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Determination of 7-hydroxycoumarin and its glucuronide and sulphate conjugates in liver slice incubates by capillary zone electrophoresis

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Abstract

The simultaneous detection of the phase I metabolite of coumarin, 7-hydroxycoumarin, and the two phase II metabolites, 7-hydroxycoumarin-glucuronide and 7-hydroxycoumarin-sulphate by capillary electrophoresis with UV detection using liver slice incubations was investigated. Separation was carried out on an untreated fused silica capillary with detection at 320 nm. Separation was achieved in under 6 min with a total run time of 8 min. Both phase two metabolites were produced following an in vitro incubation of liver slices in Krebs-Hanseleit buffer with 100 μM 7-hydroxycoumarin. Limits of detection were 5.52 μM (2 μg ml^{-1}) for the glucuronide, 2.21 μM (0.5 μg ml^{-1}) for the sulphate and 6.17 μM (1 μg $ml)^{-1}$ for 7-hydroxycoumarin. Mean inter- and intra-assay results are presented for all three analytes, respectively.

Keywords: 7-Hydroxycoumarin; 7-Hydroxycoumarin-glucuronide; 7-Hydroxycoumarin-sulphate

1. Introduction

The exact pharmacokinetic description of drug metabolism requires the determination of both phase I and phase II conjugates. Advances in HPLC, and more recently capillary electrophoresis, have allowed the determination of multiple metabolites in one analysis run [1–3]. Classical methods for the analysis of metabolites include spectrofluorometry, TLC and ELISA. Most of the methods involve the analysis of one metabolite at a time and often incur many time-consuming steps, such as deconjugation of one metabolite to a more easily analysed form (7-hydroxycoumarin-glucuronide deconjugation to 7-hydroxycoumarin and glucuronic acid and subsequent analysis).

Capillary electrophoresis offers many advantages over classical methods. It requires minute amounts of sample, therefore consuming less reagents, is easily automated for precise quantitative analysis and ease of use, and total analysis time is greatly reduced. Here we report on the use of capillary electrophoresis in the study of coumarin metabolism for the detection of one of its more common phase I metabolites, 7-hydroxycou-

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marin (7-HC), and two of its phase II metabolites, 7-hydroxycoumarin-glucuronide (7-HCG) and 7-hydroxycoumarin-sulphate (7-HS).

The coumarins belong to a group of compounds known as benzopyranones, and have long been used in the clinical field for the treatment of diseases such as lymphoedema and other high protein oedemas [4], thermal injuries [5], renal cell carcinoma [6] and prostate cancer [7]. A number of researchers have looked at the analysis of coumarin metabolites using HPLC [3,8], with limits of detection of 0.125 μM, 0.025 μM, and 0.125 μM for 7-HCG, 7-HC and 7-HS, respectively, and at coumarin metabolism using both HPLC and CE [9,10]. However this method shown here offers a significant time saving per analysis, up to five-fold in one case, [3]. More recently Cole et al., while evaluating extended light path capillaries for use in capillary electrophoresis, looked at the detection of 7-ethoxycoumarin and metabolites in liver slice incubation media using laser-induced fluorescence detection. However, not all metabolites could be detected due to significant differences in their excitation/emission wavelengths [11]. This is one of the difficulties with working with laser-induced fluorescence (LIF) detection in the direct mode. UV detection offers the advantage of being able to detect all metabolites simultaneously. However, with the superior levels of detection offered by LIF [12,13], we are currently working towards its use in the indirect mode (ILIF), for more sensitive levels of detection for the metabolites of coumarin.

Previous studies have shown that the liver is the major site of glucuronidation for drugs, catalysed by the uridine diphosphate glucuronosyl transferase (UDPGT), multigene family of enzymes. The resulting glucuronide is generally water soluble, less toxic and more easily excreted than the parent compound [15]. Further studies have shown that phenol UDPGT is also located in the epithelial cells of the bile duct and the endothelial cells of the bile duct and portal vein [16]. Other organs also have the ability to glucuronidate compounds, albeit with a more restricted substrate specificity and capacity than hepatic tissue [17]. A recent study carried out on rabbit tissues showed the liver to be the major site of glucuronidation for 7-hydroxycoumarin (2.3 nmol per min per milligram of protein), followed by the kidney [9].

As with glucuronidation, sulphation increases the water solubility of most compounds, and, therefore, their renal excretion, as well as generally decreasing their biological action. However, it can also play a role in the bioactivation of procarcinogens [18]. Even though sulphation was discovered by Baumann (1876) over a century ago, it is only within the last thirty years that the enzymology of this reaction has been studied in detail.

2. Experimental

2.1. Chemicals

7-Hydroxycoumarin sulphate was synthesised according to the method of Walsh et al. [3]. 7-hydroxycoumarin glucuronide was obtained from Salford Ultrafine Chemicals, Manchester, England. Na_2HPO_4, NaH_2PO_4 and sodium chloride were supplied by Riedel-de Haen, Hannover, Germany, and potassium chloride from Lennox Laboratories, Dublin, Ireland. Krebs-Hanseleit buffer was made according to the procedure of Hiller and Cole [8], all reagents being obtained from Sigma Chemical Co., St. Louis, MO, USA. The electrolyte buffer used for the separations was 50 mM phosphate buffer (sodium salt), pH 7.0, filter sterilised (0.22 μm). All standards and buffers were made using ultra pure water, (Millipore-Q system, Millipore Marlborough, MA).

2.2. Liver slice incubation

Mouse liver was obtained from Balb\C mice and stored at −20°C until required. Liver slices were prepared using a labratory-made razor-bladed instrument based on that described by Krumdieck et al., [14]. Incubations were carried out in blood tubes at 37°C. Tissue slices were preincubated for 90 min at 37°C in a warm room using a blood tube mixer with the revolving face of the mixer horizontal to ensure gentle mixing of the buffer. The slices were then incubated with 100 μM 7-hydroxycoumarin for 6–10 h prior to analysis.

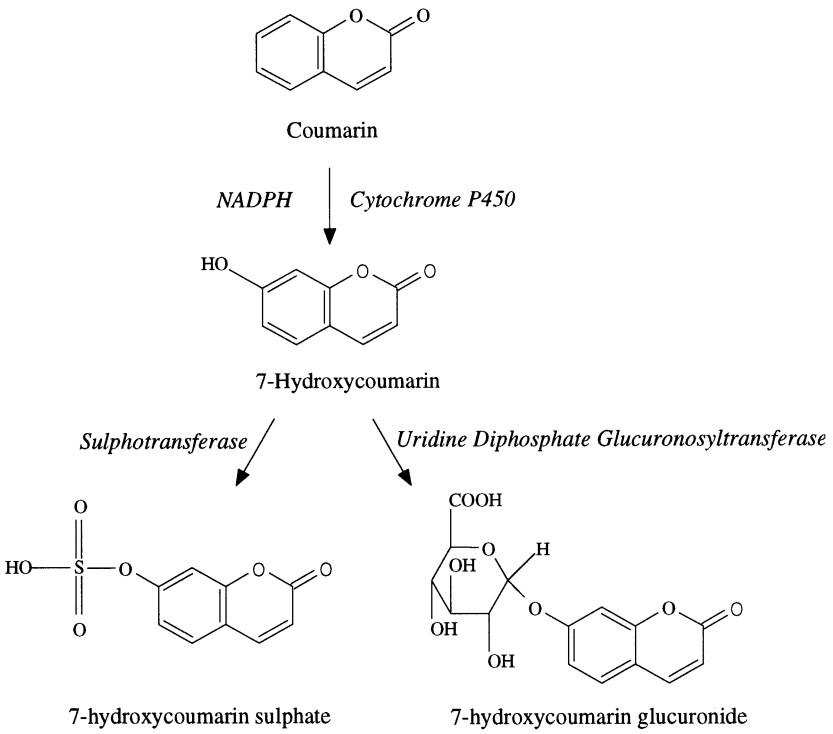

Fig. 1. Reaction scheme showing the structures of the parent drug coumarin, 7-HC, 7-HCG, 7-HS and the production of 7-HCG by UDPGT enzyme and 7-HS by sulphotransferase enzyme.

2.3. Preparation of standards

7-HC standards were prepared from a 1 mg ml^{-1} stock solution prepared in ethanol and ultra pure water (10:90 v/v). Both 7-HCG and 7-HS standards were prepared from a 1 mg ml^{-1} stock prepared in ultra pure water. All standards were diluted with Krebs-Hanseleit buffer. All three analytes had a linear detection range from 0 to 150 μM. The range of concentrations selected for the study corresponded to the lower, middle and upper portions of the standard curve.

2.4. CE separation

Separation was carried out in a fused untreated silica capillary (27 cm × 50 μm I.D. × 375 μm O.D., 19.3 cm to detector window, Beckman Instruments, Fullerton, CA, USA). The samples were analysed on a Beckman CE P/ACE 5500 instrument with detection by a P/ACE UV absorbance detector. All components were controlled by System Gold software (Beckman Instruments). On a daily basis, the capillary was conditioned with 0.1 M HCl for 10 min, 0.1 M NaOH for 10 min and finally with 50 mM electrolyte buffer (p.H. 7.0) for 5 min. At the end of each day the capillary was given a four minute rinse with both acid, (0.1 M HCl), base, (0.1 M NaOH), and a 4-min rinse in ultrapure water. It was stored overnight in ultrapure water. The capillary was conditioned between each run by a 2.0-min rinse with electrolyte buffer. Samples were applied by 5 s pressurised injection at 0.5 psi (1 psi = 6894.76 Pa). Separations were carried out at 25 kV, 25°C with detection at 320 nm. Typical running current was 170 μA. The same capillary was used for the duration of the study. Inter- and intra-assay variations were assessed to determine the accuracy and precision of the technique.

3. Results and discussion

In humans, the majority of coumarin administered is excreted in the form of 7-hydroxycoumarin-glucuronide. Fig. 1 shows the reaction scheme for both the glucuronide and sulphate conjugates. All three analytes absorb at 214 as well as 320 nm. However, due to interferents in the media also absorbing at 214 nm, it was not possible to analyse at this wavelength. Hence 320 nm was chosen as the wavelength of detection, despite a slight loss in sensitivity.

The method proved very satisfactory for the resolution of all three analytes. Fig. 2 shows an electropherogram of a standard solution of all three analytes in Krebs-Hanseleit buffer. All three analytes were seen to resolve in just over 5 min. The Krebs-Hanseleit buffer was also run without any of the three analytes added and no peaks which might interfere with results were noted. Fig. 3 shows an electropherogram following an 8-h incubation with 100 μM 7-HC in Krebs-Hanseleit buffer. It shows the production of both phase II metabolites of 7-HC, namely, 7-hydroxycoumarin glucuronide and sulphate.

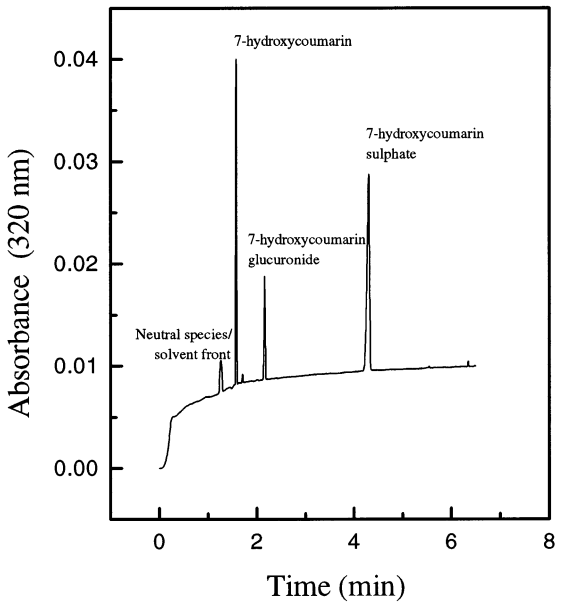

Fig. 2. Electrophereogram of a standard mixture of 7-HC, 7-HCG and 7-HS, separated as outlined under Section 2. The concentrations of the analytes were as follows: 60 μM for 7-HC and 7-HS, and 40 μM for 7-HCG.

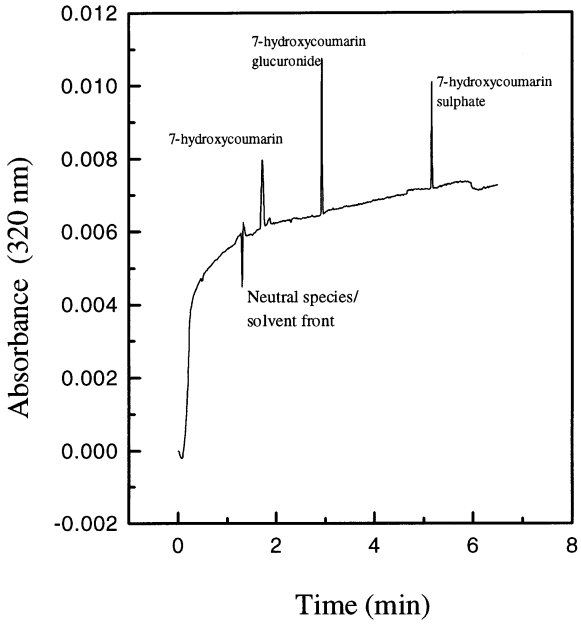

Fig. 3. Electropherogram showing the production of 7-HCG and 7-HS from 7-HC after 8 h incubation. Samples were 0.22 μm filtered prior to analysis as outlined in Section 2.

Intra-assay performance was assessed by analysing concentrations corresponding to the lower, middle and upper regions of the standard curve. The samples were analysed in replicates of five on the same day. Table 1 shows the results obtained. Precision and accuracy values are within ± 14 and $\pm 11\%$, respectively, with 80% of the values for both precision and accuracy deviating within $\pm 7\%$. As expected, precision and accuracy showed the largest deviation at the limit of detection of the method, most noticeable with 7-HC and 7-HS.

Inter-assay performance was assessed by analysis of samples in a range of four different concentrations of each analyte in triplicate over three different days. Table 2 shows the results obtained. Precision and accuracy values were between ± 13 and $\pm 6\%$, respectively. As with the intra-assay performance, both precision and accuracy showed the highest values at the limit of detection of the method. Assay specificity was assessed by analysis of incubates without any 7-HC added. No signifi-

Table 1
Intra-assay precision and accuracy results for the determination of 7-HCG and 7-HS conjugates of 7-hydroxycoumarin for liver slice incubates

	Intra-assay preformance (concentration, μM)				
	Nominal	Calculated	S.D.	% Accuracy	% Precision
7-HC	6.17	5.55	0.52	89.95	9.37
	12.34	11.52	1.60	93.35	13.89
	30.86	31.59	1.28	102.36	4.05
	61.73	63.10	2.25	102.22	3.57
	123.46	121.31	5.76	98.26	4.75
7-HCG	2.76	3.07	0.22	111.23	7.17
	5.52	5.76	0.25	104.35	4.34
	13.80	14.14	0.76	102.46	5.37
	27.60	28.55	1.75	103.44	6.13
	55.20	56.97	0.32	103.21	0.56
7-HS	4.42	5.08	0.67	101.00	13.19
	8.84	8.55	0.56	96.72	6.54
	22.10	21.59	0.47	97.69	2.18
	44.21	44.21	0.40	102.04	0.89
	88.42	89.08	2.52	100.75	2.83

cant interferents were noted in any of the migration zones of the analytes.

4. Conclusions

The method applied proved to be very simple and effective for the determination of all three analytes, with good resolution and easy sample preparation, while still keeping the run time to a minimum. Although UV detection does not offer the level of sensitivity of laser induced fluorescence detection (LIF) it still offers acceptable levels at a much lower cost.

Table 2
Inter-assay precision and accuracy results for the determination of 7-HCG and 7-HS conjugates of 7-hydroxycoumarin for liver slice incubates

	Inter-assay performance (concentration, mM)							
	Nominal	Day 1	Day 2	Day 3	Mean	S.D.	% Accuracy	% Precision
7-HC	12.34	13.64	12.34	12.09	12.69	0.83	102.84	6.54
	30.86	30.97	31.10	30.42	30.83	0.36	99.90	1.17
	61.73	61.14	62.26	61.45	61.22	0.58	99.82	0.94
	123.46	122.54	123.33	123.65	123.17	0.57	99.93	0.46
7-HCG	5.52	6.35	5.02	6.04	5.80	0.70	105.07	12.06
	13.80	13.97	13.52	14.55	14.01	0.52	101.08	3.71
	27.60	27.38	27.84	27.51	27.58	0.24	99.93	0.87
	55.20	54.95	55.26	55.37	55.19	0.22	99.98	0.40
7-HS	8.84	7.91	8.08	9.11	8.37	0.67	94.68	8.00
	22.10	22.36	23.47	21.92	22.58	0.80	102.17	3.54
	44.21	44.10	43.27	45.61	44.33	1.19	100.27	2.68
	88.42	88.09	89.33	91.58	89.67	1.78	101.41	1.98

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