Journal of Chromatography, 487 (1989) 489–495
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4530

Note

Application of high-performance liquid chromatographic analysis of scoparone and its metabolites in the study of cytochrome P450 differentiation in vitro

FRANK N.A.M. VAN PELT

Institute of Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, P.O. Box 80 176, 3508 TD Utrecht (The Netherlands) and RCC Notox B V., Hambakenwetering 7, 5231 DD 'sHertogenbosch (The Netherlands)

and

WIM C. MENNES, INE G.A.M. HASSING and BAS J. BLAAUBOER*

Institute of Veterinary Pharmacology, Pharmacy and Toxicology, University of Utrecht, P.O Box 80 176, 3508 TD Utrecht (The Netherlands)

(First received May 3rd, 1988; revised manuscript received October 4th 1988)

Scoparone (6,7-dimethoxycoumarin) is readily metabolized by the cytochrome P450 monooxygenase system, predominantly into the two monomethoxy derivatives scopoletin (7-hydroxy-6-methoxycoumarin) and isoscopoletin (6-hydroxy-7-methoxycoumarin) [1]. This regioselective O-demethylation is performed by several isoenzymes of cytochrome P450 [1–4]. Induction of cytochrome P450 by either phenobarbital or polycyclic aromatic compounds results in an increase of the O-demethylase activity and an alteration of the ratio of scopoletin to isoscopoletin formed. Therefore, scoparone is a useful substrate to study the effects of chemicals and culture conditions on the cytochrome P450 enzyme system and to determine the activity of the different isoenzymes in cultured cells.

In most reports on the regioselective biotransformation of scoparone, fluorimetric and radiochemical methods are combined to quantify the formation of the two metabolites [1–4]. Other methods described are gas chromatographic–mass spectrometric analysis [5] and spectrophotometric determination [6].

Legrum et al. [7] mentioned the use of a high-performance liquid chromatographic (HPLC) method for studying scoparone metabolism that gave results comparable with those from the radiochemical studies. Their HPLC method con-

0378-4347/89/$03.50

sisted of a non-isocratic elution system with rather long analysis times. A modification of this method, using an isocratic mobile phase, was presented by Müller-Enoch and Greischel [8].

We have modified the HPLC analysis to be suitable for simultaneous quantification of scoparone, scopoletin and isoscopoletin in biological samples. The method can easily be applied to the measurement of these compounds in cell cultures. The recovery is more than 85% and limits of determination of the compounds are ca. $5 \cdot 10^{-7}$ *M*.

EXPERIMENTAL

Chemicals

Scoparone and scopoletin were purchased from Aldrich (Brussels, Belgium). Isoscopoletin was obtained from C. Roth (Karlsruhe, F.R.G.). β-Glucuronidase/arylsulphatase from *Helix pomatia* was supplied by Boehringer (Mannheim, F.R.G.). All other chemicals were of reagent-grade purity. Scoparone was purified on a 650 mm × 22 mm I.D. column packed with Polygosil 60-4063 (silica gel 60, particle size 40–63 μm, Macherey-Nagel, Düren, F.R.G.) and eluted with *tert.*-butanol–1-pentanol–25% ammonium hydroxide (5:4:1, v/v). The effluent was acidified and extracted twice with diethyl ether. The ether fractions were combined and evaporated to dryness under reduced pressure. The purified scoparone showed no detectable amounts of its metabolites.

Incubation of cells

Rat and hamster hepatocytes were isolated as described previously [9].

Shaking cultures. Hepatocytes isolated from both untreated and phenobarbital-pretreated rats were resuspended in RPMI 1640 giving a cell density of $4 \cdot 10^6$ cells per ml. The hepatocytes were incubated under an atmosphere of oxygen–carbon dioxide (95:5) in a shaking water-bath at 37 °C. After preincubation for 10 min, scoparone was added in a final concentration of 10^{-5} *M*, and the cells were incubated for 2 h. The medium and cells were separated by centrifugation (3 min, 100 *g*). β-Glucuronidase/arylsulphatase (EC 3.2.1.31/EC 3.1.6.1; 2000 Fishman units/16 000 Roy units) dissolved in 0.5 ml of sodium acetate buffer (1 *M*, pH 4.9) was added to 1 ml of supernatant. The samples were hydrolysed for 65 h, saturated with sodium chloride and extracted twice with 1.5 ml of diethyl ether. The ether fractions were combined and evaporated to dryness, and the residue was dissolved in 1 ml of 0.05 *M* ammonium acetate buffer (pH 4.25)–acetonitrile (88:12, v/v).

Primary cultures. Primary cultures were incubated in a 9-cm culture dish (Sterilin, Feltham, U.K.) at a density of $6 \cdot 10^6$ cells per culture in 5 ml of Waymouth MB 752/1 supplemented with 3% newborn calf serum, 10^{-6} *M* insulin and 10^{-5} *M* hydrocortisone. To the hamster cultures 4 m*M* calcium chloride and 4 m*M* magnesium chloride were added. Where appropriate, the medium was changed after 24 h for the same medium. After preincubation for 4, 24 or 48 h under an atmosphere of air–carbon dioxide (95:5) at 37 °C, the cell cultures were incubated with medium containing $5 \cdot 10^{-5}$ *M* scoparone for 1 h. β-Glucuronidase/arylsul-

phatase (8000 Fishman units/64 000 Roy units) dissolved in 2.0 ml of sodium acetate buffer (1 *M*, pH 4.9) was added to 4.0 ml of the culture medium. The samples were hydrolysed for 65 h, saturated with sodium chloride and extracted twice with 4.0 ml of diethyl ether. The ether fractions were combined and evaporated to dryness, and the residue was dissolved in 1 ml of 0.05 *M* ammonium acetate buffer (pH 4.25)–acetonitrile (88:12, v/v).

Biochemical determinations

The cells were harvested in 2.5 ml of 0.1 *M* phosphate buffer (pH 7.4) containing 20% glycerol, 1 m*M* EDTA, 1 m*M* dithiothreitol and 0.2% Emulgen 913. After homogenization, the CO-difference spectrum was measured according to Rutten et al. [10]. DNA analyses were performed in 10-μl samples of the homogenates, after Hukkelhoven et al. [11], using a buffer consisting of 10 m*M* Tris, 10 m*M* EDTA and 100 m*M* sodium chloride (pH 7.0).

HPLC analysis

Analyses were carried out using an LKB 2150 HPLC pump equipped with a Rheodyne 7125 injection valve (20-μl loop) and a reversed-phase column (Chromsep C_{18}, 100 mm × 3 mm I.D., particle size 5 μm, Chrompack, Middelburg, The Netherlands) preceded by a C_{18} guard column (Chromsep C_{18}, Chrompack). In all analyses the mobile phase was 0.05 *M* ammonium acetate buffer (pH 4.25)–acetonitrile (88:12, v/v) at a flow-rate of 0.5 ml/min. Solvents were saturated with helium prior to use. Detection of scoparone and its metabolites was performed on an LKB 2151 variable-wavelength monitor at 340 nm. The amounts of the eluted compounds were calculated using a Shimadzu C-R1A Chromatopac integrator (Kyoto, Japan).

RESULTS AND DISCUSSION

A good separation and retention of scoparone and its metabolites were obtained using the described method with little interference from culture medium compounds after extraction (Fig. 1). In blank medium containing β-glucuronidase/arylsulphatase, a peak was found that coincided with scopoletin. Since this peak was not present in medium extracts from blanks without this enzyme mixture, we concluded that it can be ascribed to a compound in this mixture. The area found for scopoletin must therefore be corrected for this phenomenon. Retention times of isoscopoletin, scopoletin and scoparone were 5.4, 6.4 and 14.3 min, respectively.

Calibration curves for all three compounds were constructed in the range $5 \cdot 10^{-7}$–$5 \cdot 10^{-4}$ *M*. The least-squares analysis of the peak area versus concentration showed linear relationships with correlation coefficients greater than 0.99.

Saturation of the culture medium with sodium chloride prior to extraction resulted in an increase in the recovery varying from 13% for scoparone up to 57% for scopoletin (Table I). The recovery was independent of the concentration of the standards in the range $5 \cdot 10^{-7}$–$5 \cdot 10^{-4}$ *M*.

The HPLC method described by Legrum et al. [7] employed a 30 cm long C_{18}

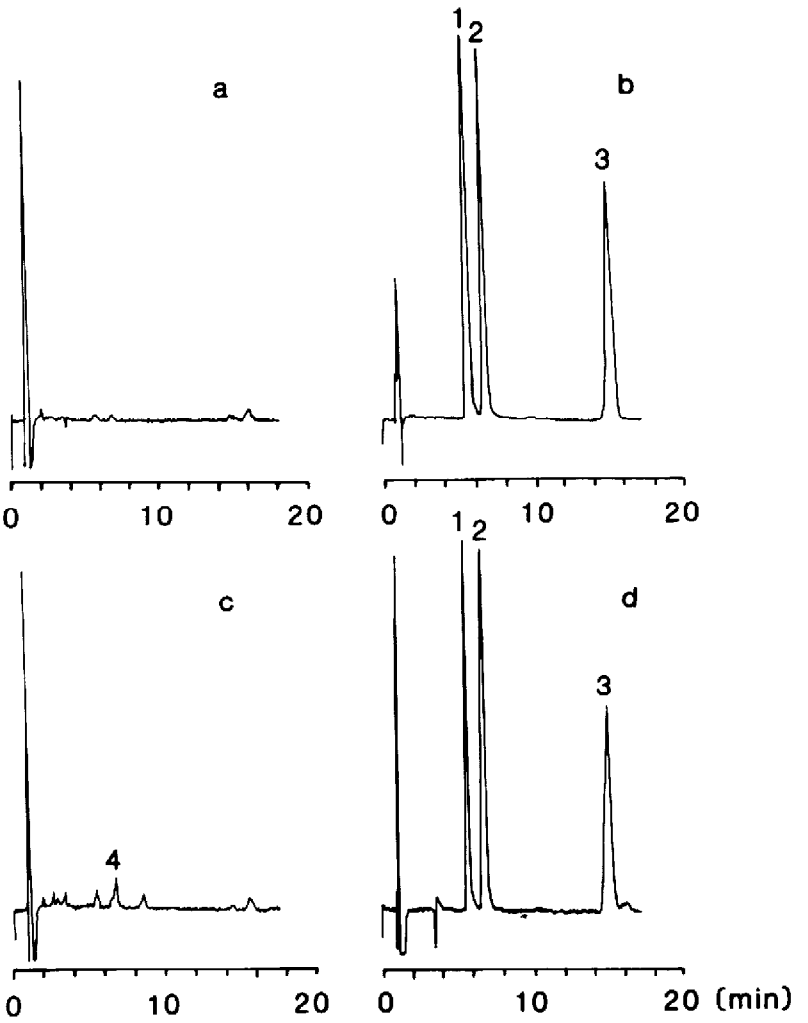

Fig. 1. Chromatograms of (a) an extract of blank culture medium, (b) a mixture of standards, (c) an extract of blank culture medium containing β-glucuronidase/arylsulphatase and (d) an extract of a mixture of standards in culture medium. Peaks: 1 = isoscopoletin; 2 = scopoletin; 3 = scoparone; 4 = interfering peak from the β-glucuronidase–arylsulphatase mixture. The analyses were performed isocratically with 0.05 *M* ammonium acetate buffer (pH 4.25)–acetonitrile (88:12, v/v) at a flow-rate of 0.5 ml/min.

column, with a flow-rate of 2.0 ml/min and a change in solvent system after 4 min of analysis. This resulted in retention times of 11, 13 and 21 min for isoscopoletin, scopoletin and scoparone, respectively. The compounds were recovered after acid hydrolysis by repeated extraction with diethyl ether. The detection was performed at a wavelength of 313 nm. As shown in Fig. 2, the maxima of absorbance for all three standards are at 340 nm. Müller-Enoch and Greischel [8] described the use of an isocratic mobile phase on an RP-18 column, resulting in shorter analysis times. After incubation of microsomes, the sample preparation consisted of deproteinization by addition of TCA and centrifugation. When this method of sample preparation is applied to culture media of hepatocytes incu-

TABLE I

RECOVERY OF SCOPARONE AND METABOLITES FROM CULTURE MEDIUM BY EXTRACTION WITH DIETHYL ETHER

Standards were extracted from acidified culture medium by diethyl ether with or without saturation with sodium chloride. Values are mean ± S.D (n = 12).

Compound	Recovery (%)	
	Without sodium chloride	With sodium chloride
Scoparone	85±6	98±2
Scopoletin	31±2	88±2
Isoscopoletin	76±3	96±2

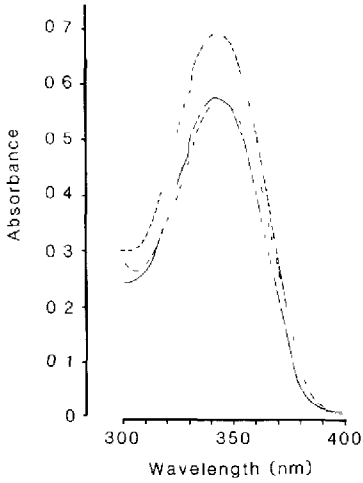

Fig. 2. Absorbance spectra of $5 \cdot 10^{-5}$ M (——) scoparone, (- - -) scopoletin and (—·—·) isoscopoletin in 0.05 M ammonium acetate buffer (pH 4.25)–acetonitrile (88·12, v/v).

bated with scoparone, a large, tailing injection peak with numerous small rider peaks was observed for the first 10 min following the injection. This makes an adequate quantification of scoparone metabolites impossible. Legrum et al. [7] extracted samples with diethyl ether. In this report we show that the addition of sodium chloride prior to extraction results in a much higher recovery (Table I) making the limits of determination as low as $5 \cdot 10^{-7}$ M for scoparone and its two major metabolites.

The biotransformation of scoparone was studied using hepatocytes isolated from both untreated and phenobarbital-induced male Wistar rats. Pretreatment with phenobarbital resulted in an alteration of the metabolism as shown in Fig. 3. From these data the ratio of scopoletin to isoscopoletin can be calculated. In hepatocytes derived from control rats the ratio was 1:3.2, whereas the ratio in liver cells isolated from phenobarbital-pretreated rats was 1:1.1.

The effect of preincubation time on the metabolism of scoparone was studied in primary cultures of both rat and hamster hepatocytes. Hamster hepatocytes appeared to be about eight times more active in metabolizing scoparone (Table

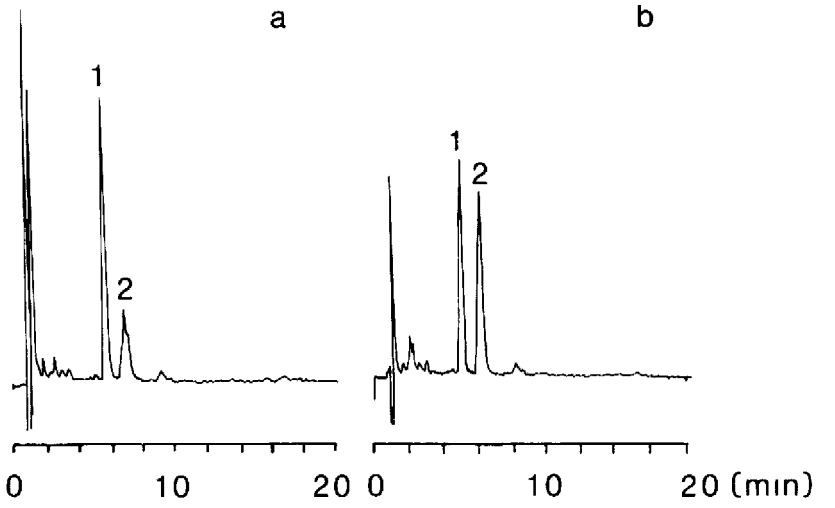

Fig. 3. Chromatograms of medium extracts from hepatocyte cultures derived from (a) untreated and (b) phenobarbital-pretreated rats. Hepatocytes were incubated for 2 h with 10^{-5} *M* scoparone. Peaks: 1 = isoscopoletin; 2 = scopoletin. The analyses were performed isocratically with 0.05 *M* ammonium acetate buffer (pH 4.25)–acetonitrile (88:12, v/v) at a flow-rate of 0.5 ml/min.

TABLE II

EFFECTS OF PREINCUBATION TIME ON THE METABOLISM OF SCOPARONE BY PRIMARY CULTURES OF RAT AND HAMSTER HEPATOCYTES

Primary cultures were incubated with $5 \cdot 10^{-5}$ *M* scoparone for 1 h. Values are mean ± S.D. for three incubations.

Preincubation time (h)	Total product formation (nmol/μg DNA/h)	Ratio[a] (*X*)	Total P450 (nmol/μg DNA)
Hamster hepatocytes			
4	1.21 ± 0.10	1.34 ± 0.02	13.60 ± 1.60
24	1.06 ± 0.10	0.77 ± 0.02	9.63 ± 0.60
48	0.32 ± 0.01	0.56 ± 0.04	3.65 ± 0.17
Rat hepatocytes			
4	0.16 ± 0.01	2.53 ± 0.07	20.64 ± 0 77
24	0.04 ± 0.01	2.12 ± 0.10	7.30 ± 0.67
48	0.03 ± 0.01	2.01 ± 0.01	3.58 ± 0.56

[a]Ratio of isoscopoletin to scopoletin = *X*:1.

II), although the total cytochrome P450 content was approximately the same in both species. The metabolic activity in the cultures was observed to decrease as the preincubation time increased. This decline was proportional to the drop in total cytochrome P450 content in the cells. In hamster hepatocytes the ratio of the two metabolites was observed to depend on the preincubation time. In contrast, rat hepatocytes did not show such a shift. Apparently, in rat hepatocytes

the decrease in activity of the isoenzymes of cytochrome P450 involved in the metabolism of scoparone is proportional to that of the total cytochrome P450 content. The shift in the ratio of the metabolites of scoparone in hamster hepatocyte cultures indicates that the isoenzyme pattern changes with the time of preincubation.

Most studies on the metabolism of scoparone in vitro used rat liver microsomes [1–4,7]. We showed that differences in the regioselective metabolism of scoparone can be detected simultaneously in the medium of cell cultures using this simple method. Therefore, scoparone may also be a useful substrate to study the effects of chemicals and culture conditions on cytochrome P450 enzyme activity and its isoenzyme pattern in cultured cells.

ACKNOWLEDGEMENTS

The receipt of financial support from the Dutch Ministry of Education and Sciences is gratefully acknowledged. We thank Professor J. Noordhoek for valuable discussions, Miss I. van Holsteijn for technical assistance and Mr. W. Geurts van Kessel for his technical advice.

REFERENCES

1 D. Muller-Enoch, H. Thomas and H Ockenfels, Z. Naturforsch., 34C (1979) 481.
2 D Müller-Enoch, N. Sato and H. Thomas, Hoppe-Seyler's Z. Physiol. Chem., 362 (1981) 1091.
3 D. Muller-Enoch, E. Büttgen and A. Nonnenmacher, Z. Naturforsch., 40C (1985) 682.
4 D. Müller-Enoch, G.A. Dannan, B. Rudolf and H.P. Friedt, Drug Res., 35 (1985) 698.
5 K. Yamamoto, S. Kato and H Shimomura, J. Chromatogr., 362 (1986) 274.
6 E. Celon, A. Cuiotto and P. Rodighiero, Analyst, 103 (1978) 1116.
7 W. Legrum, L. Kling and E. Funke, J. Pharmacol. Exp. Ther., 228 (1984) 769.
8 D. Müller-Enoch and A. Greischel, in A. Guillouzo (Editor), Liver Cells and Drugs, Vol 166, Colloque INSERM/John Libbey Eurotext, London, 1988, p. 85.
9 B J. Blaauboer and C.W.M. van Holsteijn, Xenobiotica, 13 (1983) 295.
10 A.A.J.J.L. Rutten, H E. Falke, J F. Catsburg, R. Topp, B.J. Blaauboer, I. van Holsteijn, L. Doorn and F.X.R. van Leeuwen, Arch. Toxicol., 61 (1987) 27.
11 M.W.A.C. Hukkelhoven, E. Vromans, A J.M. Vermorken and H. Bloemendal, Anticancer Res., 1 (1981) 341.