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Note

Gas–liquid chromatographic assay of polycyclic aromatic hydrocarbon mixtures: specifically modified method for rat tissues

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The metabolism of polycyclic aromatic hydrocarbons (PAHs) and mechanisms through which they induce tumours in laboratory mice and rats following administration of single compounds have been widely studied. However, little is known about the distribution, availability, elimination and interactions of PAHs when a mixture is administered. Until only a few years ago there were technical limitations to this approach: the few reported methods involved too many steps; the analyses were therefore time consuming and failed to achieve acceptable separations of some compounds. Dao *et al.*[1] described a fluorimetric method for the determination of some PAHs in various organs of rats; this method, however, could not be applied to PAH mixtures because it required the preliminary separation of all of the individual compounds. Analogous studies were reported by Flesher[2] on [^{3}H]7,12-dimethylbenzanthracene (DMBA) and by Kotin *et al.*[3] and Schlede *et al.*[4] on [^{14}C]-3,4-benzopyrene. As these methods required different isotopes for to labelling each polycyclic hydrocarbon and did not discriminate between radioactivity bound to the parent compound and its metabolites, they are difficult to use for the analysis of PAH mixtures.

Gammal *et al.*[5] applied a combined method [thin-layer chromatography + gas–liquid chromatography (GLC)] in a study of distribution of 7,12-DMBA in some organs of rats, but the separation of the isomers was not possible. Howard *et al.*[6], McGinnis and Norris[7] and Grimmer and Hildebrandt[8] developed procedures for determining PAH mixtures in smoked foods, high-protein foods, oils, fats and yeasts; because of the high specificity and sensitivity required for such studies, the methods involved many purification steps and very lengthy procedures.

Subsequently, Santoro *et al.*[9] developed a method for the determination of 11 PAHs in yeasts, involving a relatively rapid analyses and giving high sensitivity. This present paper describes modifications to this method intended to simplify extraction and to accelerate the assay of PAHs in different rat tissues for kinetic studies.

EXPERIMENTAL

Chemicals

Triphenylene and benzo[*e*]pyrene were supplied by Aldrich (Beersee, Belgium), chrysene by EGA Chemie (Steinheim, G.F.R.) and benz[*a*]anthracene by Fluka (Buchs, Switzerland). All PAHs were of 99% purity.

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N,N′-Bis(*p*-phenylbenzylidene)-α,α′-bi-*p*-toluidine (BPhBT) was prepared by condensation of toluidine and *p*-phenylbenzaldehyde as described in previous papers[9,10]. Silica gel 60 (70–230 mesh ASTM) was activated for 3 h in an oven at 120°C, then water was added (15%) with shaking. The silica gel was stored in a stoppered flask at room temperature for about 2 h in order to allow the water to distribute evenly. Alumina (Merck, 90 Aktiv) was prepared as described[9].

All other reagents were of analytical-reagent grade and the water was doubly distilled.

Animals

Tissues obtained from CD-COBS rats (Charles River, Italy) were utilized. Blood was collected in a test-tube containing 10% (v/v) of heparin (2 I.U.). Mammary and parametrial adipose tissue, liver and brain were rapidly excised and frozen on solid carbon dioxide. All samples were stored at −20°C until taken for analysis.

Apparatus

A Model 2450 gas chromatograph (Carlo Erba, Milan, Italy) with a flame-ionization detector and a column (1.20 m × 3 mm I.D.) packed with 2.5% (w/w) BPhBT on Chromosorb W 100 were used; deteails of the column preparation and conditions for the GLC assay were the same as previously described[9,10].

Initial preparation of samples for PAH assay

After thawing, blood was diluted with 10 ml of doubly distilled water; brain and liver were homogenized in 5 volumes of water (for liver homogenate only, an amount corresponding to 2.5 g of the organ was used for PAH analysis).

Mammary and adipose tissue were hydrolysed [ethanol (10 volumes) + water (3 volumes) + potassium hydroxide (1 g per g of tissue)] for 1 h under reflux at 80°C on a hot-plate with a magnetic stirrer.

Extraction

All samples were extracted three times with 10 ml of cyclohexane (at 50°C, shaking for 1 min, centrifugation for 5 min at 2500 *g*). The extracts of hydrolyzed adipose tissue were further washed twice with water (10 ml) and dried with Na_2SO_4.

Purification

For blood, brain, liver and mammary tissues, the extracts were concentrated to 0.3–0.4 ml under a flow of nitrogen in a water-bath at 40°C, then purified by chromatography on alumina [5 g with 10% (w/w) of water per sample] in a column (1 cm I.D.) with a fritted disc and a PTFE stopcock; no pre-washing of the column was needed. The concentrate and the following washes of the 50-ml test-tubes (four portions of 0.3–0.4 ml of cyclohexane) were applied with a glass pipette on the top of the column. The PAHs were eluted with cyclohexane (10 ml). For the extracts of adipose tissue purification by chromatography on alumina [5 g with 10% (w/w) of water) in a column of 1.3 cm I.D., no pre-washing of the columns was needed.

The extracts and cyclohexane washings of the 50-ml test-tubes were eluted, cyclohexane (10 ml) was added to the column and the eluate was collected in a 50-ml

test-tube, and concentrated to 0.3–0.4 ml under a stream of nitrogen in a water-bath at 40°C.

A further purification step by chromatography on silica gel[9] was always required for the samples of adipose tissue.

GLC analysis

The packing material [2.5% (w/w) of N,N′-bis(*p*-phenylbenzylidene)-α,α′-bi-*p*-toluidine on 100–120 mesh Chromosorb W HP] was prepared by the solvent slurry method, fluidized, dried with nitrogen and resieved to 100–120 mesh. The column was conditioned overnight at 290°C with a flow of carrier gas (nitrogen) at 8 ml/min. The oven temperature was 265°C, injector temperature 275°C, air flow-rate 300 ml/ml, hydrogen flow-rate 22 ml/min and carrier gas flow-rate 30 ml/min. An internal standard [benzo(*e*)pyrene] was added to the alumina or silica gel eluate and the sample was concentrated to about 100 μl under a stream of nitrogen stream at 40°C, thoroughly washing the test-tube walls; 1–2 μl of the concentrate were injected into the gas chromatograph. The correction factor for each PAH was calculated using the internal standard as described previously[9].

RESULTS AND DISCUSSION

The modifications introduced for the GLC determination of PAHs in rat tissues shorten the method of Santoro *et al.*[9] and permit the rapid analysis of many different biological samples. About 60–100 analyses of tissue PAHs can be performed by two technicians each week. The time saving in the performance did not result in a decrease in specificity and sensitivity. As indicated in Table I, the recovery was satisfactory, ranging from a minimum of 83% to a maximum of 95%. The sensitivity limits were 0.05 μg for the three isomers. The reproducibility of the results was particularly good as indicated by the low standard errors reported in Table I. The coefficient of variation ranged between 1 and 5%.

The application of this method to the determination of the kinetic parameters of PaHs administered singly or in mixtures to rats will be reported elsewhere.

TABLE I

RECOVERY OF POLYCYCLIC AROMATIC HYDROCARBON MIXTURES

10 μg of each hydrocarbon were added per gram of rat tissue. Results are means ± standard errors of 5 experiments.

Tissue	*Triphenylene (μg)*	*Chrysene (μg)*	*Benz[a]anthracene (μg)*
Blood	9.551 ± 0.091	9.541 ± 0.012	9.285 ± 0.073
Liver	8.589 ± 0.131	8.390 ± 0.130	8.342 ± 0.121
Brain	9.187 ± 0.142	8.756 ± 0.288	8.729 ± 0.229
Mammary tissue	9.355 ± 0.175	9.344 ± 0.263	9.313 ± 0.146
Parametrial adipose tissue	8.892 ± 0.135	9.478 ± 0.441	8.995 ± 0.127

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REFERENCES

1 T. L. Dao, F. G. Bock and S. Crouch, *Proc. Soc. Exp. Biol. Med.*, 102 (1959) 635.
2 J. W. Flesher, *Biochem. Pharmacol.*, 16 (1967) 1821.
3 P. Kotin, H. L. Falk and R. Busser, *J. Nat. Cancer Inst.*, 23 (1959) 541.
4 E. Schlede, R. Kuntzman, S. Haber and A. H. Conney, *Cancer Res.*, 30 (1970) 2893.
5 E. B. Gammal, K. K. Carroll, B. H. Muhlstock and E. R. Plunkett, *Proc. Soc. Exp. Biol. Med.*, 119 (1965) 1086.
6 J. W. Howard, R. T. Teague, Jr., and R. H. White, *J. Ass. Offic. Anal. Chem.*, 49 (1966) 595.
7 E. L. McGinnis and M. S. Norris, *J. Agr. Food Chem.*, 23 (1975) 221.
8 G. Grimmer and A. Hildebrandt, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 631.
9 A. Santoro, R. Modica, S. Paglialunga and I. Bartošek, *Toxicol. Lett.*, 3 (1979) 85.
10 G. N. Janini, G. M. Muschik, J. A. Schroer and W. L. Zielinski, Jr., *Anal. Chem.*, 48 (1976) 1879.