*Journal of Chromatography*, 362 (1986) 274–277
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 694

## Note

# Gas chromatographic–mass spectrometric analysis of monodemethylated metabolites of 6,7- and 7,8-dimethoxycoumarin isomers

KENJI YAMAMOTO*, SACHIKO KATO and HITOMI SHIMOMURA
*School of Pharmacy, Hokuriku University, Kanazawa 920-11 (Japan)*
(Received March 24th, 1986)

6,7-Dimethoxycoumarin is the main factor in the cholekinetic activity of *Artemisiae capillaris* Flos, which is the most representative crude drug for jaundice in Chinese medicine[1,2]. A study of the *in vivo* metabolism of 6,7-dimethoxycoumarin in rabbits by paper chromatographic analysis has shown that this compound is mainly monodemethylated to 6-hydroxy-7-methoxycoumarin (6-OH-7-$OCH_3$) and 7-hydroxy-6-methoxycoumarin (7-OH-6-$OCH_3$)[3]. In a previous paper[4] we described the gas chromatographic (GC) separation of monodemethylated derivatives of dimethoxycoumarin isomers, in their free forms, using a column packed with a phthalate–alkylene glycol polyester phase.

The present study was undertaken to develop a gas chromatography–mass spectrometry–selected-ion monitoring (GC–MS–SIM) procedure with deuterated internal standards capable of measuring accurately micro-amounts of the monodemethylated metabolites of 6,7-dimethoxycoumarin, which would be applicable to the determination of metabolites in tissue incubations. For purposes of comparison, the monodemethylated metabolites of 7,8-dimethoxycoumarin were also analyzed.

## EXPERIMENTAL

### *Standards*

6-OH-7-$OCH_3$, 7-OH-6-$OCH_3$, 7-hydroxy-8-methoxycoumarin (7-OH-8-$OCH_3$) and 8-hydroxy-7-methoxycoumarin (8-OH-7-$OCH_3$) were synthesized according to the described methods[5]. The deuterated analogues were similarly synthesized by methylation with [$^2H_6$]dimethyl sulphate (E. Merck, Darmstadt, F.R.G.).

### *GC–MS–SIM*

GC–MS–SIM was performed on a JEOL Model JMS DX-300 gas chromatograph–mass spectrometer system equipped with a data processing system. Chromatographic separation was performed on a 1 m × 2 mm I.D. glass column packed with 5% Thermon 3000 on Chromosorb W AW DMCS (80–100 mesh), supplied by Shimadzu (Kyoto, Japan). The column temperature was maintained at 250°C. The injector port, separator and transfer line were operated at 270°C. Helium was used as the carrier gas at a flow-rate of 40 ml/min. The electron-impact energy was set at 70 eV. The selected-ion monitor was focused on the molecular ions at $m/z$

0021-9673/86/$03.50 

192 for the monodemethylated derivatives of dimethoxycoumarin isomers and at *m/z* 195 for the corresponding deuterated internal standards.

*Sample preparation*

To 2.5 ml of an incubation mixture were added 750 ng each of deuterated 6-OH-7-$OCH_3$ and 7-OH-6-$OCH_3$ or 750 ng each of deuterated 7-OH-8-$OCH_3$ and 8-OH-7-$OCH_3$ dissolved in 20 $\mu$l of acetone, and the pH was adjusted to 2.0 with 10% hydrochloric acid. The mixture was extracted three times with 3 ml of diethyl ether by mechanical shaking for 5 min, followed by centrifugation at 1000 *g*. The organic phase was dried over anhydrous sodium sulphate and then evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 $\mu$l of acetone, and a 1.0-$\mu$l aliquot was subjected to GC–MS–SIM.

*Calibration graphs*

Calibration graphs for 6-OH-7-$OCH_3$ and 7-OH-6-$OCH_3$ were constructed by analyzing a series of 2.5-ml samples of the incubation mixtures (minus cofactors) to which 150–3000 ng of each of the unlabelled standards and 750 ng of each of the deuterated internal standards had been added. These standards were then subjected to the same extraction procedure as used for unknown samples.

Calibration graphs for 7-OH-8-$OCH_3$ and 8-OH-7-$OCH_3$ were constructed as described above.

## RESULTS AND DISCUSSION

Table I shows a comparison of the electron-impact mass spectral data for 6-OH-7-$OCH_3$, 7-OH-6-$OCH_3$, 7-OH-8-$OCH_3$ and 8-OH-7-$OCH_3$ with those for 6,7-dimethoxycoumarin[6]. The four monodemethylated metabolites of dimethoxycoumarin isomers present in incubation mixtures containing the supernatant obtained by centrifugation of rat liver at 9000 *g* were identified by GC–MS and comparison with authentic samples.

Figs. 1 and 2 show the GC–MS–SIM traces of 6-OH-7-$OCH_3$ and 7-OH-6-$OCH_3$ and of 7-OH-8-$OCH_3$ and 8-OH-7-$OCH_3$, respectively, and the corresponding deuterated internal standards. Using the described sample preparation, no interfering substances were present in the analyzed samples from tissue incubations. The detec-

TABLE I

ELECTRON-IMPACT MASS SPECTRAL DATA FOR MONODEMETHYLATED DERIVATIVES OF DIMETHOXYCOUMARIN ISOMERS

| *Compound* | *Relative intensity (%)* | | | | |
|---|---|---|---|---|---|
| | *m/z 192* $[M]^{+\cdot}$ | *177* $[M-CH_3]^+$ | *164* $[M-CO]^{+\cdot}$ | *149* $[M-CH_3-CO]^+$ | *121* $[M-CH_3-2CO]^+$ |
| 6-OH-7-$OCH_3$ | 100 | 11 | 35 | 48 | 11 |
| 7-OH-6-$OCH_3$ | 100 | 55 | 21 | 33 | 11 |
| 7-OH-8-$OCH_3$ | 100 | 21 | 16 | 19 | 14 |
| 8-OH-7-$OCH_3$ | 100 | 10 | 14 | 28 | 12 |


Fig. 1. Selected-ion monitoring of 6-OH-7-$OCH_3$ (1) and 7-OH-6-$OCH_3$ (2) and the deuterated internal standards.

Fig. 2. Selected-ion monitoring of 7-OH-8-$OCH_3$ (1) and 8-OH-7-$OCH_3$ (2) and the deuterated internal standards.

tion limits of these analyses were *ca.* 40 ng/ml of an incubation mixture, and the detection limits for the pure substances were *ca.* 2 ng per injection.

Calibration graphs for these four monodemethylated metabolites were constructed in the range of 60–1200 ng/ml of incubate as shown in Fig. 3. Least-squares analysis of the peak area ratio *versus* weight ratio of the unlabelled and labelled standards gave a linear relationship with a correlation coefficient of greater than 0.99 for each metabolite. The average recovery for each metabolite at a concentration of 300 ng/ml was found to be 96.2–99.9%, with a coefficient of variation of $\leqslant 5.1\%$ ($n = 4$).

The proposed method has been used successfully to monitor the *in vitro* metabolism of 6,7- and 7,8-dimethoxycoumarin isomers by the rat liver supernatant obtained by centrifugation at 9000 *g* (Table II). Metabolite levels in the incubation mixtures ranged from *ca.* 100 to *ca.* 1000 ng/ml, values which are within the dynamic range of the assay described.

From the data shown, the proposed method for the determination of mono-

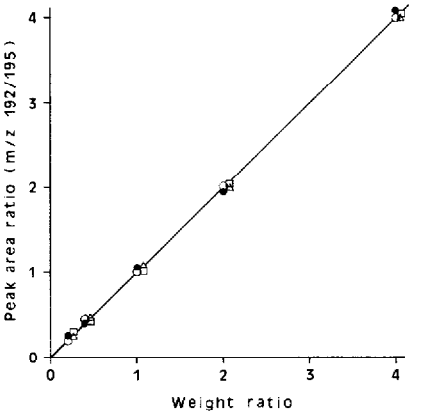


Fig. 3. Calibration graphs for 6-OH-7-$OCH_3$ (●), 7-OH-6-$OCH_3$ (○), 7-OH-8-$OCH_3$ (□) and 8-OH-7-$OCH_3$ (△). The amount of unlabelled standard was varied between 60 and 1200 ng/ml; the amount of deuterated internal standard was fixed at 300 ng/ml.

TABLE II

*IN VITRO* FORMATION OF MONODEMETHYLATED METABOLITES FROM DIMETHOXYCOUMARIN ISOMERS BY RAT LIVER SUPERNATANT

Incubation was carried out at 37°C for 20 min with 0.5 m*M* dimethoxycoumarin, 0.5 m*M* NADP, 5 m*M* glucose-6-phosphate, 5 m*M* magnesium chloride, the supernatant obtained by centrifugation of rat liver at 9000 *g* (1 ml/0.25 g of liver) and 0.1 *M* phosphate buffer (pH 7.4) in a total volume of 2.5 ml.

| *Substrate* | *Metabolite* | *(nmol/g liver · min)** |
|---|---|---|
| 6,7-Dimethoxycoumarin | 6-OH-7-$OCH_3$ | 2.62 ± 0.12 |
| | 7-OH-6-$OCH_3$ | 0.86 ± 0.01 |
| 7,8-Dimethoxycoumarin | 7-OH-8-$OCH_3$ | 1.37 ± 0.08 |
| | 8-OH-7-$OCH_3$ | 0.32 ± 0.01 |

* Values are the means ± S.E. for $n = 4$.

demethylated metabolites of dimethoxycoumarin isomers is specific, accurate, precise and easy to use. Since 7-ethoxycoumarin was introduced by Ullrich and Weber[7] as an interesting substrate for measuring mixed-function monooxygenase activity, the O-dealkylation activities of several 7-alkoxycoumarins have been measured to characterize the different species of cytochrome P-450[8,9]. Further studies are planned for the analysis of the regioselective monodemethylation of dimethoxycoumarin isomers by microsomal preparations.

REFERENCES

1 M. Aburada, H. Sasaki and M. Harada, *Yakugaku Zasshi*, 96 (1976) 147.
2 M. Kimura, *Proc. 10th Symposium on WAKAN-YAKU, Toyama, August 28-29, 1976*, Research Institute for WAKAN-YAKU, Toyama, 1977, pp. 121–126.
3 T. Furuya, *Chem. Pharm. Bull.*, 6 (1958) 696.
4 S. Kato and K. Yamamoto, *J. Chromatogr.*, 333 (1985) 175.
5 R. D. H. Murray, J. Méndez and S. A. Brown, *The Natural Coumarins*, Wiley, Chichester, 1982.
6 R. H. Shapiro and C. Djerassi, *J. Org. Chem.*, 30 (1965) 955.
7 V. Ullrich and P. Weber, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 1171.
8 T. Kamataki, M. Ando, Y. Yamazoe, K. Ishii and R. Kato, *Biochem. Pharmacol.*, 29 (1980) 1015.
9 M. Komori, Y. Imai and R. Sato, *J. Biochem (Tokyo)*, 95 (1984) 1379.