Journal of Chromatography, 196 (1980) 501–505
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,901

Note

High-performance liquid chromatography of coumarin and its metabolites

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(Received April 21st, 1980)

Coumarin is a naturally occurring flavouring and fragrance material that has been used in food, tobacco, cosmetics and toiletries[1]. Histological evidence of hepatotoxicity was observed in the rat[2–4] and the dog[4], but not in the baboon[5,6] on chronic administration of coumarin in the diet. It has been reported to produce "bile duct carcinomas" in rats[3], although this diagnosis of the lesions observed has been questioned[1].

The metabolism of coumarin has been extensively studied in a variety of animal species and a number of metabolites identified[1], including 3-, 4-, 5-, 6-, 7- and 8-hydroxycoumarin, *o*-hydroxyphenylacetic acid, *o*-hydroxyphenylpropionic acid, *o*-hydroxyphenyllactic acid and *o*-coumaric acid (Fig. 1). The mechanism by which coumarin exerts its hepatotoxic effects is, however, unclear. It is still a matter of debate whether coumarin itself or one of its metabolites is responsible for the biological activity observed[1].

The present investigations were undertaken in order to develop a high-

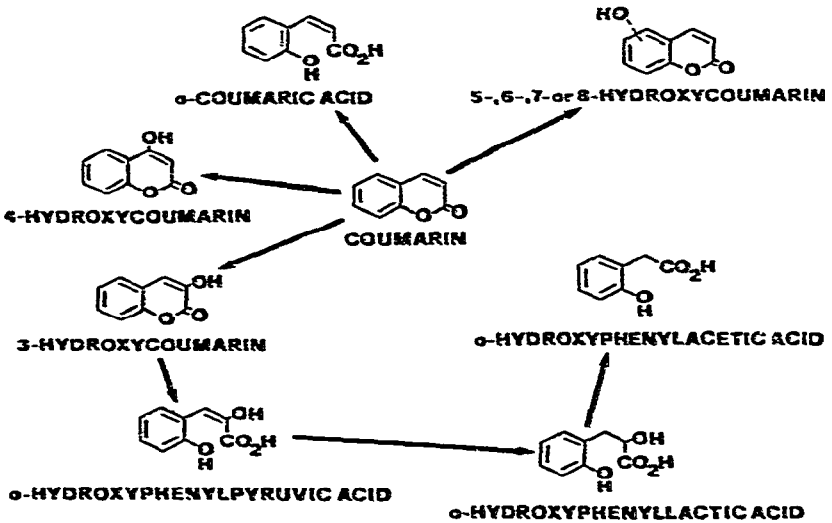

Fig. 1. Possible metabolic pathways of coumarin in mammals.

0021-9673/80/0000–0000/$02.25

performance liquid chromatographic (HPLC) procedure capable of resolving and quantitating the known metabolites of coumarin, which would be applicable to the determination of metabolites in biological samples and tissue incubates.

EXPERIMENTAL

Standards

[3-^{14}C]Coumarin (Radiochemical Centre, Amersham, Great Britain) was purified to > 99.5% radiochemical purity by thin-layer chromatography on silica gel G using benzene–chloroform (1:1) as solvent. Coumarin (Aldrich, Gillingham, Great Britain), 4- and 7-hydroxycoumarin, *o*-hydroxyphenylacetic acid (Sigma London, Poole, Great Britain) and *o*-coumaric acid (Koch-Light Labs., Colnbrook, Great Britain) were obtained commercially. 5-, 6- and 8-hydroxycoumarin were the generous gift of Dr. J. Caldwell, Department of Biochemical and Experimental Pharmacology, St. Mary's Hospital Medical School, London, Great Britain. 3-Hydroxycoumarin was prepared by the method of Erlenmayer and Stadlin[7].

Equipment

Reversed-phase chromatography was performed using an ACS LC750 high-performance liquid chromatograph (Applied Chromatography Systems, Luton, Great Britain) fitted with 25 cm × 4.6 mm I.D. columns. The eluent was monitored at 273 nm with a Cecil 2012 variable-wavelength detector. Radioactivity in the column eluent was determined by collecting fractions with an LKB 2112 Redirac fraction collector followed by scintillation counting[8]. For biological samples a precolumn of CO:PELL:ODS (5 cm × 4.6 mm I.D.) was fitted (Whatman Labsales, Maidstone, Great Britain).

Stationary phases and solvents

Prepacked columns containing Partisil-10 ODS, ODS-2 (Whatman), Li-Chrosorb 10 RP-8, 10 RP-2 and Hypersil 5 ODS (supplied by HPLC Technology, Wilmslow, Great Britain) were used. HPLC grade methanol and tetrahydrofuran (unstabilised) were obtained from Rathburn Chemicals, Walkerburn, Great Britain. Distilled water was stored in glass bottles. Prepared solvent mixtures were sparged with helium and maintained under a helium atmosphere while in use. A flow-rate of 2 ml/min was used for all experiments and the columns were maintained at 25 ± 0.5°C

RESULTS AND DISCUSSION

Initially a Partisil-10 ODS stationary phase was selected with mixtures of methanol and water as eluent. The separation of coumarin and *o*-hydroxyphenylacetic acid was examined as representative of the range of polarities expected amongst the metabolites. Difficulty was experienced in finding a system which would retain the acid without generating an inordinately long retention time for the parent compound. The inclusion of 0.1% acetic acid in the eluent improved the peak shape for *o*-hydroxyphenylacetic acid and allowed its retention time to be altered at will by varying the methanol concentration. Promising, but incomplete, separation was observed when a mixture of all the chromatographic standards available to us was

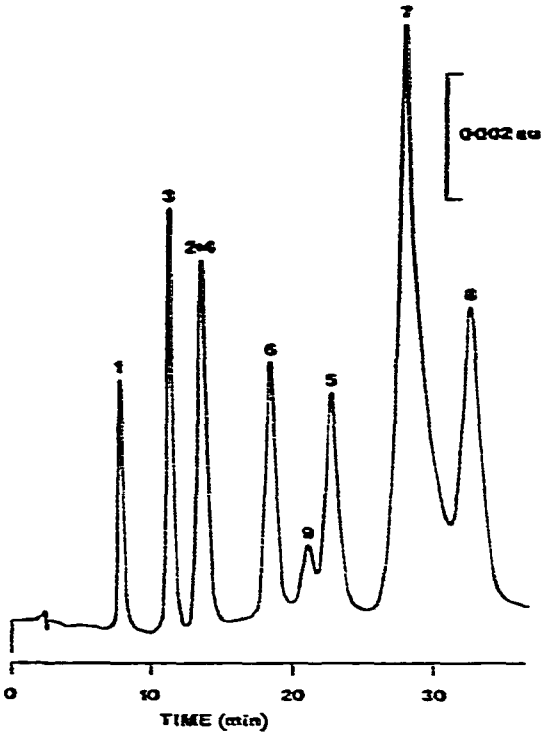

Fig. 2. Chromatogram obtained using a Partisil-10 ODS-2 column eluted isocratically with methanol–water–acetic acid (400:600:1). Injection (9.5 μl) of a test mixture containing 29 ng/μl *o*-hydroxyphenylacetic acid (1), 18 ng/μl 8-hydroxycoumarin (2), 10 ng/μl 6-hydroxycoumarin (3), 30 ng/μl 7-hydroxycoumarin (4), 16 ng/μl coumarin (5), 15 ng/μl 5-hydroxycoumarin (6), 54 ng/μl 3-hydroxycoumarin (7), 12 ng/μl 4-hydroxycoumarin (8) and 29 ng/μl *o*-coumaric acid (9).

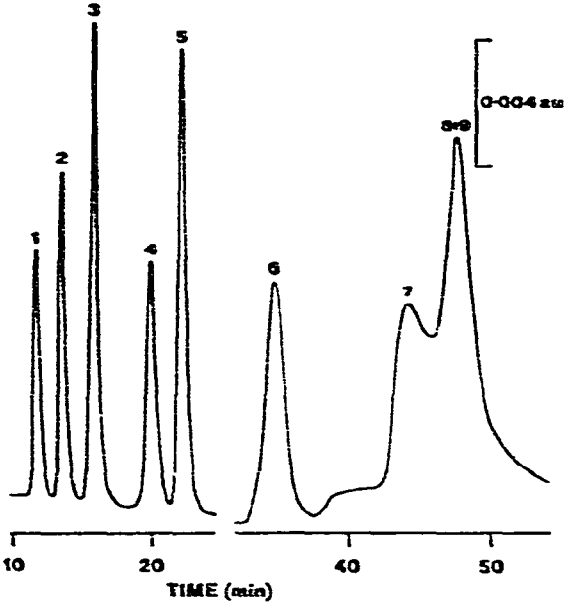

Fig. 3. Chromatogram obtained using a Partisil-10 ODS-2 column eluted isocratically with tetrahydrofuran–water–acetic acid (120:880:1). 50 μl injection of the same mixture as Fig. 1.

injected, with the exception of 7- and 8-hydroxycoumarin which were unresolved. Subsequent examination of the alternative stationary phases Partisil-10 ODS-2, LiChrosorb 10 RP-8, 10 RP-2 and Hypersil 5 ODS failed to resolve these metabolites using methanol–water–acetic acid mixtures as eluents. Fig. 2 shows a separation using methanol–water–acetic acid (400:600:1) as eluent and Partisil-10 ODS-2 as stationary phase. Peaks were identified in the mixture injected by increasing the quantity of each component present individually.

The use of tetrahydrofuran–water–acetic acid mixtures was found to yield the desired separation of 7- and 8-hydroxycoumarin on Partisil-10 ODS-2 columns. The only remaining problem was to elute all the metabolites from the column within a reasonable time without concomitant loss of resolution among the more polar compounds.

Fig. 3 shows the pattern of peaks obtained when an isocratic elution with tetrahydrofuran–water–acetic acid (120:880:1) was used while Fig. 4 shows the effect of introducing two linear gradient steps from 9.7% to 12.8% and then to 20% tetrahydrofuran. Clearly this last system provides a useable and convenient method as a basis for metabolic studies.

Biological application

The development of an adequate procedure for the preparation of samples originating from tissue incubations in a form suitable for direct injection into the

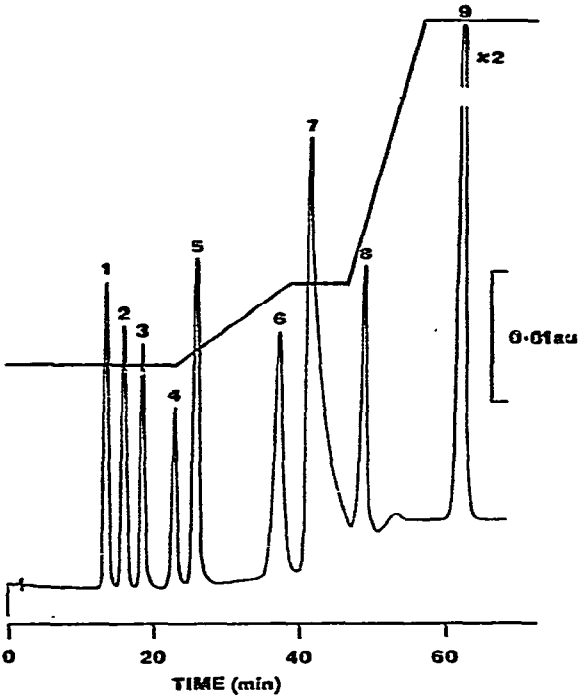

Fig. 4. Chromatogram obtained using a Partisil-10 ODS-2 column eluted with a two-stage linear gradient. Reservoir A contained tetrahydrofuran–water–acetic acid (10:900:1); reservoir B contained tetrahydrofuran–water–acetic acid (200:800:1). Elution with 46% B for 16 min followed by a linear increase of 1%/min in B up to 62% B (*i.e.*, over 16 min). Held for 8 min then 5%/min increase in B up to 100% B. 80-μl injection of the same substances as in Fig. 1 but at the following concentrations: 49 ng/μl (1), 11 ng/μl (2); 6 ng/μl (3); 19 ng/μl (4); 10 ng/μl (5); 28 ng/μl (6); 96 ng/μl (7); 8 ng/μl (8) and 18 ng/μl (9).

HPLC system proved more difficult than expected. Solvent extraction yielded incomplete recoveries of radioactivity when [3-^{14}C]coumarin was used, as would be anticipated from the widely varying polarity of the metabolites. The clear supernatant obtained from ultracentrifugation (average 158,000 *g* for 40 min) of incubation mixtures of [3-^{14}C]coumarin with washed rat liver microsomes to which an NADPH generating system had been added was found to contain > 95% of the added radioactivity and to be suitable for direct injection provided a precolumn was used. Fig. 5 shows the pattern of radioactivity observed from an incubation of rat liver microsomes. Essentially quantitative recoveries of injected radioactivity were obtained. The ultracentrifuged sample was spiked with the same mixture of standards as for Fig. 4. The labelled peaks corresponded to UV absorbing peaks attributable to the compounds indicated.

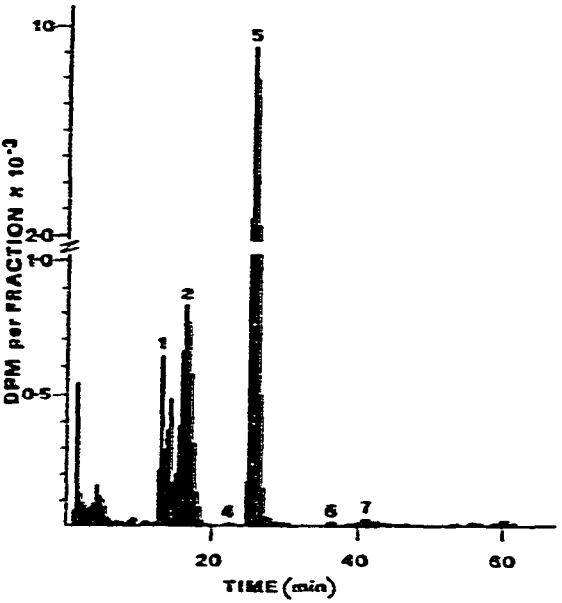

Fig. 5. Pattern of radioactive metabolites observed from incubations of [3-^{14}C]coumarin with rat liver microsomes. Incubations contained 1 m*M* coumarin (1.4 μCi/μmole), 1.5 m*M* NADP, 7.5 m*M* DL-isocitric acid, 0.5 units/ml isocitrate dehydrogenase, 2.5 mg/ml microsomal protein and 0.1 *M* phosphate buffer pH 7.6 in a total volume of 2 ml. The peaks identified correspond to the metabolites listed in the legend to Fig. 2. Chromatographic conditions as Fig. 4.

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