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Note

Analysis of coumarin and its urinary metabolites by high-performance liquid chromatography

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Coumarin is a naturally occurring benzopyran (2H-1-benzopyran-2-one) which is found in many plants and essential oils [1]. In 1936, Szent-Gyorgi et al. [2] described a vitamin-like activity of a group of benzopyrans (including coumarin) – the flavones. He called this activity vitamin P. Subsequently, the group was renamed the bioflavanoids.

In vitro coumarin has been shown to activate macrophages [3, 4]. Clinically, this property is utilized in the treatment of high protein lymphoedemas [5]. The activated macrophages cause an increase in the rate of phagocytosis of proteins. Peptide fragments formed by the action of proteolytic enzymes on these phagocytosed proteins can pass back into the plasma as their concentration gradient is towards the extra-cellular fluid [6–8]. Activation of macrophages gives rise to an increase in the immune response. This has resulted in the use of coumarin in cancer therapy [9, 10] and also for the treatment of brucellosis and immune suppression [11]. Coumarin has also been shown to have an inhibitory effect on the induction of cancer [12, 13].

Due to its extensive use in the food industry as a flavour enhancer, much research was done on the toxicology of coumarin. Indications that the compound was potentially hepatotoxic in rats [14, 15] resulted in a ban on the use of cou-

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marin in foodstuffs. Subsequently, this hepatotoxicity was shown to be species-specific, as coumarin is metabolised differently in primates than in rats [16, 17] and it is this difference in metabolising abilities that gives rise to inter-species differences in toxicity.

Coumarin is in use for some years at the Colman K. Byrne's Research Centre, St. Laurence's Hospital (Dublin, Ireland) in the treatment of cancer and chronic brucellosis. A study was therefore undertaken to analyse the urinary metabolites of coumarin in humans and a high-performance liquid chromatographic (HPLC) procedure capable of resolving and quantitating the known metabolites found in the urine was developed. The method used was a modification of that used by Walters et al. [18].

EXPERIMENTAL

Standards

Coumarin was obtained from Eastman Kodak (Rochester, NY, U.S.A.). 7-Hydroxycoumarin, 4-hydroxycoumarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin and scopoletin (7-hydroxy-6-methoxycoumarin) were obtained from Sigma (Poole, U.K.). *o*-Hydroxyphenylacetic acid and 4H-pyran-4-one were obtained from Aldrich (Gillingham, U.K.) and *o*-coumaric acid from Koch-Light Labs. (Colnbrook, U.K.). Coumarin tablets (100 mg) were manufactured by Schaper and Brümmer (F.R.G.).

Equipment, stationary and mobile phases

Reversed-phase chromatography was performed using a Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph fitted with a pre-packed μBondapak radial compression type column (particle size 10 μm). Chromatography was carried out at room temperature. The eluent was monitored at 280 nm using a Waters fixed-wavelength UV detector (Model No. 440).

All solvents used were HPLC grade and were obtained from Fisons (Loughborough, U.K.). The solvents were filtered through Sartorious membrane filters, pore size 0.45 μm, and degassed using a Millipore vacuum pump while being stirred continuously on a magnetic stirrer. Solvents were prepared freshly each day and stored at room temperature in glass bottles.

Sample preparation and treatment

A pretreatment urine sample was collected and each subject was then given an oral dose of 200 mg coumarin. Urine samples were collected 1 h after administration and thereafter every 2–4 h for a total of 24 h. Total volumes of all urine samples were recorded. Aliquots (5 ml) of all urine samples were taken, freeze-dried and redissolved in 2.5 ml of distilled water. A 0.4-ml volume of this aqueous extract was added to 0.4 ml of 1 *M* acetate buffer pH 3.8. A 0.2-ml volume of β-glucuronidase (EC 3.2.1.31; type H-2; Sigma) was added at a concentration of 20 000 U/ml (=0.7 E.C. U/ml). The mixture was incubated at 37°C for 16 h. The samples were then evaporated to dryness and redissolved in 1.0 ml of HPLC-grade methanol prior to application to HPLC.

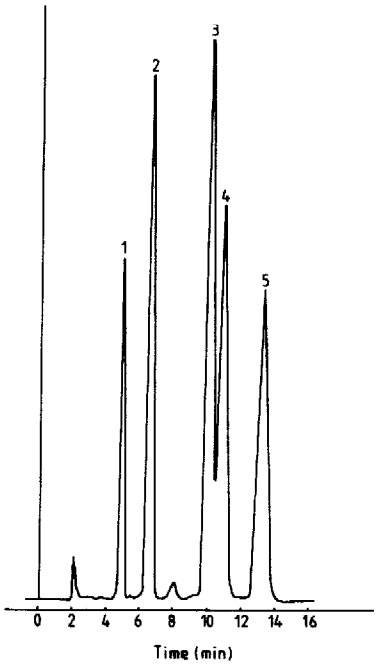

Fig. 1. HPLC profile of authentic standards using as mobile phase methanol–water–acetic acid (200:300:1) on a μBondapak C_{18} reversed-phase column. Flow-rate, 2 ml/min; chart-rate, 5 mm/min; sensitivity, 0.05; absorbance measured at 280 nm. Peaks: 1 = *o*-hydroxyphenylacetic acid; 2 = 7-hydroxycoumarin; 3 = coumarin; 4 = *o*-coumaric acid; 5 = 4-hydroxycoumarin.

Quantitation of the main metabolite (7-hydroxycoumarin) was made by measuring peak heights. It was, therefore, necessary to prepare a standard curve for 7-hydroxycoumarin. This was carried out by injecting known quantities of authentic 7-hydroxycoumarin standard in triplicate, measuring peak heights and a standard curve was then drawn up.

RESULTS AND DISCUSSION

Various mobile phases using different combinations of methanol–water–acetic acid were used. The aim of these experiments was to find a system which would give complete separation of all coumarin standards available to us without giving an inordinately long overall retention time. The first two mobile phases, i.e. methanol–water–acetic acid (150:350:1) and (125:375:1), were found to be unsatisfactory; the former because of incomplete resolution of all standards and the latter because of long overall retention time. A third mobile phase, methanol–water–acetic acid (200:300:1) was considered satisfactory with respect to separation and total retention time and was used for further studies (Fig. 1).

Three internal standards were tested for suitability, 4-H-pyran-4-one, 3-(α-acetonylbenzyl)-4-hydroxycoumarin and 7-hydroxy-6-methoxycoumarin. None proved successful, as they either eluated too close to the standards or had an extremely long retention time.

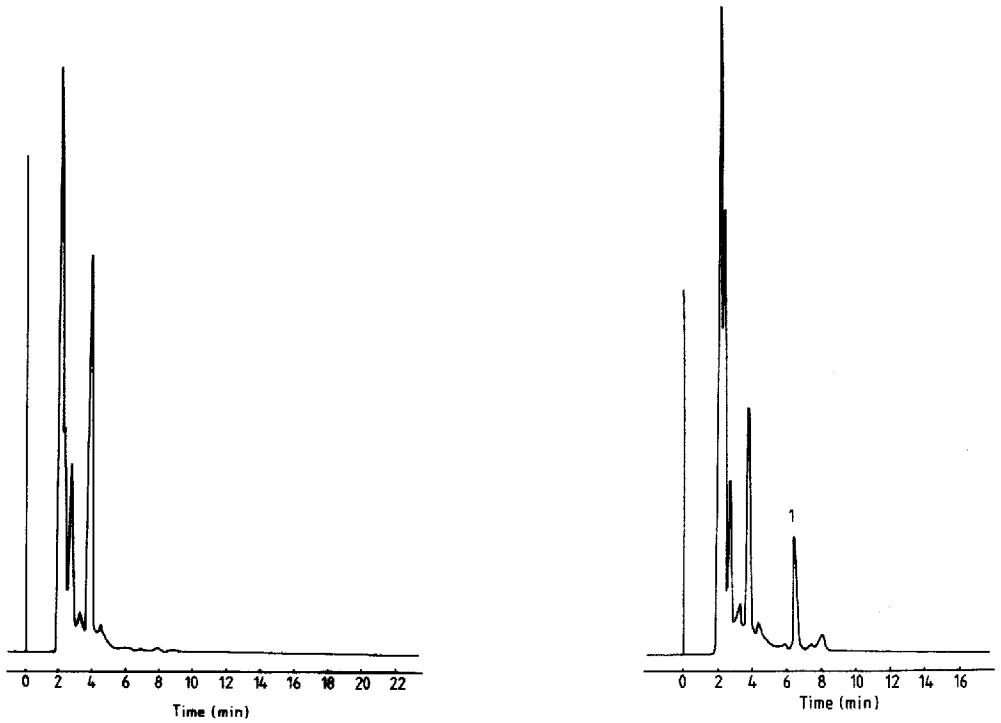

Fig. 2. HPLC profile of urine sample prior to administration of coumarin. Mobile phase, flow-rate, chart-rate, sensitivity and absorbance are same as for Fig. 1.

Fig. 3. HPLC profile of urine sample obtained 7 h after administration of 200 mg of coumarin. Peak 1 = 7-hydroxycoumarin (identified from authentic standard run under identical conditions). Mobile phase, flow-rate, chart-rate, sensitivity and absorbance as for Figs. 1 and 2.

Two extraction procedures were tried. The first method, that of Shilling et al. [16], was found to be unsuitable due to losses of 7-hydroxycoumarin during the extraction procedure. The second method, that of Waller and Chasseaud [19], was used successfully. The enzyme β-glucuronidase was used to convert the 7-hydroxycoumarin glucuronide conjugate to free 7-hydroxycoumarin.

A study of the recovery rate of 7-hydroxycoumarin was carried out by spiking normal urine samples with various concentrations of 7-hydroyxcoumarin standard prior to following the extraction procedure outlined above. Quantitation was again made by measuring peak heights. Recovery rates were excellent (97–100%).

Fig. 2 shows the HPLC profile of a normal pretreatment urine sample. Fig. 3 shows the HPLC profile of a sample, taken 7 h after administration of 200 mg coumarin, indicating the presence of 7-hydroxycoumarin.

The main metabolite of coumarin in urine is 7-hydroxycoumarin, present mainly as the conjugate form, 7-hydroxycoumarin glucuronide. Very small amounts of the coumarin have been reported to be metabolised to and excreted as *o*-hydroxyphenylacetic acid [16]. However, our studies did not detect any of this metabolite.

Overall results showed that 63.4% of the total coumarin administered was recovered as 7-hydroxycoumarin. This is the mean value for seven different subjects. The major portion, 95%, was recovered within 10 h following administra-

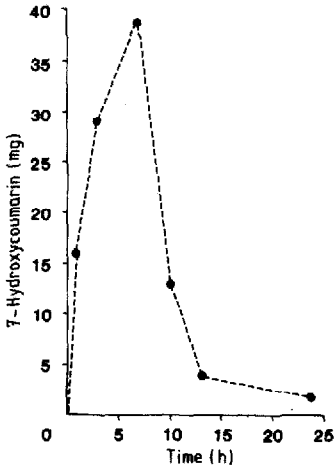

Fig. 4. Excretion of 7-hydroxycoumarin over a period of 24 h, following the ingestion of 200 mg coumarin. Each point on the graph represents mean value of seven different samples.

tion (Fig. 4). It was found that there was little variation in the metabolism of coumarin between the same subjects examined at different times, e.g. for one subject the percentage recovered as 7-hydroyxcoumarin for five different occasions was 56.6 ± 7.3 (mean $\pm$ S.D.).

The method outlined in this paper may be successfully applied to the study of the metabolism of coumarin in patients undergoing treatment.

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