Journal of Chromatography, 448 (1988) 127–133
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 590

CHROMATOGRAPHIC AND CYTOGENETIC ANALYSIS OF *IN VIVO* METABOLITES OF FLUORANTHENE

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(Received April 22nd, 1988)

SUMMARY

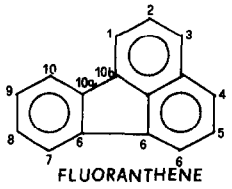
Fluoranthene metabolites in rat serum were analysed by high-performance liquid chromatography (HPLC) with UV and fluorescence detection and compared with *in vitro* metabolites obtained by incubation with microsomal fraction of rat hepatocytes. In order to resolve very polar fluorescent compounds present in rat serum, a modification of HPLC existing methods for *in vitro* metabolites separation was necessary. Mutagenic 2,3-dihydrodiol was identified in both *in vitro* sample and rat serum: this result is in good accord with cytogenetic analysis on rats bone marrow cells, that shows a slight but significant increase of sister chromatide exchanges.

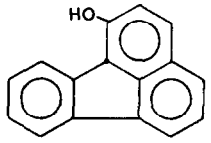
INTRODUCTION

Fluoranthene (Ft) is a widespread polycyclic aromatic hydrocarbon which shows a high mutagenic activity after metabolic activation in *Salmonella typhimurium*[1] and in human lymphoblasts[2]. It is inactive in rodent bioassays for cancer[3], but has recently been shown to be tumorigenic in a newborn mouse lung adenoma bioassay[4].

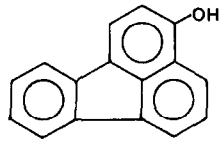
In vitro metabolites of Ft activated by the microsomal fraction of Aroclor-treated rat hepatocytes (S9) have been separated by high-performance liquid chromatography (HPLC) and identified by spectroscopic techniques by LaVoie *et al.*[5] and Babson *et al.*[6] as 1-hydroxy-Ft, 3-hydroxy-Ft, 8-hydroxy-Ft and 2,3-dihydroxy-2,3-dihydro-Ft. 7-Hydroxy-Ft[5] and Ft-2,3-quinone[6] have also been found, but Ft-7,8-diol and Ft-1,10b-diol, which are possible oxidation products[7], have not been

0021-9673/88/$03.50

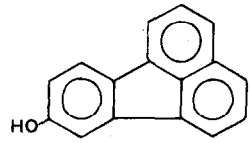




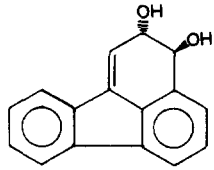
1-HYDROXYFLUORANTHENE



3-HYDROXYFLUORANTHENE



8-HYDROXYFLUORANTHENE



2,3-DIHYDROXY-2,3-DIHYDROFLUORANTHENE

detected[5,6]. Ft-2,3-diol yields the direct-acting mutagen 2,3-dihydroxy-1,10b-epoxy-1,2,3-trihydro-Ft, which is able to react with DNA, forming an N^2-deoxyguanosine adduct[8].

In a previous study[9], it was observed that Ft induces, after metabolic activation with S9 from rats, sister chromatide exchanges (SCE) in Chinese hamster ovary cells grown *in vitro*, whereas no mutagenic activity was observed in bone marrow cells of Ft-treated mice. HPLC analysis of the mice serum showed that Ft metabolism was very slow; in fact, even 24 h after administration Ft was still present as a large peak with a very low formation of polar metabolites, among which isomeric phenols were identified. Therefore, an *in vivo* study of Ft metabolism in rats was performed in order to investigate more exactly the correlation between the *in vivo* and *in vitro* Ft activation in two comparable enzymatic systems.

The aim of this work was the HPLC separation of Ft metabolites in the serum of *in vivo*-treated rats, in order to compare the chromatographic profiles of such *in vivo* samples with those obtained *in vitro* using S9 derived from rats as the metabolic-activating system. *In vivo* chromatographic data were also compared with cytogenetic assays on bone marrow cells of rats.

EXPERIMENTAL

Chromatography

Sample preparation. For *in vitro* metabolic activation, Ft (Merck, Darmstadt, F.R.G.) was dissolved in dimethyl sulphoxide (DMSO) and incubated for 3 h with rat liver homogenate prepared from the livers of young male Sprague-Dawley/CD rats, whose hepatic enzymes were induced with Aroclor (S9). The S9 mixture was prepared according to Natarajan *et al.*[10]. The concentration of Ft was 36 μg/ml.

For the *in vivo* test, male Fisher 344 rats, weighing 200–220 g, were treated

orally with 1 g/kg body weight of Ft dissolved in corn oil, 3, 6 and 24 h after treatment, samples of blood were taken from the aorta of the rats and centrifuged at 150 *g* for 10 min. A 24-h control sample was obtained utilizing a rat that was given only corn oil. About 2 ml of serum were obtained from each rat.

Every experiment was repeated three times.

In vivo and *in vitro* samples were extracted five times with a 5-fold excess volume of ethyl acetate, dried with anhydrous sodium sulphate and evaporated to dryness. The initial volumes were replaced with methanol.

Chemicals were purchased from Carlo Erba (Milan, Italy). The solvents, except DMSO, were of HPLC grade.

Apparatus. A Perkin-Elmer apparatus was used with a Series 4 pumping system, an LC 75 variable-wavelength detector with Autocontrol, a Perkin-Elmer 650-10 S spectrofluorimetric detector and a Rheodyne 7105 valve as injector. A Vydac C_{18} (5 μm) 201 HS 104 prepacked column (25 cm × 4.6 mm I.D.) and a 5-cm precolumn packed with Supelco C_{18} pellicular packing were used. The mobile phase flow-rate was 1 ml/min. The gradient programme is given in Table I.

Cytogenetic assay

In order to perform cytogenetic analysis of SCEs on differently stained chromatids in bone marrow cells, two agar-coated 5-bromodeoxyuridine tablets (50 mg) (Fluka, Buchs, Switzerland) were implanted subcutaneously in rats. Two hours after implantation the rats were treated by gavage with Ft; 22 h later they received an intraperitoneal injection of colchicine (Merck) (3 μg/g body weight) and, after 2 h, their blood was taken from the aorta for analysis by HPLC, as described before (24-h sample). Simultaneously air-dried preparations of bone marrow cells were made according to the technique of Ford and Hamerton[11] and staining was performed according to the method of Perry and Wolff[12]. For each rat 40 well spread metaphases were analysed for SCEs.

RESULTS AND DISCUSSION

To achieve the HPLC separation of *in vivo* rat serum samples, a specific chromatographic system had to be set up. This system represents an improvement over the method of Babson *et al.*[6] for the separation of S9 *in vitro* activated Ft. In fact

TABLE I

GRADIENT PROGRAMME USED IN HPLC

Step	*Acetonitrile (vol.-%)*	*Water (vol.-%)*	*Time (min)*	*Gradient shape*
1	10	90	0	
2	30	70	15	Linear
3	30	70	5	
4	70	30	20	Linear
5	100	0	5	Linear
6	100	0	15	

the elution programme of Babson *et al.*[6] could not resolve the polar metabolites formed in the direct *in vivo* activation, whereas the present gradient profile, starting from a low (10%) proportion of acetonitrile in water and slowly reaching the final 100% concentration of organic modifier shows a good selectivity for these compounds, without altering the resolution of the more retained compounds.

Fig. 1 shows the chromatographic separation of ethyl acetate extracts of *in*

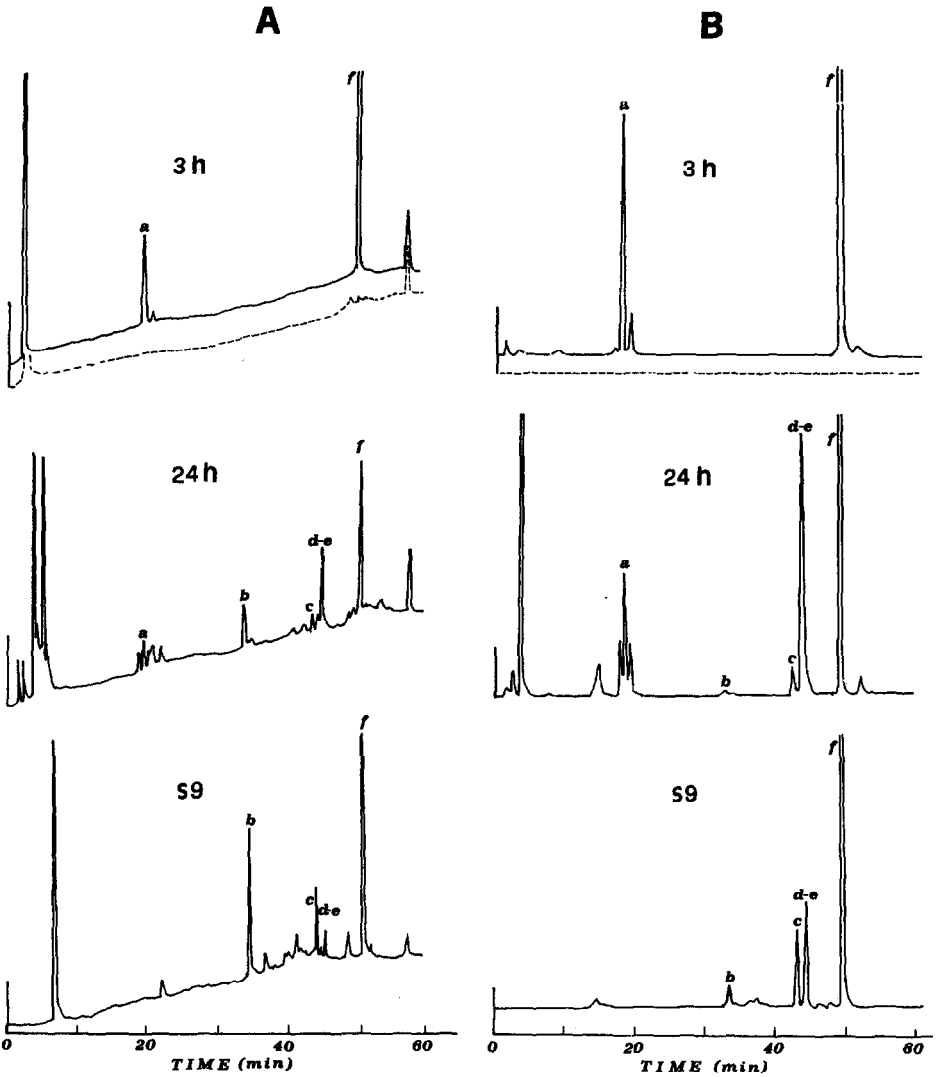


Fig. 1. Chromatographic profiles of *in vitro* S9 activated Ft and *in vivo* rat serum samples taken 3 and 24 h after Ft administration obtained with (A) UV (at 254 nm) and (B) fluorescence (at $\lambda_{ex.}$ 305 nm, $\lambda_{em.}$ 460 nm) detection. The control sample chromatogram is shown by the broken line. Column, Vydac C_{18} (5 μm) (25 cm × 4.6 mm I.D.); mobile phase, acetonitrile–water, gradient as described in Table I; flow-rate, 1 ml/min; temperature, ambient. Peaks: a = unidentified compound; b = 2,3-dihydroxy-2,3-dihydro-Ft; c = 8-hydroxy-Ft; d = 1-hydroxy-Ft; e = 3-hydroxy-Ft; f = Ft.

vitro S9 activated Ft and *in vivo* rat serum. Rat serum samples were taken 3 and 24 h after Ft administration. The chromatogram of the 6-h sample is similar to that of the 3-h sample, apart from an increase in the amount of polar metabolites.

The "*in vivo*" 24-h sample elution pattern is similar to the *in vitro* pattern. In fact, there are peaks with the same retention times of 2,3-diol and phenolic metabolites, which have been isolated and identified in the *in vitro* sample by mass, UV and fluorescence spectrometry (as described elsewhere)[9].

In order to identify these compounds in rat serum, their on-line scanned UV and fluorescence spectra were recorded, stopping the eluent flow during chromatography. The 2,3-diol spectra obtained for the *in vivo* and *in vitro* samples are shown in Fig. 2; in the same way the fluorescence spectrum allowed the identification of 8-hydroxy-Ft (maximum emission wavelength, $\lambda_{em.}$ = 385 nm at excitation wavelength, $\lambda_{ex.}$ = 305 nm).

Unfortunately, 1-hydroxy- and 3-hydroxy-Ft are not resolved with the new gradient program, so the peak corresponding to these substances was analysed as follows. Fluorescence emission spectra of the head and the tail of the peak were scanned at $\lambda_{ex.}$ = 305 nm, giving respectively maxima at $\lambda_{em.}$ = 435 nm (head), characteristics of 1-hydroxy-Ft, and at $\lambda_{em.}$ = 477 nm (tail), characteristic of 3-hydroxy-Ft.

Further, the peaks corresponding to phenolic isomeric metabolites were collected in a single fraction and submitted to mass spectrometric analysis, giving the molecular peak at *m/z* 218, in accordance with the literature and data obtained for the *in vitro* metabolites.

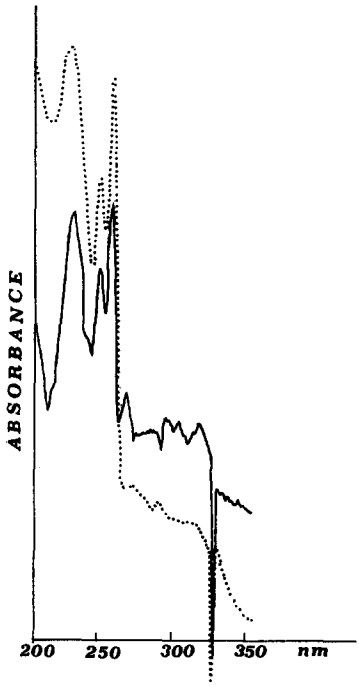


Fig. 2. UV–visibile spectra of 2,3-dihydroxy-2,3-dihydro-Ft in *in vivo* (solid line) and *in vitro* (broken line) samples.

However, the 24-h *in vivo* sample and the S9 *in vitro* activated fluoranthene chromatograms show some differences, particularly the presence of very polar, highly fluorescent compounds in the rat serum sample, whereas no fluorescent peak is present in the initial part of the S9 sample chromatogram. In fact, in the 24-h serum chromatogram it is possible to observe a group of three peaks, including a major compound with a retention time of 19 min 40s, which is indicated in Fig. 1 as peak a, and a group of very polar peaks, eluting near the solvent front, probably due to the decomposition of compound a.

Comparing the 3- and 24-h *in vivo* samples, there is evidence that compound a is formed very early. In three series of *in vivo* samples which were examined, it is almost the only metabolite present at the 3 and 6 h, whereas it is absent from the control sample.

In different serum samples taken 24 h after Ft treatment, compound a and the solvent front eluting peaks both occur, but both are present in varying amounts. The UV and fluorescence spectra of peak a were scanned on-line, giving the following results: fluorescence spectrum, $\lambda_{em.} = 465$ nm at $\lambda_{ex.} = 305$ nm; UV spectrum (0.04 a.u.f.s. range), $\lambda_{max.}$ 214 ($a=0.022$), 238 ($a=0.022$), 282 ($a=0.01$), 290 ($a=0.014$), 350 ($a=0.004$), 365 ($a=0.004$) and $\lambda_{min.}$ 225 ($a=0.016$), 270 ($a=0.008$), 300 ($a=0.0036$).

Up to now, attempts to isolate this compound by HPLC in order to analyse it by fast atom bombardment mass spectrometry have been unsuccessful, because of both the small amount of the sample and the high decomposition rate of the substance under examination.

Isomeric phenolic derivatives of Ft are also present in varying amounts, but 2,3-diol peak is approximately constant. At the same time, cytogenetic analysis showed that fluoranthene induces a slight but significant increase of SCEs in bone marrow cells of 24-h treated rats: $\bar{x}$(SCEs/cell) = 4.5 ± 1.8 in treated rats *versus* $\bar{x}$(SCEs/cell) = 2.6 ± 1.4 in the control.

CONCLUSION

Chromatographic analysis of *in vivo* and *in vitro* metabolites of suspected mutagenic compounds is a necessary approach to the investigation of their effective activation behaviour. With fluoranthene, fluorescence detection was particularly useful for the selective identification of metabolites. The HPLC system showed the presence in the *in vivo* samples of some metabolic products that have not been detected previously in *in vitro* samples. The fact that these compounds are present even a few hours after fluoranthene administration to the rats seems to exclude the possibility that they may be detoxification products of other metabolites. Apart from this, it is possible that the *in vitro* mutagenic 2,3-diol metabolite is formed in rat blood even after a long time. The presence of the 2,3-diol is in accord with the results of a cytogenetic study on bone marrow cells of the same animals, showing a slight increase in SCEs. In a previous study[9] it was shown that fluoranthene was able to induce SCEs in Chinese hamster ovary cells in an *in vitro* cytogenetic test in the presence of S9 mixture from rats. This is consistent with the formation of the active metabolite fluoranthene-2,3-diol. The data from this study show that the direct *in vivo* activation of fluorantene leads to a smaller mutagenic effect than that observed *in vitro*. The

presence of the 2,3-diol in *in vivo* serum samples can explain the small mutagenic effect found *in vivo*.

ACKNOWLEDGEMENTS

The Mass Spectrometry Service of the Area della Ricerca di Roma CNR is acknowledged for measuring the mass spectra. This investigation was supported by Progetti Strategici del CNR "Chimica dei Processi Biologici" e "Mutagenesi".

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