A DOUBLE ISOTOPE DILUTION METHOD FOR ASSAYING OF POLYCYCLIC AROMATIC HYDROCARBONS IN CIGARETTE SMOKE CONDENSATE

Abstract — This report describes a double isotope dilution method for analysis of the polycyclic aromatic hydrocarbons (PAH) phenanthrene, fluoranthene, pyrene, and benzo[a]pyrene in cigarette smoke particulates. The first isotope dilution used deuterated analogues of the first three PAH as internal standards. The second isotope dilution, for benzo[a]pyrene, used the tritiated analogue as an internal standard. The PAH were isolated from extracts of cigarette smoke particulates using a two-step procedure based on selective extraction from aqueous dimethyl sulfoxide (DMSO) followed by chromatography on silica gel extraction columns. After isolation, aliquots of the samples were analyzed for phenanthrene, pyrene, and fluoranthene by gas chromatography with mass spectrometric detection (GC/MS). Separate aliquots of the samples were analyzed for benzo[a]pyrene by high pressure liquid chromatography with fluorescence detection followed by liquid scintillation spectrometry. PAH levels from cigarette smoke condensates collected from different exposure modes were compared; no exposure-related differences were found.

PRINCIPAL INVESTIGATORS

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Studies are being initiated at this Institute to determine the influence of chronic exposure to cigarette smoke on lung cancer risk from other toxicants. In the past, exposures of laboratory animals to cigarette smoke have mainly been conducted using nose-only techniques; these are costly and labor intensive. Alternatively, exposures to cigarette smoke may be conducted using whole-body exposure chambers; this approach permits longer daily exposure times and more total smoke deposition in the animals. However, the whole-body exposure approach must be evaluated for the impact of aging of the smoke as it passes through the larger volume of the exposure chambers. To compare the atmospheres during whole-body vs. nose-only smoke exposures, selected chemicals in the two smoke atmospheres were quantified. The chemical class of polycyclic aromatic hydrocarbons (PAH) was investigated because several members of the class are known carcinogens.

A number of previous studies have attempted to measure PAH in cigarette smoke, despite the low levels present (ppb to ppm).[1-4] For early studies, it was necessary to obtain the smoke condensates from as many as 100 to 2000 cigarettes for PAH quantitation.[1,2] As analytical techniques have become more sophisticated, the amount of condensate necessary for quantitation of selected PAH has decreased, and recent studies have used the condensate from as few as one cigarette.[3,4] However, the studies to be performed here at the Institute posed even more stringent constraints, because only 5-10 mg of condensate could be conveniently collected. Thus, methods were needed for quantitation of selected PAH using only a fraction of the condensate delivered by a typical reference cigarette.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from commercial vendors and used as acquired. Type 1R3 research cigarettes obtained from the Tobacco Health Research Institute (Lexington, KY) were used to

produce mainstream cigarette smoke in an AMESA Mark III automatic smoke generation machine (AMESA Electronics, Geneva, Switzerland). The different smoke generation and exposure methods were similar to those described previously (1986-87 Annual Report, LMF-120, pp. 68-71). The nose-only, intermittent (NOI) mode consisted of 10, 10-min exposures at 360 or 720 mg total particulate matter (TPM)/m^3 during each 6 h day. The nose-only, continuous (NOC) and whole-body continuous (WBC) modes consisted of continuous exposure to 100 or 200 mg TPM/m^3 during each 6 h exposure period.

The isolation scheme for PAH in cigarette smoke particulates is shown in Figure 1. Glass fiber filters with tobacco smoke condensate were placed into seven mL vials. About five mL of DMSO were added to each vial, followed by approximately 0.01 µCi (0.5 kBq) of ^{3}H-BaP and 100 ng each of perdeuterated phenanthrene, pyrene, and fluoranthene. The filters were sonicated for 1 h, and the DMSO decanted into a 15 mL centrifuge tube. Five mL of hexane were added to each tube and thoroughly mixed. The hexane was allowed to separate, and was decanted and discarded. Five mL of water were slowly added to the DMSO, followed by another five mL of hexane. After mixing, the hexane was decanted and collected into a separate vial. The extraction was repeated two more times, and the organic phases combined.

The hexane extracts were then passed through a silica gel extraction cartridge pre-equilibrated with hexane. The cartridge was eluted with three mL of 10% dichloromethane in hexane. The original hexane eluate and the 10% dichloromethane wash were combined, and evaporated in increments in a three mL reacta-vial with dry nitrogen. Care was taken to stop the nitrogen flow when the extract was just dried. The residue was taken up in 100 µL of acetonitrile for chromatographic analysis.

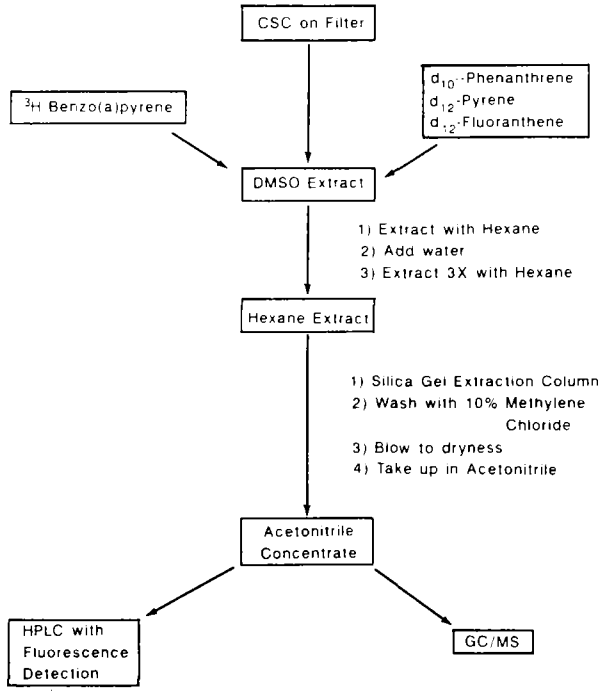

Figure 1. Fractionation of cigarette smoke particulate for PAH.

High Pressure Liquid Chromatography

We used a fluorescence detector for high pressure liquid chromatographic (HPLC) analysis of benzo(a)pyrene in the acetonitrile concentrate. Excitation and emission wavelengths were 365 and 420 nm, respectively. We used C18 absorbant, 150 mm x 4.6 mm, with 3 µm particle size in the chromatographic column. The mobile phase was 95% acetonitrile and 5% water, isocratic, at 1

mL/min. Ten microliters of the acetonitrile mixtures were injected. Standard curves were created by injecting ^{14}C-BaP (21.7 mCi/mmole) and collecting eluate corresponding to the BaP peak. The eluate was counted for ^{14}C radioactivity by liquid scintillation spectrometry, and the amount of BaP was calculated from the known specific activity. Peak areas due to the BaP were plotted against the mass to create the standard curve. For injections of cigarette smoke particulates, the peak with a retention time corresponding to BaP was collected and counted for tritium by liquid scintillation spectrometry. The total BaP in the original particulate was computed from the chromatographic peak area and the standard curve, and was corrected for the percent recovery determined from the quantities of triated BaP added before and measured after HPLC analysis.

Gas Chromatography/Mass Spectroscopy

Analyses of phenanthrene, fluoranthene, and pyrene were carried out using a GC/MS equipped with an HP Ultra 1 methyl silica capillary column, 25 m x 0.22 mm, with 0.11 μm film thickness. The transfer line was heated to 280°C. Electron multiplier voltage was 1800 EV. GC conditions were an initial temperature of 140°C held for 3 min, followed by a 20°C/min ramp to 300°C. Injections were split/splitless, with the purge on at 0.5 min. The injector temperature was held at 250°C.

The mass spectrometer was tuned to the calibrant ions 69, 219, and 502. Selected ion monitoring was performed using the ions 178 and 188 from 5-8 min (phenanthrene and d_{10}-phenanthrene), and ions 202 and 212 from 8-12 min (pyrene, fluoranthene, d_{12}-pyrene and d_{12}-fluoranthrene). The dwell time was 100 ms.

RESULTS AND DISCUSSION

The amount of particulate used in this study, about 6-10 mg, yielded a measurable quantity of the selected PAH. Attempts to analyze less particulate (2 mg or below) usually resulted in an insufficient quantity of the three and four ring PAH for analysis by GC/MS. No effort was made to systematically determine the best extraction solvent for optimal recovery of PAH from filters; however, very little mass appeared to be associated with the filters after extraction using DMSO. In addition, recoveries of the added tritiated BaP were consistently high (> 90%). Use of DMSO for extraction of the filters was ideal, as the first step in the isolation procedure was based on the selective extraction of the PAH from aqueous mixtures of this solvent. Recoveries of the PAH in the remaining steps of the isolation procedure were not determined, as the internal standard naturally corrects for any losses which take place.

Contrary to previous reports,[4] the DMSO extraction proved to be effective as an initial sample clean-up. Attempts to apply filter extracts directly to the silica gel extraction cartridges resulted in overloading of the adsorbant. The DMSO extraction procedure has also been used in fractionation of a variety of other complex chemical mixtures.[5]

Tomkins *et al.*[3] have effectively used silica gel extraction cartridges for the isolation of BaP from smoke particulates. The elution conditions used in this work were similar to those previously described.[3]

The BaP selectivity of the final analytical procedure has been reported previously.[3] Briefly, the most likely interferences in the reverse phase separation include benzo[e]pyrene, benzo[b,j or k]fluoranthene, perylene, and alkylated BaP analogs. With the exception of the alkylated analogues, standards were available for all of the above. When each of these were injected onto the HPLC, using the conditions described above, BaP could be distinguished by either a difference in retention time, or by significantly higher fluorescence response factor (> 100) at the excitation and emission wavelengths. The alkylated analogues were expected to have retention times significantly greater than BaP.[3]

Few interferences are likely for the GC/MS assay. No other PAH share the common molecular weight of fluoranthene and pyrene (202 amu), and only anthracene is isomeric to phenanthrene (178 amu). Anthracene is easily separable from phenanthrene, and can be seen as a small peak eluting immediately after phenanthrene in Figure 2 (7.2 and 7.25 min). The selectivity of the assay is further established by the close elution of the non-deuterated analytes with the perdeuterated internal standards.

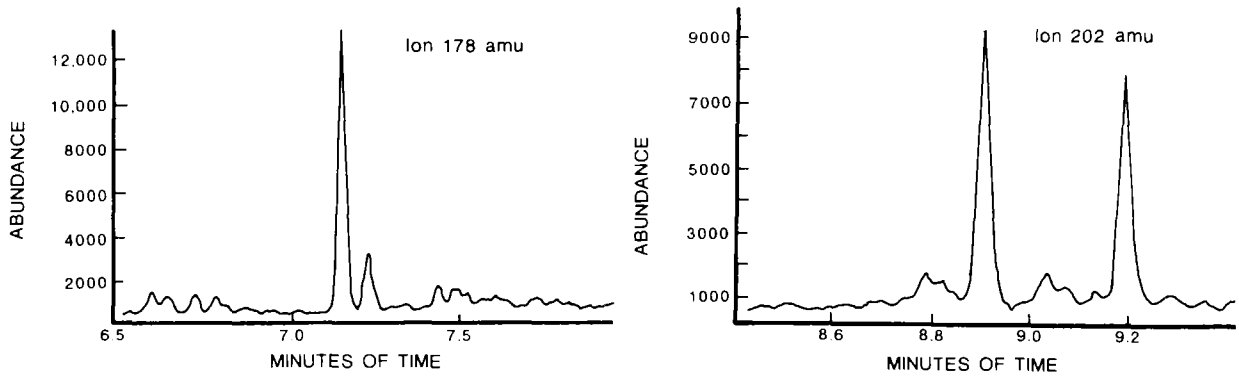

Figure 2. GC/MS with selected ion monitoring for PAH in cigarette smoke particulate.

The concentrations of the selected PAH found in cigarette smokes generated from the different exposure modes is shown in Table 1. The results indicate no significant differences ($p > 0.05$, two-tailed t test). These results are similar to those found for a variety of other chemicals measured in both the vapor and particulate phases of the cigarette smokes.

Table 1
Polycyclic Aromatic Hydrocarbon (PAH) Concentrations of Smoke Atmospheres in the Nose-Only Intermittent (NOI), Nose-Only Continuous (NOC), and Whole-Body Continuous (WBC) Exposure Modes

PAH[a]	NOI	NOC	WBC
Phenanthrene	17.9 ± 1.8	18.1 ± 2.2	14.7 ± 2.1
Pyrene	4.9 ± 0.5	4.9 ± 0.6	4.1 ± 0.1
Fluoranthene	6.1 ± 0.7	5.8 ± 0.5	5.0 ± 0.1
Benzo[a]pyrene	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1

[a]Measured during exposures, n = 4. Values are means ± standard errors. Units are ng PAH/mg extract.

REFERENCES

1. Griest, W. H., H. Kubota, and M. R. Guerin. Resolution of Polynuclear Aromatic Hydrocarbons by Packed Column GLC, Anal. Lett. 8: 949-957, 1975.

2. Davis, H. J., L. A. Lee, and 1. R. Davidson. Fluorometric Determination of Benzo[a]pyrene in Cigarette Smoke Condensate, Anal. Chem. 38: 1752-1755, 1966.

3. Tomkins, B. A., R. A. Jenkins, W. H. Griest, R. A. Reagan, and S. K. Holladay. Liquid Chromatographic Determination of Benzo[a]pyrene in Total Particulate Matter of Cigarette Smoke, J. Assoc. Off. Anal. Chem. 68: 935-940, 1985.

4. Risner, C. H. The Determination of Benzo[a]pyrene in the Total Particulate Matter of Cigarette Smoke, J. Chromatogr. Sci. 26: 113-120, 1988.

5. Natusch, D. F. S. and B. A. Tomkins. Isolation of Polycyclic Organic Compounds by Solvent Extraction With Dimethyl Sulfoxide, Anal. Chem. 50: 1429-1434, 1978.