Journal of Chromatography, 276 (1983) 189—196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1729

Note

Rapid high-performance liquid chromatographic method for detection of interindividual differences in carcinogen metabolism

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(First received December 1st, 1982; revised manuscript received March 24th, 1983)

Interindividual variation in metabolism of carcinogens [mostly polycyclic aromatic hydrocarbons (PAH)] has been studied using various human tissues and cells [1—9]. The observed variation seems to be primarily under genetic control [10]. Differences in metabolic capacity to activate environmental carcinogens may result in differences in susceptibility to these carcinogens. However, all the studies indicated above have used biopsy material that is not convenient for screening of populations because of practical reasons (e.g. with bronchus) or because of considerable different metabolic capacities compared to tissues susceptible to chemical carcinogenesis.

Human hair follicles have been suggested as a convenient biopsy tissue for screening individual differences in carcinogen metabolism since: (1) They are of epithelial origin, which is important in view of the fact that 90% of human cancers arise in epithelial cells (i.e. are carcinomas). (2) They are available from a large number of volunteers without any risk. (3) They have been shown able to activate and metabolise benzo[*a*]pyrene (BP), a widely distributed carcinogen in our environment and a possible health hazard to humans [11]. (4) Metabolism of BP to dihydrodiol derivatives, the direct precursors of the suspected carcinogens, the diol-epoxides, has been shown to be genetically determined for a large part in hair follicles [12]. (5) Human hair follicle keratinocytes can be brought in culture [13], which enables the study of the effect of inducers and inhibitors of carcinogen-metabolising enzymes on the metabolite pattern of carcinogens [14]. (6) The response of BP metabolism in cultured hair follicle keratinocytes towards pre-exposure to PAH is comparable to that in cultured epithelial cells of the human bronchus [15], the target tissue for PAH-induced neoplasia.

0378-4347/83/$03.00

In the present report the high-performance liquid chromatographic (HPLC) analysis of the whole spectrum of organic solvent-soluble metabolites of BP in freshly isolated hair follicles and in cultured hair follicle keratinocytes is described. This methodology gives the opportunity to detect individual differences in carcinogen metabolism, using an epithelial biopsy tissue.

EXPERIMENTAL

Chemicals

[G-^{3}H]BP was purchased from The Radiochemical Centre (Amersham, Great Britain). BP was from Aldrich (Beerse, Belgium). NADPH was obtained from Boehringer (Mannheim, G.F.R.) and gentamycin sulfate from Schering (Kenilworth, Great Britain). Fetal calf serum, Minimal Essential Medium (with Earle's salts, MEM) and glutamine were purchased from Gibco (Glasgow, Great Britain). Hydrocortisone was from Sigma (St. Louis, MO, U.S.A.), insulin from Organon (Oss, The Netherlands), and epidermal growth factor from Collaboratorive Research (Waltham, MA, U.S.A.). LiChrosob RP-18 was obtained from Merck (Darmstadt, G.F.R.). Synthetic BP derivatives were kindly provided by the NCI Chemical Repository at the IIT Research Institute (Chicago, IL, U.S.A.). Aquasol was obtained from New England Nuclear (Boston, MA, U.S.A.).

Collection of tissue and cell culture

Human hair follicles were obtained from the scalp of healthy volunteers using a pair of tweezers. Only hair follicles with visible bulb and sheath were used. Human hair follicle keratinocytes were cultured as described earlier [13] using a natural basement membrane-like extracellular matrix as growth substrate (bovine eye lens capsules) as described [16]. Lens capsules and culture dishes (Epicult) were obtained from Sanbio B.V. (Nistelrode, The Netherlands). In short, hair follicles were placed on the lens capsules in the Epicult dishes and one drop of medium (MEM containing 15% fetal calf serum, 0.4 μg/ml hydrocortisone, 4 μg/ml bovine insulin and 10 ng/ml epidermal growth factor) was added. The cultures were placed in a humidified atmosphere of 5% carbon dioxide in 95% air. After three days when initial outgrowth started to appear, 0.3 ml of fresh medium was added. From then on the medium was changed twice a week. After 2—3 weeks the cultures had grown to confluency (about $2 \cdot 10^5$ cells/dish) and experiments were started.

Equipment

Throughout the study the following equipment was used: a liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a U6K universal injector, two pumps (Model 6000A), a solvent programmer (Model 660), a UV—visible variable-wavelength detector (Model 450), a reversed-phase LiChrosorb RP-18 (5 μm) column (120 × 4.6 mm) and an Omniscribe recorder (Houston Instruments, Houston, TX, U.S.A.). Fractions were obtained with a programmable fraction collector $FRAC_{3000}$ (Pharmacia, Uppsala, Sweden) and the radioactivity in the samples was analysed with an LKB_{1215} Rackbeta liquid scintillation counter (LKB, Stockholm, Sweden).

Analysis of [^{3}H]BP metabolism

[^{3}H]BP (5 μCi/ml culture medium) was purified by thin-layer chromatography [11], dissolved in ethanol, diluted with unlabeled BP and added to the cultures in a final concentration of 0.5 μM. In the case of freshly isolated hair follicles, incubation was performed with 60 hair follicles in 1 ml of 50 mM Tris—HCl, pH 8.5, containing 0.1 M sucrose, 3 mM $MgCl_2$, 10 μg/ml gentamycin sulfate, 2 mM NADPH and 0.5 μM [^{3}H]BP (5 μCi). After the incubation period (1 h for freshly isolated hair follicles and 24 h for the cultures) cells or hair follicles and medium were extracted three times with an equal volume of ethyl acetate. For this purpose cultured cells were scraped in the medium with a bent Pasteur pipette and transferred to an Eppendorf tube. The collected organic phases were evaporated to dryness under a nitrogen stream, dissolved in 50 μl of methanol and loaded on the HPLC column. From the time of injection the column was eluted with a linear gradient of 65—100% methanol in water. The gradient change was completed in 20 min. The constant flow rate was 0.8 ml/min. Eighty fractions of 0.4 min each (0.32 ml) were collected in minivials. As a consequence the last 30 fractions were obtained with the elution gradient in the end condition (100% methanol). The radioactivity in each fraction was determined with Aquasol as counting medium. After the collection of fractions background radioactivity could be washed out of the column within 10 min, and the column could be equilibrated for the next chromatographic analysis. A mixture of synthetic BP derivatives was used for the determination of the retention times of the various metabolites. For this purpose detection was carried out by UV spectroscopy at 254 nm. Identification of the [^{3}H]BP metabolites was achieved by comparison with the position of the authentic standards.

DNA assay

DNA in cultured hair follicle cells and freshly isolated hair follicles was determined by the mithramycin technique. After the metabolite extraction the tubes were centrifuged and the medium changed for distilled water. Then the cells or hair follicles were treated with pronase and the DNA measured as described earlier [17]. Metabolite formation was expressed as fmol metabolite per μg DNA per h.

RESULTS AND DISCUSSION

Fig. 1 represents the separation of a mixture of BP and synthetic BP derivatives under the conditions described. The fraction numbers and the corresponding retention times of these BP metabolites are listed in Table I. Fig. 2 shows the HPLC profile of organic-solvent-soluble [^{3}H]BP metabolites after incubation of 60 freshly isolated hair follicles for 1 h (A) and of cultured hair follicle keratinocytes incubated for 24 h (B). In addition to diols, quinones and phenols, two early eluting components can be identified, especially in the cultured cells. The first one is a polar compound which hardly has any retention delay and which possibly is BP-3-yl hydrogen sulfate, an ethyl acetate extractable sulfate conjugate. This metabolite has been identified in, for example, human and rodent lung cultures [18]. However, certain tetrols can

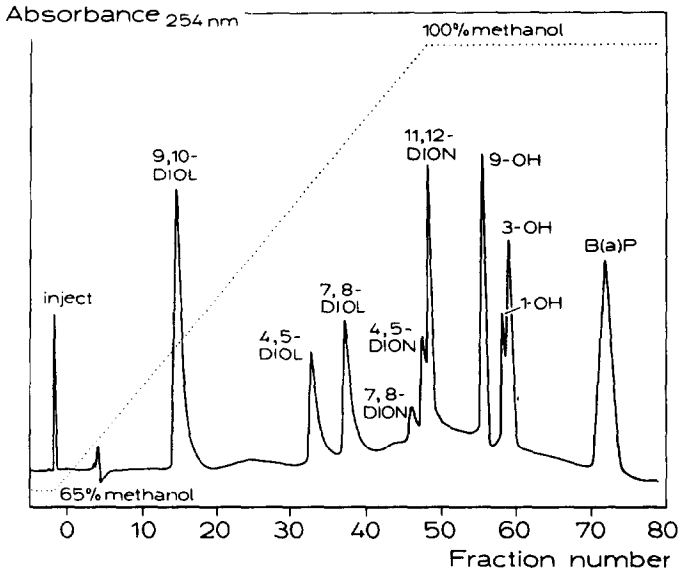

Fig. 1. Separation of a mixture of BP and synthetic BP derivatives under the conditions described in Experimental. Abbreviations: diol = *trans*-dihydro-dihydroxy-BP; OH = hydroxy-BP.

TABLE I

PEAK FRACTION NUMBERS AND CORRESPONDING RETENTION TIMES OF VARIOUS REFERENCE BP METABOLITES IN THE HPLC METHODOLOGY DESCRIBED

Compound*	Peak fraction number	Corresponding retention time (min)
9,10-Diol	16	6.4
4,5-Diol	33	13.2
7,8-Diol	38	14.8
7,8-Dion	46	18.8
4,5-Dion	48	19.2
11,12-Dion	49	19.6
9-OH	57	22.4
1-OH	59	23.6
3-OH	60	24.0
BP	72	28.8

*Abbreviations as in Fig. 1.

also elute in this region. The second early eluting component (fraction number 10—13) has been reported frequently in various tissues [7, 19, 20] and probably is one or more tetrol derivatives of BP. However, since absolute proof of the identity of this component is not available, we have denoted this peak "pre-9,10-diol". It should be noted that tetrols and triols are evidence for the formation of diol-epoxides, the proposed ultimate carcinogens of PAH [21].

Although several quinone and phenol isomers of BP can be separated under the conditions described (Fig. 1), it is difficult to identify each peak in

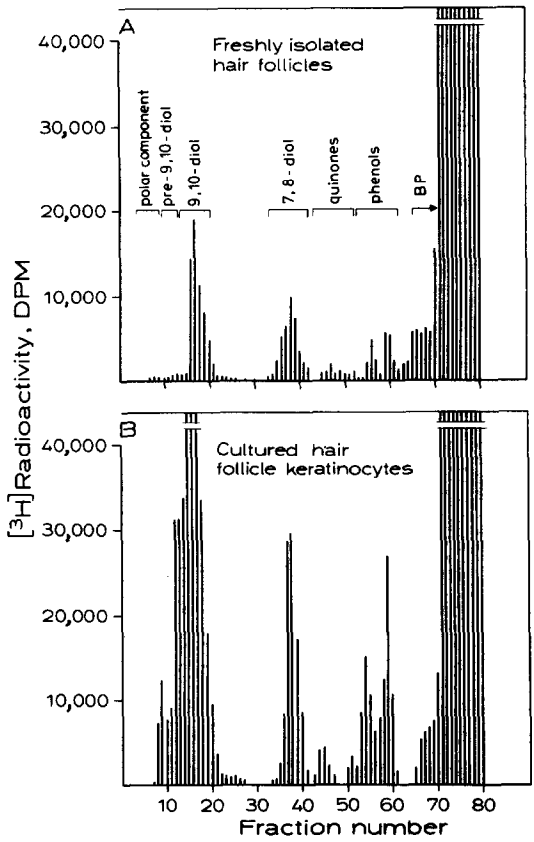

Fig. 2. HPLC profile of organic-solvent-soluble [^{3}H]BP metabolites of freshly isolated hair follicles (incubated for 1 h) (A) and cultured hair follicle keratinocytes (incubated for 24 h) (B). Fractions 4—9 contain a polar component, possibly BP-4-yl hydrogen sulfate. Fractions 10—13 probably represent one or more tetrol metabolites of BP, called "pre-9,10-diol". The *trans*-9,10- and 7,8-dihydrodiol derivatives of BP elute between fractions 14 and 20, and 34 and 42, respectively. Quinones appear between fractions 44 and 52, and phenols between fractions 53 and 62. Unmetabolised [^{3}H]BP elutes from fraction 65.

the quinone and phenol region exactly as one specific isomer. Therefore, all the metabolites which elute between fractions 44 and 52 have been taken together as quinones and the peaks between fractions 53 and 62 as phenols. The HPLC methodology described results in good separation of all the three dihydrodiol metabolites which have been isolated and characterized from various sources [21] as the (—)-*trans*-4,5-dihydrodiol, (—)-*trans*-7,8-dihydrodiol and the (—)-*trans*-9,10-dihydrodiol. Fig. 2A shows that in freshly isolated hair follicles more than 70% of the organic-solvent-soluble metabolites are represented by the 7,8- and 9,10-dihydrodiol metabolites. In contrast, freshly isolated hair follicles hardly metabolise BP to the 4,5-dihydrodiol derivative. Two other important groups of organic-solvent-soluble BP metabolites formed by freshly isolated hair follicles are represented by quinones and phenols although they are formed to a lesser extent than the dihydrodiols.

We have analysed BP metabolism in freshly isolated hair follicles from a

TABLE II

RANGE OF FORMATION OF VARIOUS [^{3}H]BP METABOLITES AND GROUPS OF METABOLITES IN FRESHLY ISOLATED HAIR FOLLICLES OF A NUMBER OF VOLUNTEERS

n = 4. Abbreviations as in Fig. 1. For further explanation see text.

Compound	Range of variation*	Mean ± S.D.
Polar component	0.6—1.5	1.1 ± 0.5
Pre-9,10-diol	1.3—2.6	1.8 ± 0.7
9,10-Diol	41.1—46.3	44.4 ± 2.6
7,8-Diol	29.1—31.5	29.9 ± 1.4
Quinones	3.6—8.5	5.3 ± 2.7
Phenols	10.7—23.1	17.5 ± 6.3

*Expressed as the percentage of the total amount of organic-solvent-soluble metabolites.

number of volunteers. The range of formation of each of the metabolite groups is illustrated in Table II. It is obvious that the variation in dihydrodiol formation is much smaller than the range in variation in phenol formation. Since dihydrodiols result from epoxide hydratase activity and phenols reflect aryl hydrocarbon hydroxylase activity, these findings suggest that the interindividual variation in epoxide hydratase is lower than that for aryl hydrocarbon hydroxylase. In fact, low interindividual variation for epoxide hydratase and large interindividual differences in aryl hydrocarbon hydroxylase activity have been reported for various human tissues (see, for example, ref. 22 and references therein) including human hair follicles [23]. Interindividual variation in total BP metabolism to organic-solvent-soluble metabolites was about three-fold as analysed in the present study.

The metabolite pattern of cultured human hair follicle keratinocytes is qualitatively comparable with freshly isolated hair follicles with dihydrodiols representing the major metabolite group, phenols and quinones the minor ones. Due to the longer incubation time the total level of metabolism is higher than in freshly isolated hair follicles (Table III). However, the mean rate of BP metabolism in cultured cells is somewhat lower than in freshly isolated hair follicles. This can be ascribed to the greater formation of dihydrodiols, quinones and phenols in freshly isolated hair follicles. In contrast, the amount of the more polar organic-solvent-soluble metabolites is greater in cultured hair follicle keratinocytes than in freshly isolated hair follicles. The observation that at longer incubation times there is about the same amount of 9,10-dihydrodiol and much more tetrols indicates that 9,10-dihydrodiol is an end-point in BP metabolism and 7,8-dihydrodiol can be further metabolised to the diol-epoxides.

Cultured hair follicle keratinocytes offer the opportunity to investigate BP metabolism after induction of the enzyme aryl hydrocarbon hydroxylase. High levels of induced activity of this enzyme have been correlated with genetic susceptibility to PAH-induced neoplasia in some studies [9, 24], while other authors failed to confirm this [25, 26]. One of the sources of controversy can be the choice of human biopsy tissue, peripheral lymphocytes. In

TABLE III

FORMATION OF [^{3}H]BP METABOLITES OR METABOLITE GROUPS IN FRESHLY ISOLATED HUMAN HAIR FOLLICLES AND CULTURED HAIR FOLLICLE KERATINOCYTES FROM THE SAME DONOR

Abbreviations as in Fig. 1. The data represent the mean values for three persons and are expressed as fmol product per μg DNA per h.

Compound	Freshly isolated hair follicles	Cultured hair follicle keratinocytes
Polar component	4 ± 1	22 ± 5
Pre-9,10-diol	4 ± 1	30 ± 5
9,10-Diol	130 ± 20	96 ± 16
7,8-Diol	92 ± 17	33 ± 6
Quinones	13 ± 3	10 ± 3
Phenols	73 ± 27	31 ± 12
Total	316 ± 43	222 ± 35

view of the prevalence of carcinomas, the use of human keratinocytes seems to be a more appropriate alternative. It has already been shown that the response of BP metabolism towards pre-exposure to benz[*a*]anthracene in cultured hair follicle keratinocytes, is qualitatively comparable with that in cultured human bronchial epithelial cells, the target tissue of PAH-induced neoplasia [15]. In contrast, murine epidermal cells, a frequently studied cell type in chemical carcinogenesis, do respond differently to pre-exposure to benz[*a*]anthracene as compared with human epidermal keratinocytes [14].

The method presented here offers the advantage of rapid analysis of BP metabolism in an easily available human biopsy tissue of epithelial origin, the hair follicle. Both differences in the profile of the various BP metabolites and variations in the rate of BP metabolism can be monitored easily. The application of the methodology on cultured hair follicle cells gives the opportunity to measure inducibility of BP metabolites after pre-exposure to PAH. Together with the recently developed assays for carcinogen-metabolising enzymes in hair follicles [27, 28], the method may contribute to identification of high-risk populations.

ACKNOWLEDGEMENT

The present investigation was supported by the Netherlands Cancer Society (Koningin Wilhelmina Fonds).

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