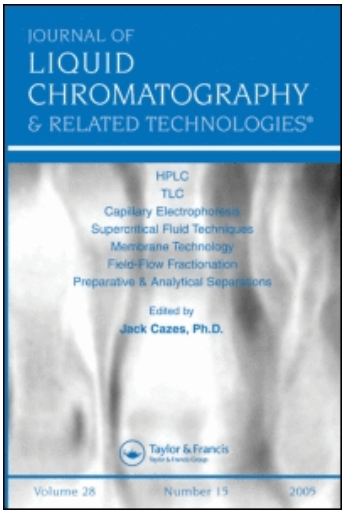

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Rosier, Jan A.(1987) 'A Micropreparative HPLC Purification Procedure for (G-^{3}H)-Benzo(a)-Pyrene and 3-(6-^{14}C)-Methylcholanthrene', Journal of Liquid Chromatography & Related Technologies, 10: 10, 2105 — 2114
To link to this Article: DOI: 10.1080/01483918708068898
URL: http://dx.doi.org/10.1080/01483918708068898

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(10), 2105-2114 (1987)

A MICROPREPARATIVE HPLC PURIFICATION PROCEDURE FOR (G-^{3}H)-BENZO(a)PYRENE AND 3-(6-^{14}C)-METHYLCHOLANTHRENE

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ABSTRACT

This paper describes a micropreparative purification method of (^{3}G)-benzo(a)pyrene and 3-(6-^{14}C)-methylcholanthrene employing high pressure reversed phase chromatography. The method can be used immediately prior to the use of these compounds as exposure agents in metabolism studies, thereby assuring the highest possible degree of purity during exposure and thereby eliminating the need for batch purification and repetitive sampling from the purified material.

INTRODUCTION

Study of the metabolism and the intrinsic differences among cultured cell lines in their ability to metabolize (G-^{3}H)-benzo(a)pyrene or 3-(6-^{14}C)-methylcholanthrene requires pure standards of these polycyclic aromatic hydrocarbons to interpret reliably the metabolite profiles obtained by HPLC analysis of the cell culture extracts.

(G-^{3}H)-Benzo(a)pyrene and 3-(6-^{14}C)-methylcholanthrene are not stable during storage and frequently require purification before their use in metabolism studies. (G-^{3}H)-Benzo(a)pyrene is so sensitive to light and air that the degree of purity of a sample can decrease by as much as 5 % per day if degradation is not carefully avoided. Therefore, the repetitive sampling of a batch purified sample cannot be recommended if the purity of these compounds is of crucial importance. The same remarks hold for 3-(6-^{14}C)-methylcholanthrene, which is not as sensitive to oxidation as (G-^{3}H)-benzo(a)pyrene, but whose purity is nevertheless dependant on storage conditions and sample handling. We therefore describe a method that allows one to purify that amount of (G-^{3}H)-benzo(a)pyrene or 3-(6-^{14}C)-methylcholanthrene needed for immediate use during an experiment.
This avoids batch purification (and the risks of breakdown associated with repetitive sampling) and laborious extraction techniques with KOH/DMSO (1) or purification by means of benzene elution from silica (2) which does not allow direct control of purity.

MATERIALS

Micropreparative High Pressure Liquid Chromatography.

Use was made of a Perkin Elmer Series 2 liquid chromatograph equipped with a Perkin Elmer LC 75 Spectrophotometric detector set at 280 nm and a Zorbax Dupont Reversed Phase Column (dimensions : 9,4 mm I.D. x 25 cm length) packed with 10 µm particles. The eluting solvent was acetonitrile. The sample was injected by means of a Rheodyne injector (type 7125) equipped with a sample loop of 1 ml. Fractions were collected at a flowrate of 1,0 ml/minute. The radioactivity profile was measured by mixing 0.01 ml aliquots of each fraction with 3 ml of Universal LSC-Coktail (Dupont, Aquasol) and counted in a Minaxi Tricarb 4000 Series Liquid Scintillation Counter (Packard).

Analytical High Pressure Liquid Chromatography

Use was made of a Varian 5000 high pressure liquid chromatograph equipped with a gradient elution controller, a variable wavelength detector (Waters, model 450) set at 280 nm and a LKB 2112 Redirac fraction collector. The analytical column used was a μBondapak RP 18^{tm} (dimensions : 4.6 mm I.D. x 30 cm langth) packed with 10 μm particles. The sample was applied on the column by means of a Rheodyne injector (Type 7201) equipped with a sample loop of 1.1 ml and eluted by gradient elution starting with 40 % methanol in water to 100 % methanol during 60 minutes. The flow rate was 2 ml/minute.

Chemicals

(G-^{3}H)-Benzo(a)pyrene (65 Ci/mmol) was purchased from Amersham Inc. as a solution in toluene and was diluted with unlabeled benzo-(a)pyrene (Sigma, Chem. Co.) to a specific activity of 50 mCi/mmol. 3-(6-^{14}C)-Methylcholanthrene was purchased from New England Nuclear (56 mCi/mmol) as a solution in benzene-ethanol (9-1) and was diluted to approximately 50 mCi/mmol.
Dimethylsulfoxide (Fisher Scientific).
Acetonitrile (HPLC-grade, Fisher Scientific).
Methanol (HPLC grade, Fisher Scientific).
Water (deionized, distilled and filtered).

METHODS

The volume of a solution of (G-^{3}H)-benzo(a)pyrene (diluted with unlabeled benzo(a)pyrene to the required specific activity) that corresponds to the activity (in cpm) necessary to carry out the experiments was applied on the preparative reversed phase column and eluted with acetonitrile. Based on the retention time of a benzo(a)-

pyrene standard and on the radioactivity in the eluted fractions, the benzo(a)pyrene peak was identified, collected, transferred to a glass tube and evaporated under N_2 at 30 C under red light. The residue was dissolved in 1,0 ml acetonitrile ; 0.01 ml of this solution was mixed with with 3 ml LSC coktail and the total activity (in cpm) of the solution measured. The acetonitrile was evaporated under N_2 at 30 C under red light and dissolved in an appropriate amount of dimethylsulfoxide. This solution was used during the exposure experiments. A small fraction of this solution (0.010 ml) was diluted with methanol to 0.5 ml and injected on the analytical column.

The purification of 3-(6-^{14}C)-methylcholanthrene proceeds in a similar way : of the benzene-ethanol solution of 3-(6-^{14}C)-methylcholanthrene, a volume that corresponds with the total required activity (in cpm) for the experiment is pipetted into a glass tube and the solvent was evaporated under N_2 at 30 C and under red light. To the residue 0.2 ml of acetonitrile was added. This solution was applied on the preparative reversed phase column and eluted with acetonitrile as described. Based on the retention time of a 3-methylcholanthrene standard and on the radioactivity in the eluted fractions, the 3-(6-^{14}C)-methylcholanthrene is identified , collected and transferred to a glass tube. The solvent is evaporated and the residue dissolved in an appropriate amount of dimethylsulfoxide.

Total time for chromatographic elution for each compound is 15 minutes and total preparation time for chromatographically pure (G-^{3}H)-benzo(a)pyrene or 3-(^{14}C 6)-methylcholanthrene is approximately 1 hour. The radiochemical purity (in %) of the unpurified sample was calculated, by determining the ratio of counts present in the 3MC peak eluting from the HPLC column to the counts in the sample applied on the column.

RESULTS

Figure 1 shows the radioactivity present in the eluting fractions and the A^{280} absorbance plot after injection of approximately 0.6 mg of

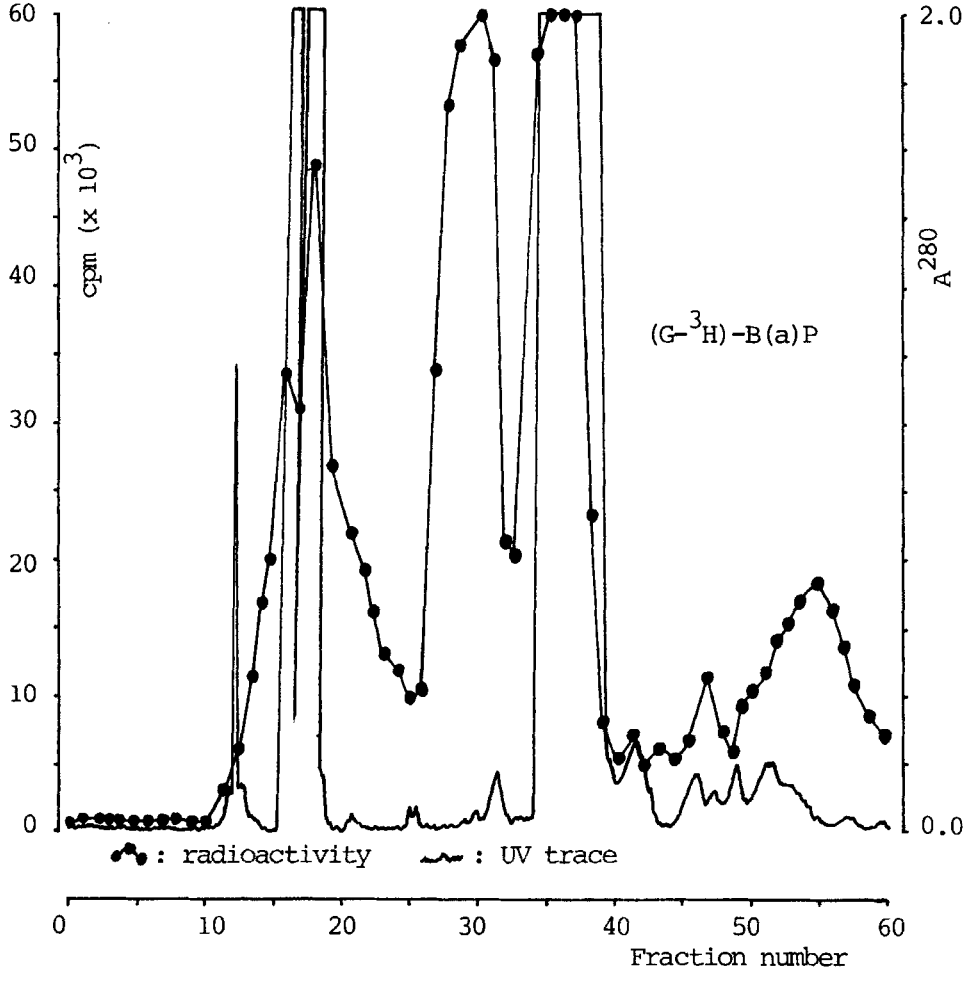

Figure 1 HPLC chromatogram of a commercial (G-^{3}H)-benzo(a)pyrene sample. The peaks eluting in fractions 11 to 34 were not identified but are probably oxidation products of (G-^{3}H)-benzo(a)pyrene. Chromatographic conditions as in text.

(G-^{3}H)-benzo(a)pyrene on the preparative reversed phase column. Fractions 35 to 37 were collected and concentrated as described.

The radiochemical purity of the commercial (G-^{3}H)-benzo(a)pyrene was determined as 30 %. Figure 2 shows the radioactivity and UV absorbance plot of the elution of the purified sample of (G-^{3}H)-benzo(a)pyrene in a water-methanol gradient on the analytical HPLC column.

Figure 3 shows the HPLC chromatogram of 0.06 mg of 3-(6-^{14}C)-methylcholanthrene detected by both radioactivity and UV absorbance detection. Fractions 33 tot 35 were collected and concentrated as described. The radiochemical purity of the commercial 3-(6-^{14}C)-methylcholanthrene was calculated as 89 %. An aliquot of the purified 3-(6-^{14}C)-methylcholanthrene was then applied on the analytical column (Figure 4). The radiochemical purity of the purified samples of (G^{3}H)-benzo(a)pyrene an 3-(6-^{14}C)-methylcholanthrene as determined by analytical HPLC was calculated as 91.5 % and 99.3 %, respectively.

The recovery of the radioactivity applied on the analytical column was determined as 97,5 % for (G-^{3}H)-benzo(a)pyrene and 95.1 % for 3-(6-^{14}C)-methylcholanthrene.

DISCUSSION

Purification of (G-^{3}H)-benzo(a)pyrene or 3-(^{14}C-6)-methylcholanthrene by means of the described method allows for the fast and efficient removal of impurities present in the commercial products. The combination of a reserved phase HPLC column and acetonitrile as eluting solvent offers a compromise between the separation of these polycyclic aromatic hydrocarbons from their impurities and the speed of the separation. This method has permitted purification of these polycyclic aromatic hydrocarbons less than 2 hours before the actual exposure of the cells in cell culture experiments, thereby assuring the high purity during exposure. Applicaton of this method to other polycyclic aromatic hydrocarbons (DMBA etc.) appears straight forward and of potential usefulness.

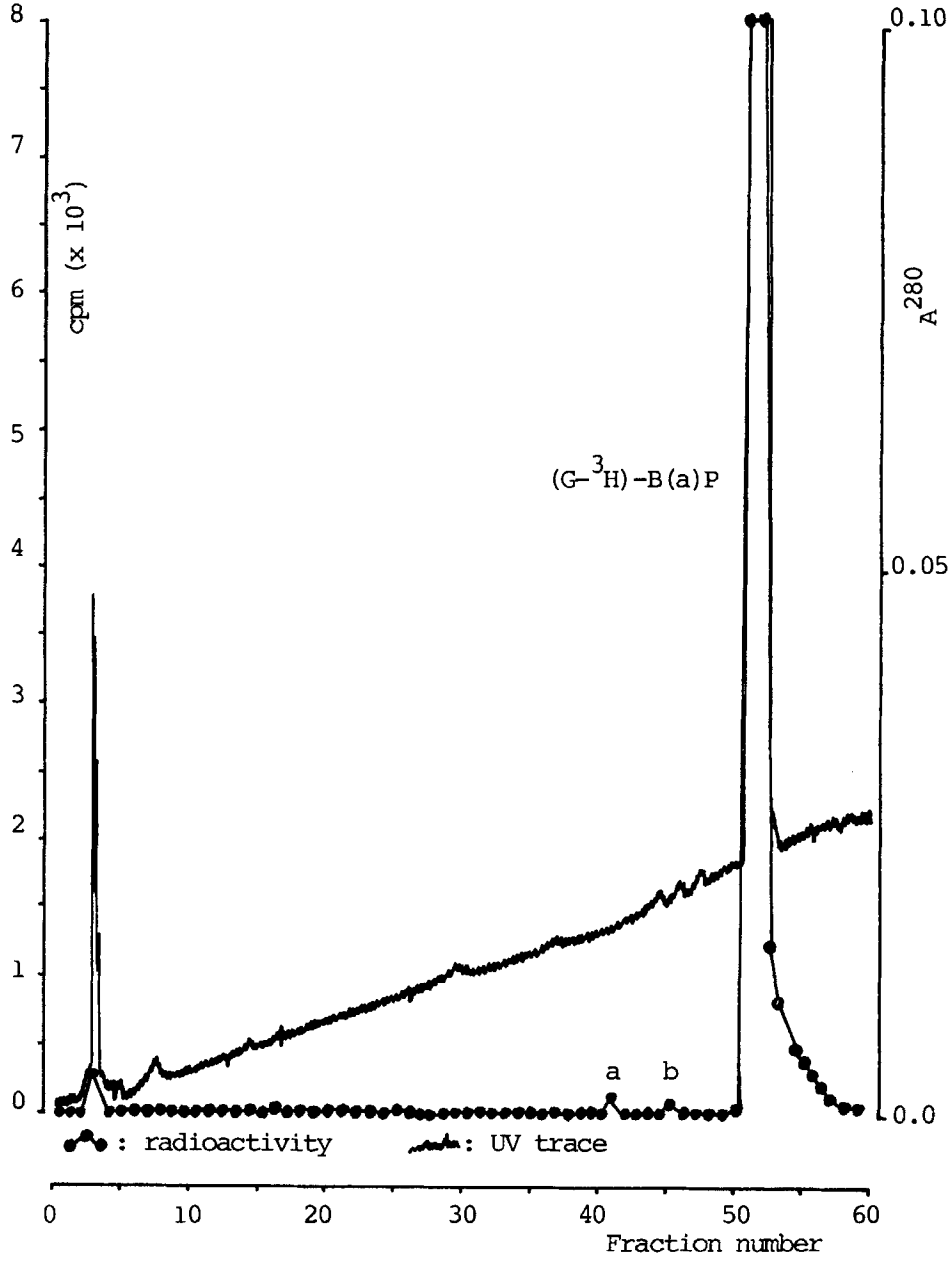

Figure 2 HPLC chromatogram of a purified sample of (G-^{3}H)-benzo-(a)pyrene on an analytical column. The peaks a and b which are still present in the purified sample contain less than 8,5 % of the total radioactivity. Chromatographic conditions as in text.

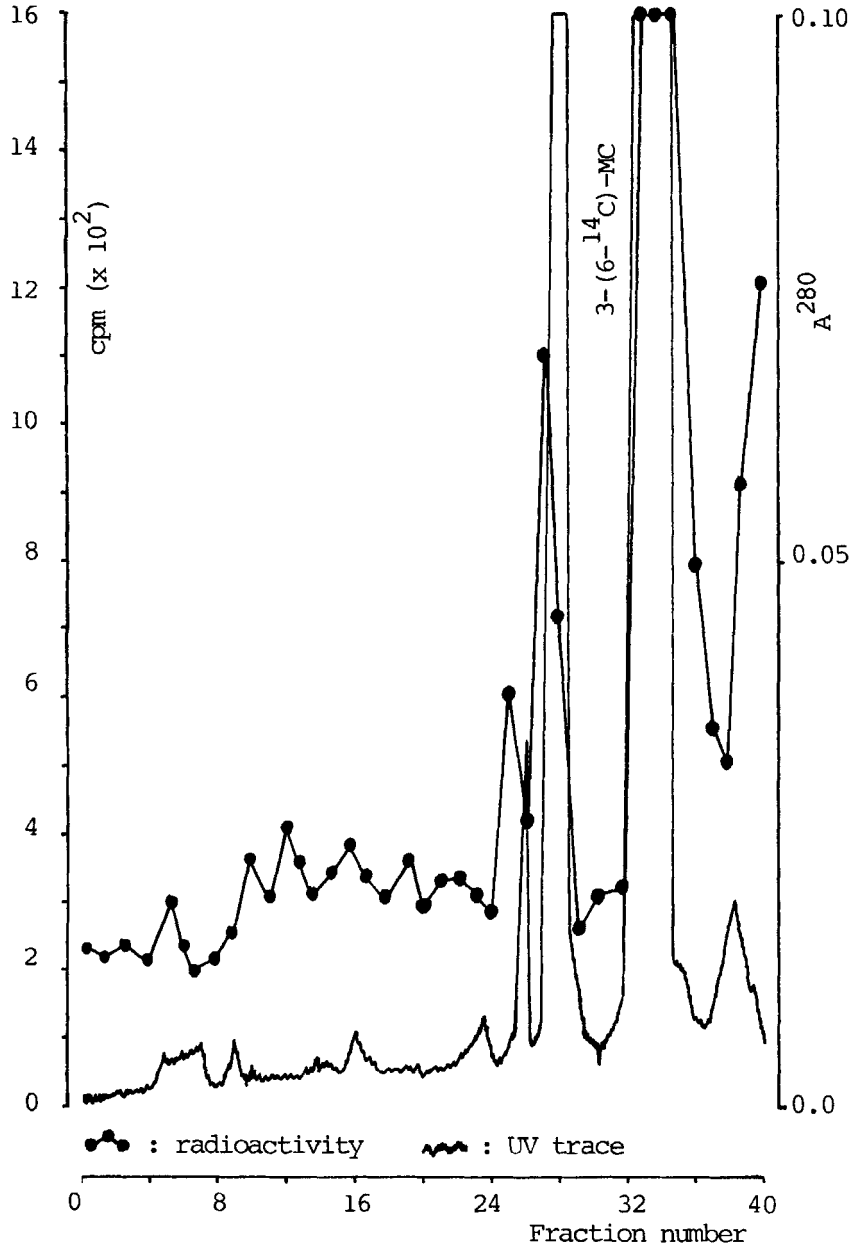

Figure 3 HPLC chromatogram of a commercial 3-(6-^{14}C)-methylcholanthrene sample. Chromatographic conditions as in text.

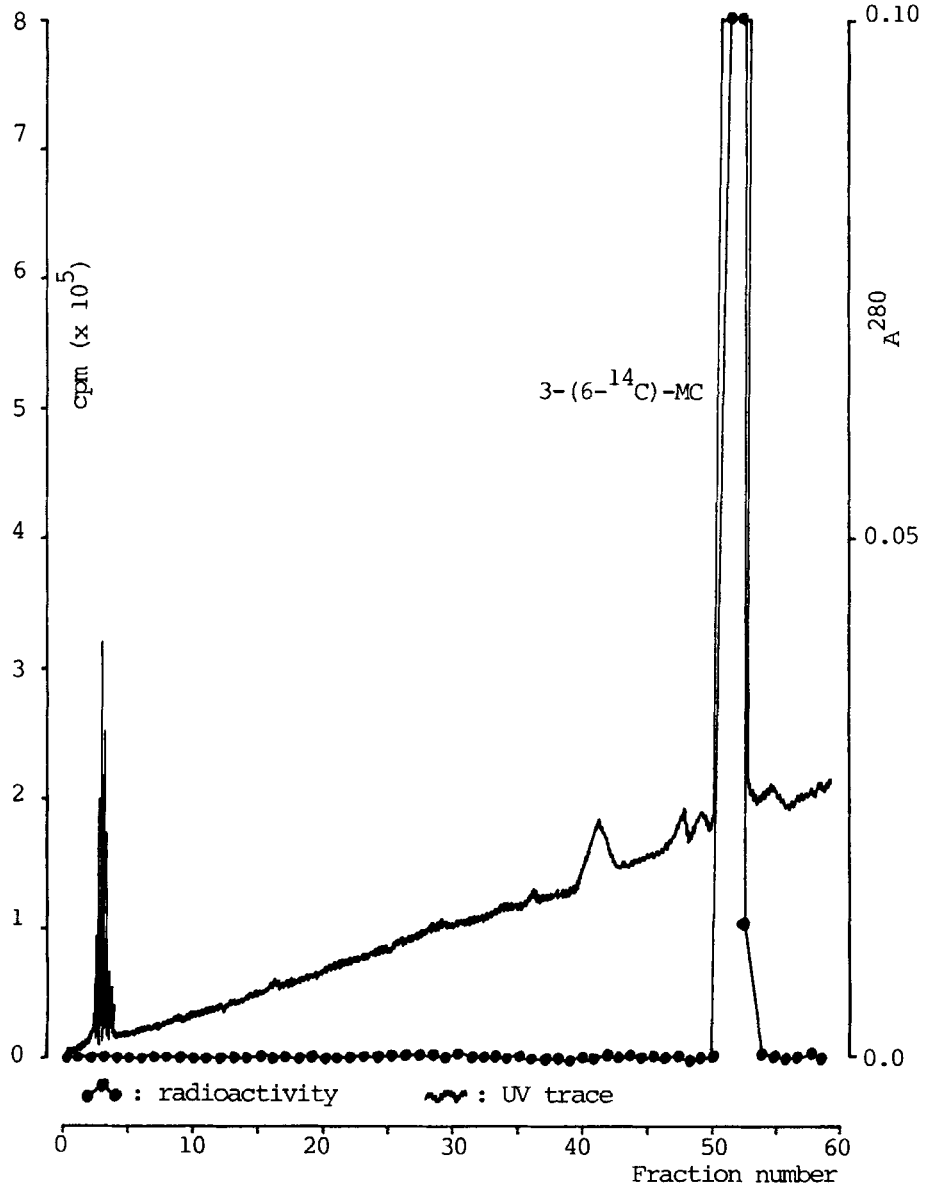

Figure 4 HPLC chromatogram of a purifeid sample of 3-(6-^{14}C)-methylcholanthrene sample on an analytical column. Chromatographic conditions as in text.

ACKNOWLEDGMENTS

This research was supported bij U.S. Puplic Health Service Grant N° ES 03250 from the National Institute of Environmental Health Sciences.

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