, in the first 70 min, was 3.1 ± 0.13 nmol/mL/min/mg protein. The method was fast and reliable with percentage relative standard deviations for concentration of 7-hydroxycoumarin-glucuronide, over time, of less than 10%.

The UDPGTs are a family of closely related membrane-bound enzymes that are responsible for the transfer of the glucuronyl group from UDPGA to many endogenous and exogenous molecules having functional groups of oxygen, nitrogen, sulfur, and carbon. The liver has been identified as the most important site of glucuronidation, both qualitatively and quantitatively. Other tissues are also known to possess glucuronyl transferase activity.[1] Glucuronidation is a major detoxification pathway, and it functions in rendering xenobiotics more water soluble, which makes them more readily excreted in the urine.[2] Some glucuronides are now known to have clinical relevance,[2] and epirubicin-glucuronide has been investigated as a prodrug for specific treatment of cancer.[3] Other glucuronide conjugates are routinely monitored to determine pregnancy.[4,5]

In many plants, 7-hydroxycoumarin (umbelliferone) is present. It is the principal product of Phase I metabolism of coumarin in humans.[6] It has been used in the treatment of high protein edemas[7] and as an anticancer agent.[8] It has also been investigated in relation to its inhibitory effect on human malignant cell lines in vitro[9] and in inhibiting oncogene-induced transformation of murine fibroblasts.[10] It is also used as a pH-sensitive fluorescent indicator, in fluorescence-based immunoassays, and as a blood-brain barrier probe.[11] The 7-hydroxycoumarin is converted by UDPGT to 7-hydroxycoumarin-glucuronide (*Figure 1*). Conway et al. have shown that glucuronidation of 7-hydroxycoumarin is threefold greater in the pericentral areas than the periportal areas of the liver.[12] The majority of coumarin administered is excreted as 7-hydroxycoumarin-glucuronide.[13] Any pharmacological role of the glucuronide conjugate has not yet been determined. Casley-Smith has suggested that, due to the active transport shown to exist for glucuronides, it is possible that 7-hydroxycoumarin-glucuronide is transported into the cells. Glucuronidases 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.[7]

CE has been used for the determination of 7-hydroxycoumarin following both in vivo[14,15] and in vitro[16,17] metabolism of coumarin and for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in plasma and urine.[15] The method of Bogan et al.[15] for the direct determination of both compounds

Indexing terms

7-hydroxycoumarin-glucuronide, UDP-glucuronyl transferase, UDP-glucuronic acid

Abbreviations

UDP, uridine diphosphate; UDPGT, uridine diphosphate glucuronyl transferase; UDPGA, uridine diphosphate glucuronic acid

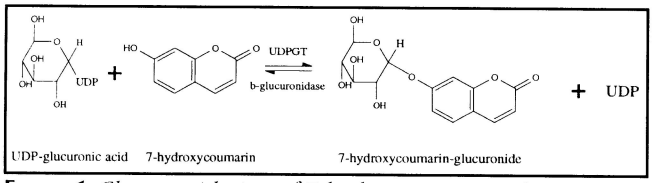

FIGURE 1 *Glucuronidation of 7-hydroxycoumarin by UDPGT.*

in urine and plasma without any sample preparation was applied to the determination of 7-hydroxycoumarin-glucuronide in an in vitro metabolic assay. Other researchers have carried out similar studies on glucuronide production with analysis by HPLC.[18–20] However, it was necessary to stop the reaction by precipitating the protein out of solution. A method similar to that of Deasy et al.[16] that did not require sample cleanup would allow the direct determination of the metabolite in real time without any interference with the reaction mixture. CE has also been used for the determination of glucuronides in urine and in Chinese medicines.[21–23]

Materials and methods

Chemicals

UDPGA, 7-hydroxycoumarin, magnesium chloride, deoxycholic acid (sodium salt), and D-saccharic acid 1,4-lactone were purchased from Sigma (St. Louis, MO, U.S.A.); 7-hydroxycoumarin-glucuronide was kindly donated by Schaper and Brummer (Salzgitter, Germany). The electrolyte solution was 90% (100 m*M* phosphate buffer/11 m*M* deoxycholic acid [sodium salt]):10% acetonitrile (Labscan, Dublin, Ireland). The phosphate buffer was prepared by adding 100 m*M* K_2HPO_4 and 100 m*M* KH_2PO_4 (Riedel de-Haen, Hanover, Germany) solutions together to a pH of 7.0. The deoxycholic acid (sodium salt) was prepared in this buffer, and the acetonitrile was then added. The pH remained at 7.0. Stock solutions of electrolyte buffer were prepared and stored at 4 °C until required.

UDPGT preparation

A bovine liver was obtained and stored at –80 °C until required. The crude protein solution was prepared by the method of Tegtmeier et al.[24] The protein solution was stored at –80 °C until required. The protein concentration was determined by the bicinchoninic acid assay (BCA, Pierce Chemical Co., Rockford, IL, U.S.A.) using bovine serum albumin as standard prepared in 50 m*M* Tris-HCl. The concentration of protein was 4 mg/mL.

Incubation solution

The incubation solution consisted of eight components (*Table 1*). The principal components for the production of 7-hydroxycoumarin-glucuronide are 7-hydroxycoumarin, the enzymatic solution containing the UDPGT, and UDPGA. A 1 mg/mL solution of 7-hydroxycoumarin was prepared in 10% ethanol (Merck, Darmstadt, Germany): 90% deionized water. All other solutions were prepared in deionized water. The reaction will not occur without the Tris-HCl buffer. Reactions were carried out in open 2-mL test tubes (Sarstedt Ltd., Wexford, Ireland) at 37 °C. The reaction was initiated by the addition of the protein solution. A sample was immediately removed and analyzed by CE. It was not necessary to add any material to stop the reaction as the application of the 30,000 V separates the components on the reaction mixture, thus stopping the opportunity for further reaction. Samples were removed approximately every 12 min and analyzed immediately. The reaction was repeated four times. Control studies were carried out in the absence of 7-hydroxycoumarin, UDPGA, and enzyme, respectively.

Standard curve preparation

The 0–1000 µg/mL standards of 7-hydroxycoumarin-glucuronide were prepared in water, and 10 µL was spiked into 40 µL of protein solution and 50 µL of 50 m*M* Tris-buffer pH 7.4. It was necessary to denature the protein solution to remove any interference of endogenous β-glucuronidase present. This was achieved by boiling the solution for 5 min. Concentrations of 7-hydroxycoumarin-glucuronide produced were de-

Table 1
COMPONENTS IN THE REACTION MIXTURE, INCLUDING STOCK CONCENTRATIONS AND FINAL CONCENTRATIONS IN THE INCUBATION SOLUTION

Component	Stock solution concentration	Volume µL	Final concentration
7-hydroxycoumarin	6.2 m*M*	200	0.77 m*M*
Enzyme solution	4 mg/mL	400	1 mg/mL
Magnesium chloride	1 *M*	10	6.25 m*M*
Saccharic acid 1,4-lactone	50 m*M*	200	6.25 m*M*
UDPGA	10 m*M*	200	1.25 m*M*
Absolute ethanol		50	
Tris-HCl (pH 7.4)	1 *M*	200	125 m*M*
Deionized water		340	

termined from a mean standard curve (n = 3) of concentration of 7-hydroxycoumarin-glucuronide versus peak area.

CE separation

Separation was carried out on a P/ACE System 5500 CE instrument (Beckman Instruments, Fullerton, CA, U.S.A.). The capillary used was a 57 × 50 µm i.d. untreated silica capillary (Beckman Instruments) with a capillary-to-detector distance of 50 cm. It was conditioned initially each day by 10-min rinses with 0.1 *M* hydrochloric acid, followed by 0.1 *M* sodium hydroxide, then deionized water, and finally electrolyte buffer. Between each run, the capillary was rinsed for 0.8 min with 0.1 *M* NaOH, then for 2.5 min with electrolyte solution. Samples were applied by 10-sec pressurized injections at 0.5 psi. Separation was achieved at 30 kV (rinse time 0.2 min) at 25 °C with detection at 320 nm. Typical running current was 170 µA. The same capillary was used without any deleterious effects on performance or reproducibility during the duration of the study.

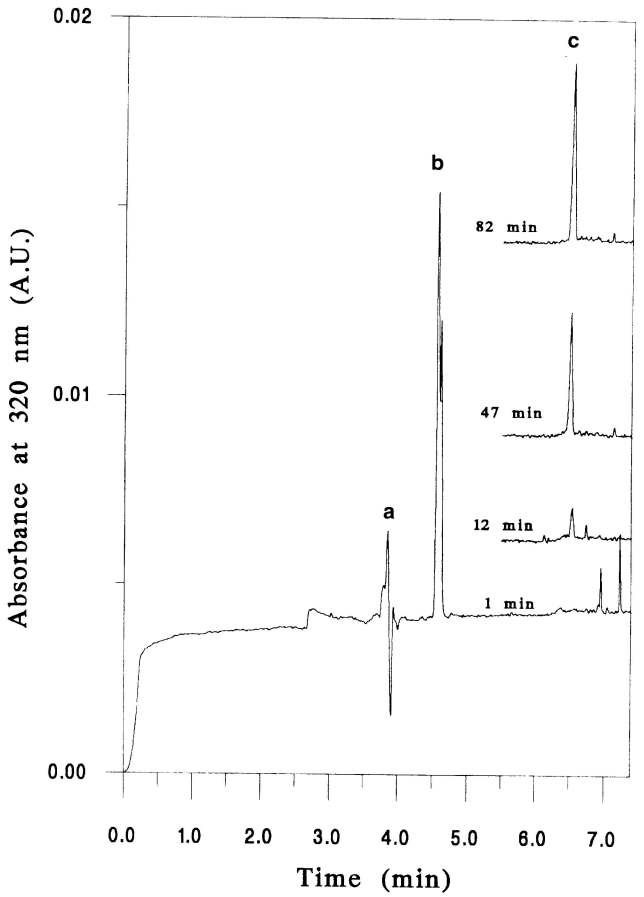

FIGURE 2 *CE electropherogram of metabolic solution as outlined in "Materials and methods." It shows an overlay of electropherograms of samples taken at 1 min, 12 min, 47 min, and 82 min, respectively. The peaks are: a) solvent front containing all neutral components; b) magnesium chloride and 7-hydroxycoumarin (shoulder); and c) 7-hydroxycoumarin-glucuronide. Electropherograms for 12 min, 47 min, and 82 min are shown in offset to illustrate the increase in peak area and are displayed between 5.5 min and 7.5 min only. The remainder of the electropherogram was typical of the 1-min profile.*

Results and discussion

The majority of coumarin administered is excreted as 7-hydroxycoumarin-glucuronide, in humans.[14] It is the principal Phase II metabolite and is produced from the main Phase I metabolite 7-hydroxycoumarin (Figure 1). The method utilized for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine and plasma[15] was applied to the determination of 7-hydroxycoumarin-glucuronide in an in vitro metabolic assay.

Separation of 7-hydroxycoumarin-glucuronide from endogenous species present in the metabolic matrix and from 7-hydroxycoumarin is achieved in less than 7 min (*Figure 2*). It is clearly seen in Figure 2 that

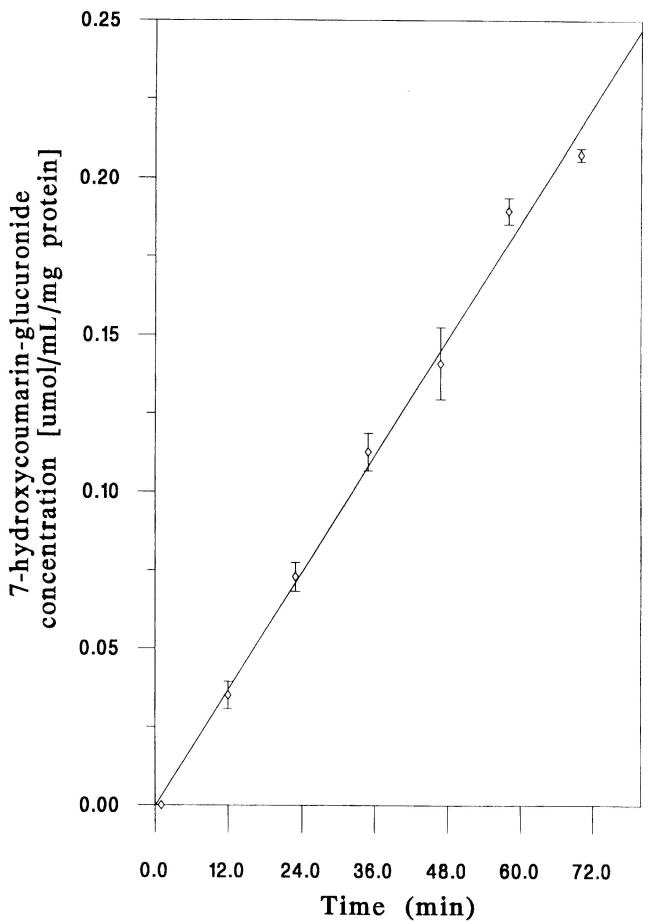

FIGURE 3 *A plot of time (min) versus mean 7-hydroxycoumarin-glucuronide concentration (n = 4) (µmol/mL/mg protein) from incubation assay study. The equation of the line is* $y = -0.00032 + 0.00309x$ *with an* $r^2 = 0.99348$.

at 1 min no 7-hydroxycoumarin-glucuronide was present, but after 12 min, the UDPGT had already started to produce 7-hydroxycoumarin-glucuronide. At 47 and 82 min, there was a visible increase in the 7-hydroxycoumarin-glucuronide (peak c) content in the matrix. The reaction proceeded in a linear manner for the first 70 min (*Figure 3*). The mean rate of reaction for the first 70 min was found to be 3.09 ± 0.13 nmol of glucuronide produced per minute per mL of solution per mg of protein (n = 4). After 70 min (results not shown), the enzyme efficiency slows down, and after 180 min, no more 7-hydroxycoumarin-glucuronide is formed. The reaction rate was slowed due to substrate limitation, enzymatic deactivation, β-glucuronidase deconjugation, or a combination of the factors.

In the absence of enzyme, 7-hydroxycoumarin, or UDPGA, no 7-hydroxycoumarin-glucuronide was produced. It was possible to monitor the breakdown of the glucuronide conjugate over time to 7-hydroxycoumarin, when the undenatured protein solution was used as the matrix for the standards. The limit of detection for 7-hydroxycoumarin-glucuronide was 2 µg/mL with a linear range of 0–100 µg/mL. Percentage relative standard deviations for each standard peak area were all below 10%.

Conclusion

The CE method developed was found to be very fast and reliable for the direct determination of 7-hydroxycoumarin-glucuronide as the in vitro metabolite of 7-hydroxycoumarin and UDPGA by the enzymatic action of UDPGT. The method was reproducible with excellent precision and accuracy. No sample cleanup was necessary, and injection of the neat mixture onto the capillary allows results to be obtained in nearly real time without any interference from errors due to sample preparation (extraction steps, precipitation steps, etc.). The method would be ideal for the investigation of UDPGT inhibitors or promoters. Thus, CE has been demonstrated to be applicable to the in vitro determination of both Phase I and Phase II metabolism studies of coumarin.

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