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A FAST AND SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ARYL HYDROCARBON HYDROXYLASE

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SUMMARY

A simple high-performance liquid chromatographic method using fluorescence detection of the remaining substrate is described for the determination of benzo[*a*]-pyrene hydroxylase activity. This assay is far simpler than the previous ones, as it does not require extraction or centrifugation and the measurement occurs directly after dilution of the total incubation medium. The aryl hydrocarbon hydroxylase (AHH) activities in rat liver microsomes are in agreement with those obtained by radioactive assays. Moreover, this assay allows the routine determination of the AHH activity in animal tissues.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have attracted a great deal of attention because they are widely distributed in the environment and have been shown to cause carcinogenic, mutagenic and cytotoxic effects in various species and tissues[1–3]. For many PAHs, biologically active arene oxides are the initial products formed by the microsomal mixed function oxidase system; epoxide hydrolase (E.C. 3.3.2.3) catalyses the hydration of the arene oxides to biologically inactive dihydrodiols, which may undergo further metabolism, by the microsomal Cyt P_{450}-dependent system, to form highly reactive metabolites.

Although the relationship between tissue inducibility of aryl hydrocarbon hydroxylase (AHH: 1.14.14.2) activity and susceptibility to PAH carcinogenesis is still not clear, several reports have shown that there is a correlation. Thus it is of interest to establish simple and reliable methods for the measurement of AHH activity in the different tissues.

Benzo[*a*]pyrene (B*a*P) is the commonest PAH used for the determination of AHH activity, and in most work the assay procedure used is the fluorimetric method described by Nebert and Gelboin[4], which measures a fraction of the metabolites of B*a*P, as only products that fluoresce in a manner similar to 3-hydroxybenzo[*a*]pyrene are taken into account. More recently, De Pierre *et al.*[5] and Van Cantfort *et al.*[6] developed radiometric assays in which the water-soluble metabolites of B*a*P were measured directly by liquid scintillation counting.

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In this paper, we report an assay consisting of a very simple and reproducible high-performance liquid chromatographic (HPLC) quantification of the unreacted B*a*P, using fluorimetric detection; it allows direct measurement in the incubation medium and is very convenient for the routine determination of AHH activity.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (150–200 g), bred at the laboratory, were allowed an AO3 diet (UAR, Villemoisson, France) and water *ad libitum*. They were starved for 12–15 h before killing.

Chemicals

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were obtained from Sigma (St. Louis, MO, U.S.A.); B*a*P was purchased from Fluka (Buchs, Switzerland). The solvents used for extraction and HPLC analysis were obtained from commercial sources and purified by distillation

Enzyme preparation

Animals were killed by exsanguination to aid removal of blood from organs. The liver was immediately excised, weighed, then chilled and homogenized with a Potter-Elvehjem instrument, fitted with a PTFE pestle, in 4 volumes of ice-cold 0.1 *M* phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 *g* for 20 min. The mitochondria-free supernatant was further centrifuged at 105,000 *g* for 60 min. The microsomal pellet was resuspended in 0.1 *M* phosphate buffer to give final protein concentrations ranging from 2 to 10 mg/ml. The protein concentration of the microsomal suspension was determined by the biuret method of Gornall *et al.*[7] using bovine serum albumin as the standard.

Aryl hydrocarbon hydroxylase assay

The reaction mixture, which contained 250 μmoles of Tris buffer (pH 7.4), 12.5 μmoles of glucose-6-phosphate, 2.5 μmoles of NADP, 2 units of glucose-6-phosphate dehydrogenase and 0.5 ml of microsomes in a final volume of 2.5 ml, was preincubated at 37°C for 5 min. The reaction was initiated by the addition of B*a*P in 50 μl of acetone, and the concentrations used varied from 2 to 100 μ*M*. The mixture was shaken for 30 min at 37°C and the reaction was stopped by the addition of 1 ml of cold acetone; the flasks were stored at 4°C until taken for quantification of B*a*P.

The assays were carried out in duplicate and the AHH activity was obtained by comparing the remaining B*a*P in the incubation flasks with B*a*P measured in identical assays to which acetone had been added prior to incubation.

High-performance liquid chromatography

The HPLC arrangement consisted of a Spectra-Physics Model SP 3500 B pump equipped with a Valco Model 60 sample injection valve (10-μl sample loop) and connected to a Schoeffel FS 970 spectrofluorimetric detector fitted with a 5-μl cell. The excitation monochromator was set at 366 nm and fluorescence emission was measured through a cut-off filter ($\lambda_{em} > 385$ nm). The column consisted of a stainless-

steel tube (250 × 4.6 mm I.D.) packed with 5-μm LiChrosorb RP 18 (Merck, Darmstadt, G.F.R.) using a Chromatem apparatus (Touzart et Matignon, France). The mobile phase consisted of 10% water in acetonitrile, the eluent flow-rate was 1.2 ml/min and all manipulations were performed at ambient temperature.

The incubates were adjusted to bring the volume to 4 ml and, after homogenization on a vortex mixer, a 200-μl aliquot was diluted with acetonitrile in order to obtain concentrations of BaP in the 1 μM range. After shaking, 10 μl of the solution were injected for HPLC analysis and the AHH activity was determined by comparing the peak heights obtained in assays in which acetone had been added before or after incubation at 37°C; AHH activity was expressed in nanomoles of hydroxylated BaP per minute per milligram of microsomal protein.

RESULTS

BaP assay

As the determination of remaining BaP was conducted on an aliquot of the total incubation medium, it was necessary to ascertain that the fluorimetric detection was specific; from this point of view, it was shown that none of the components of the incubation medium gave interfering peaks with the same retention time as BaP. It was also observed (Fig. 1) that the same response was obtained when a 200-μl sample was

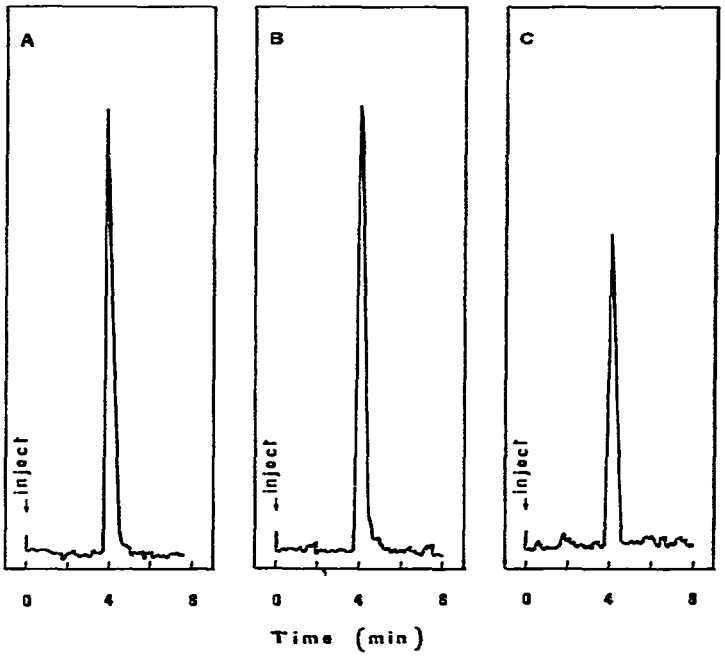

Fig. 1. (A) Liquid chromatogram of a 10-μl sample from a 1 μM solution of BaP in acetone–acetonitrile. (B) and (C) Liquid chromatograms of 10-μl samples from diluted incubation mixtures (B = blank incubation; C = assay incubation). After the 30-min incubation period, dilution with acetonitrile was such that the BaP concentration in the control flask (B) was 1 μM; the same dilution was applied to the assay flask (C). The difference in the responses gives the hydroxylation rate.

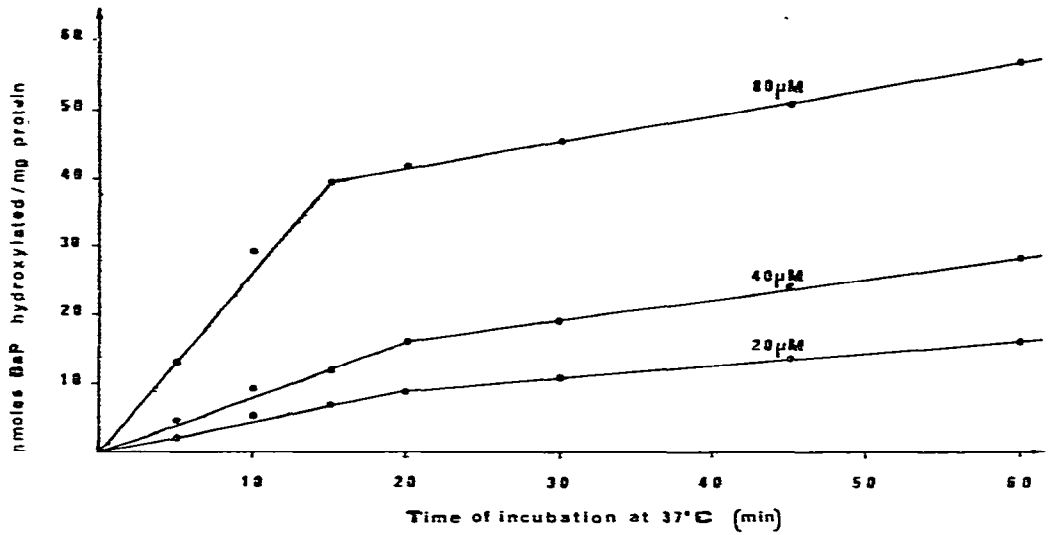

Fig. 2. Relationship between time and B*a*P hydroxylase activity in rat microsomes. Details of the assay procedure are described under Materials and methods, and each point represents the mean of duplicate determinations. The incubation systems contained rat microsomes at a concentration of 6 mg protein per millilitre. The reaction was started by adding sufficient B*a*P (dissolved in acetone) to produce final concentrations of 20, 40 and 80 μM. Incubations were carried out at 37°C for 5, 10, 15, 20, 30, 45 and 60 min.

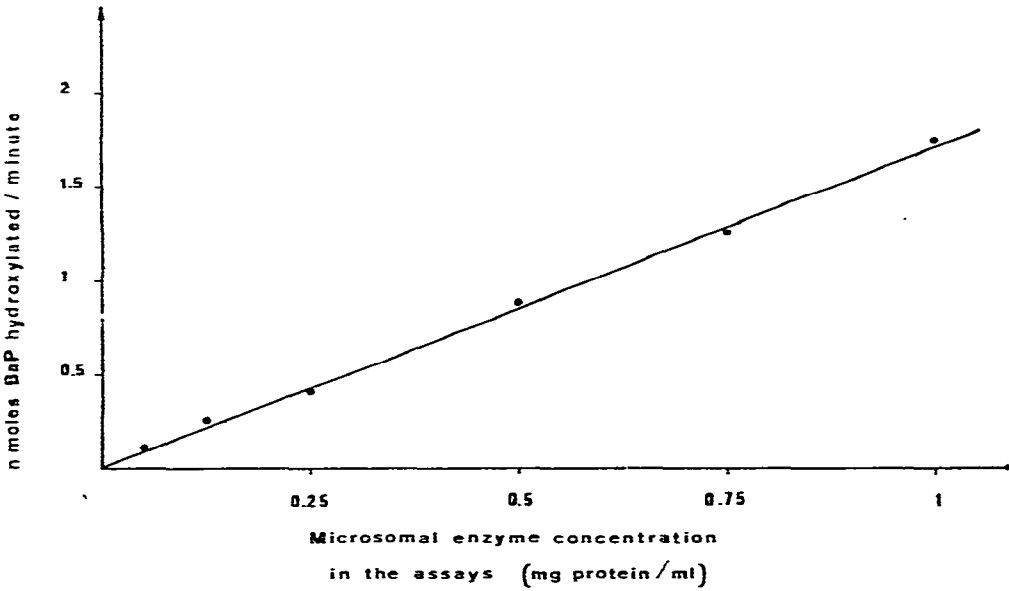

Fig. 3. Dependence of B*a*P hydroxylation on enzyme concentration. The incubation systems used rat microsomes at a concentration of 6 mg protein per millilitre. B*a*P was used at a final concentration of 20 μM and incubations were carried out at 37°C for 30 min.

diluted in acetonitrile, whether it came from a pure solution of B*a*P in acetone at the concentration used in the incubation flasks or from an incubation medium. The possibility of erroneous measurements of the remaining B*a*P after incubation, due to interfering metabolites with the same retention time, was eliminated after analysis by HPLC under the same conditions, after total removal of B*a*P by extraction with *n*-hexane.

All of the assays were carried out in duplicate, and the differences between the duplicate results were less than 2%; under the conditions described, the retention time of B*a*P was about 4 min and the response was linear for the range 0–0.05 nmole injected.

Linearity with time and protein

Fig. 2 shows the relationship between enzyme activity and time; linearity of hydroxylation was observed for at least 15 min; as has been described for the radioactive assay[5], the extent of linearity depends on the rate of formation of the reaction products.

Fig. 3 shows the effect of liver microsomal protein on the AHH activity; hydroxylation of B*a*P is linear when concentrations in the range 0–1 mg of protein per millilitre of incubation mixture are used.

Saturation with substrate

It is of great importance, when comparing in animal tissues the effects of various treatments on enzymatic activity, to use a saturating concentration but not a too high one (which would decrease the precision of the comparison); thus the depen-

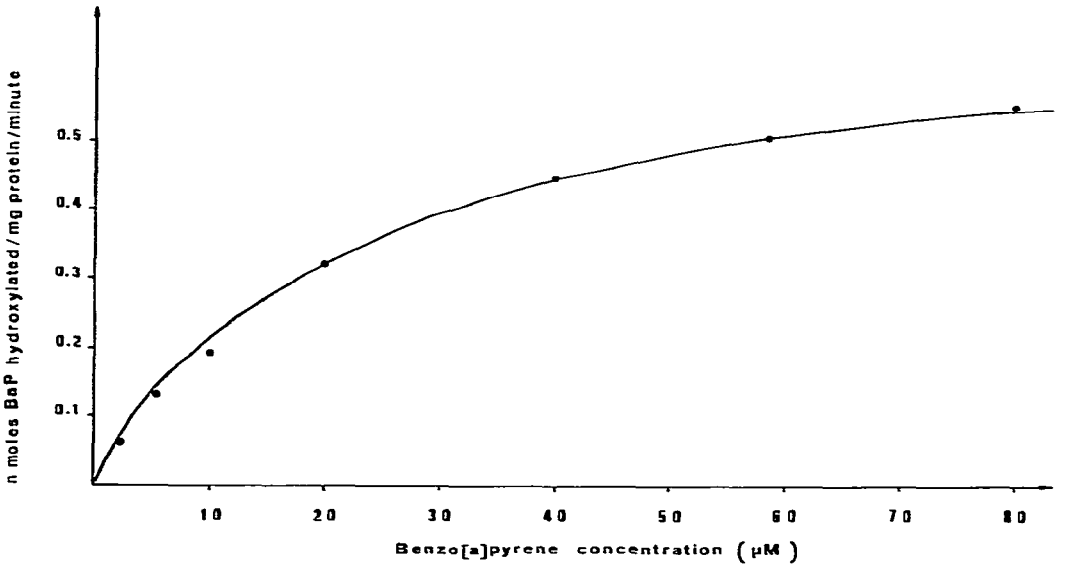

Fig. 4. Dependence of B*a*P hydroxylase on substrate concentration. The assay procedure is described under Materials and methods. The incubation systems used rat microsomes at a concentration of 6 mg protein per millilitre. The reactions were started by adding B*a*P to produce final concentrations of 2, 5, 10, 20, 40, 60 and 80 μM. Incubations were carried out at 37°C for 30 min.

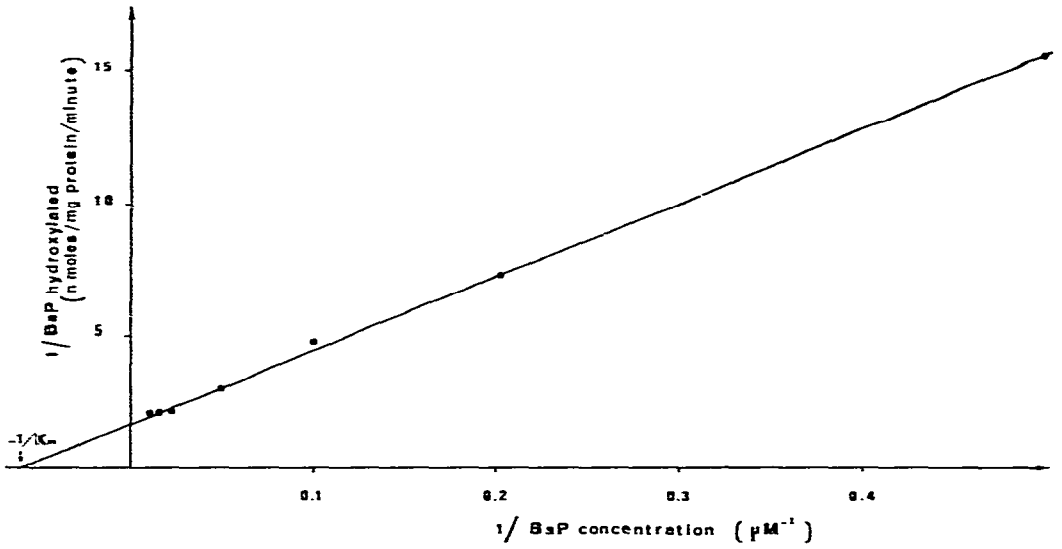

Fig. 5. Lineweaver–Burk plot of B*a*P hydroxylation. Incubation conditions as in Fig. 4. The abscissa indicates the reciprocal of the B*a*P concentrations employed and the ordinate shows the reciprocal of the apparent rate of metabolism.

dence of B*a*P hydroxylase on substrate concentration was determined and is shown in Fig. 4.

It was possible to obtain the apparent K_M for B*a*P hydroxylase by displaying the data on a Lineweaver–Burk plot (Fig. 5). The value of 16 μM is in good agreement with those obtained in other laboratories for similar microsomal protein concentrations.

DISCUSSION

The procedure described allows the determination of unreacted substrate without interference from any of the reaction products. The enzyme activity is linear over reasonable ranges of time and protein concentration, and thus this assay appears to be useful for AHH measurement. Nevertheless, it should be compared with the fluorimetric assay of Nebert and Gelboin[4] and with the radioassay described by De Pierre *et al.*[5] and modified by Van Cantfort *et al.*[6].

Up to now, the fluorescence assay was the most commonly used, owing to its very high sensitivity; however, it is a questionable technique for numerous reasons. It is based on an acetone–*n*-hexane extraction of the products formed but only a portion of the B*a*P metabolites are extracted; on the other hand, spectrofluorimetric measurement of products extractable in alkaline medium (emission peak at 522 nm produced by activation at 396 nm) is limited to the determination of products fluorescing under the same conditions as 3-hydroxybenzo[*a*]pyrene. This method does not assay diols, epoxides, quinones or other poorly fluorescent metabolites, and it has been observed that it measures not more than half of the alkali-soluble metabolites. From this point of view, the radiometric assay is better, as it measures, after *n*-hexane extraction of the

remaining B*a*P, the whole of the radioactivity associated with the water-soluble metabolites formed.

Van Cantfort *et al.*[6], using HPLC analysis, showed that dihydrodiols and quinones were present in non-negligible proportions in the *n*-hexane extract. After modification of the polarity of the aqueous phase, complete removal of B*a*P from its metabolites (>95%) was obtained. Nevertheless, they specified that a second extraction with *n*-hexane was necessary in order to achieve minimal contamination of the aqueous phase by unchanged substrate, but without any indication on the new yields of the measured metabolites.

Our method is much simpler, as measurement is effected after direct dilution of the incubation medium. Of course, all the cofactors and all the B*a*P metabolites are present but the specificity of the HPLC determination associated with the high dilution used (50- or 100-fold when 80 μM of B*a*P was used) allows a very accurate quantification of the unchanged B*a*P. In addition to specificity, very good reproducibility is achieved as measurements on duplicates did not differ by more than 2%.

In other respects, the classical fluorimetric assay is limited, owing to the decreasing fluorescence of the metabolites when they are stored after alkali extraction from the organic phase; the samples must be extracted one at a time and the fluorescence determined as quickly as possible; with our method, the samples can be stored, before or after dilution of an aliquot with acetonitrile, for several days without affecting the results if protected from sunlight. Thus it is possible to perform a routine determination of AHH activity with an HPLC apparatus fitted with an automatic injector.

If we consider the sensitivities of the methods, the radiometric and fluorimetric assays seem very similar; the lower limit as indicated by Van Cantfort *et al.* is about $2 \cdot 10^{-11}$ moles of metabolites formed per millilitre of incubation medium. It is impossible to compare these sensitivities with that of our assay in which we measure the rate of disappearance of the substrate.

Although we are able to detect $8 \cdot 10^{-14}$ moles of B*a*P, our HPLC measurement of AHH activity is limited by the lowest significant difference measurable between the assays in which acetone is added before or after incubation. Owing to the good reproducibility of the measurements on duplicates, a 5% difference can be considered as significant, corresponding to the hydroxylation of the substrate by the mixed function oxidase (MFO) system. Thus, if we utilize substrate concentrations in the range 40–80 μM, our assay can be used for tissues in which the lower limit of the hydroxylation route is about 100 pmole per minute per milligram of microsomal protein (which is 10-fold lower than the value observed for rat liver).

It has been observed that PAH metabolism differs qualitatively and quantitatively according to the organs or animal species considered[8–11]. Moreover, it has been shown that the relative distribution between the different metabolites of B*a*P (phenols, diols, epoxides, quinones) was not the same when the animal had been treated or not by inducers such as 3-methylcholanthrene or other PAHs[11,12]. As a result, it is impossible to compare the AHH activities using the fluorimetric assay, but the radioisotopic method and our assay are very satisfactory.

Nevertheless, the best method would in fact be the identification and quantification of all of the metabolites of B*a*P produced, in order to determine how patterns of metabolism may vary (between animal species or chemical treatments), and thus

indications could be obtained of the factors influencing carcinogenic risk. If HPLC fulfills the requirements for such an analysis it is impossible to apply it in routine experiments. In addition to the separation of the water-soluble metabolites and of the organic solvent-soluble metabolites, it would be necessary to determine the various metabolites that are covalently bound to protein and which may represent 4–9% of the total metabolites of B*a*P[13,14]. Moreover, taking into account that non-enzymatic conversions may occur, which modify the relative distribution between the metabolites, the systematic quantification of each metabolite is of little interest.

Thus it is necessary to have a simple technique for AHH determination; the fluorimetric assay can be discarded as it measures only part of the mixed function oxidase activity. The radiometric method and our HPLC assay are similar, and give a good determination of the AHH activity. The former method requires two extraction and centrifugation steps, whereas in our method measurement is effected after dilution of the incubation medium. Utilization of labelled B*a*P in the radiometric assay makes it more expensive and requires frequent checking of chemical and radiochemical purities; indeed, the more serious limitation to the sensitivity of a radioactive enzymatic assay is often the background from contaminants in the substrate and from non-enzymatic reactions.

Finally, the HPLC method described here is much simpler and reproducible than the previous assays; the possibility of automation makes it more convenient for large-scale analysis and is of great interest when studying the induction of liver AHH by various xenobiotics in many animal species or when utilizing the AHH measurement as an indicator of petroleum hydrocarbon pollution in the marine environment[15].

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