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SHORT COMMUNICATION

Detection of metabolites of polycyclic aromatic hydrocarbons in human urine

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A non-invasive assay has been developed for the recovery of *r*-7,*t*-8,*t*-9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[α]-pyrene (BP-7,10/8,9-tetrol) from human urine. This tetrol is excreted as a metabolite of benzo[α]pyrene (BP) in a process catalyzed by cytochrome P450 enzymes and epoxide hydrolases. Urine was hydrolysed to release activated benzo[α]-pyrene-diol-epoxides covalently bound to macromolecular species or conjugated tetrols. The relatively non-polar organic molecules from urine hydrolysates were collected on octadecasilane chromatography columns (Sep-Paks). Materials eluted in solvent (80% CH_3OH), were further purified on immunoaffinity columns with antibodies raised against anti-N2-[10(7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[α]pyrenyl)]-guanosine. HPLC was then used to isolate BP-7,10/8,9-tetrol, which was quantitated by synchronous fluorescence spectroscopy (SFS). This assay detected 0.24–3.12 pmol BP-7,10/8,9-tetrol per ml urine (limit of detection 0.01 pmol/ml, given 10 ml urine), in four study subjects. Reproducibility was assessed by adding tritium labeled BP-7,10/8,9-tetrol (1500 fmol) to a urine sample previously identified to contain the tetrol at levels below the limit of detection of the fluorescence assay; a recovery of >30% of the added radioactivity was achieved (510 ± 64 fmol, mean ± SD, *n* = 3). Because HPLC alone was not sufficient to isolate materials for quantitation by SFS directly from human urine, immunoaffinity chromatography was found to be a necessary preparatory step in BP-7,10/8,9-tetrol isolation. These data demonstrate the presence of tetrahydrotetrol metabolites of BP in human urine and suggest that measurement of BP-7,10/8,9-tetrol and other polycyclic aromatic hydrocarbon-tetrols may prove to be valuable dosimeters of human internal exposure to polycyclic aromatic hydrocarbons.

The polycyclic aromatic hydrocarbon (PAH*) benzo[α]pyrene BP has been measured in food consumed by humans (char-broiled hamburger 1–133 μg/kg) (1–3), in the polluted air of urban and work environments (1–2220 μg/m^3 and 1–7300 μg/m^3 respectively) (3) and in human urine (4–10 μg/l) (4). Even though these levels of BP are readily detectable by mass spectrometry, metabolites have not been isolated or measured in human urine. However, the presence of BP in human urine, and measures of urine mutagenicity (with microsomal S-9 metabolic activation) imply internalization of procarcinogens (5). Therefore, measurement of *r*-7,*t*-8,*t*-9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BP-7,10/8,9-tetrol) in human urine would give a more useful indication of genotoxic exposure to the parent carcinogen than simply monitoring for BP or total BP-metabolites. An additional advantage would be improved specificity, compared to urine mutagenesis measurements, in a non-invasive bioassay. This report describes validation of an assay for identification and measurement of BP-7,10/8,9-tetrol in human urine using synchronous fluorescence spectroscopy (SFS).

The urine samples analyzed here were obtained from individuals consuming a diet rich in char-broiled food in order to increase the likelihood of PAH detection. No attempt has been made to describe a dose response in humans with such a small sample set. The data presented demonstrate the feasibility of measuring BP-7,10/8,9-tetrol in human urine and establish a practical method for this.

Urine samples were collected from four non-smoking volunteers. These volunteers had consumed a diet consisting of high levels of char-broiled food for 7 days, (details of which have been published previously (6)). Additional samples were collected from several laboratory personnel. To one of these additional samples, for which BP-7,10/8,9-tetrol was not detectable by SFS, [^{3}H]-BP-7,10/8,9-tetrol (sp. act. 1.6 Ci/mmol, prepared by hydrolysis from [±]-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydro[1,3-^{3}H]benzo[α]pyrene, National Cancer Institute, Chemical Carcinogen Reference Standard Repository, Bethesda, MD) was added (1.5 pmol/10 ml urine). Portions of the urine samples (10 ml) were acidified by the addition of HCl (concentrated (37%), 80 μl). The samples were heated (90°C, 3 h) and purified using octadecasilane chromatography columns (C18 Sep-Paks, Waters Associates, Milford, MA). Polar materials were removed by eluting with an aqueous solution of methanol (30%) while relatively non-polar materials, including tetrahydrotetrol metabolites of PAHs, were eluted with a higher methanol concentration (80%). The eluates (80% methanol) were dried under reduced pressure and redissolved in water (1 ml). Water soluble materials were then subjected to immunoaffinity chromatography using monoclonal antibodies produced by an hybridoma cell-line (8E11), that was prepared from the spleens of animals challenged with a mixture of anti-N2-[10(7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[α]pyrenyl)]-guanosine (BPDE-dG) and key-hole limpet haemocyanin (7). The preparation and development of immunoaffinity columns for the isolation of BPDE-dG has been described previously (8). An identical protocol was used for BP-7,10/8,9-tetrol. Materials not recognized by the antibodies were eluted with (Tris–HCl, 100 mM, pH 7.4), and materials captured by the antibodies were removed with

*Abbreviations: PAH, polycyclic aromatic hydrocarbon; BP, benzo[α]pyrene; BP-7,10/8,9-tetrol, *r*-7,*t*-8,*t*-9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[α]-pyrene; BPDE-dG, anti-N2-[10(7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[α]-pyrenyl)]-guanosine; SFS, synchronous fluorescence spectroscopy; BP-7/8,9,10-tetrol, *r*-7,*t*-8,*t*-9,*t*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[α]pyrene;BPDE I, *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene.

methanol. Methanol fractions were evaporated under reduced pressure, redissolved in water (500 μl) and subjected to HPLC on octadecasilane columns (250×4.6 mm) that were eluted with a water methanol gradient (30–100%, flow rate 1 ml/min). Fractions (500 μl) were collected, evaporated to dryness, redissolved in water (500 μl) and analyzed by SFS. For SFS the excitation and emission monochromators of an MPF-66 spectrophotofluorimeter (Perkin–Elmer Corp., Rockville, MD) were driven simultaneously with a wavelength difference of 34 nm, monochromator slits were set at 5 nm (8).

The reproducibility of the assay was tested by introducing 1500 fmol of tritium labeled BP-7,10/8,9-tetrol into each of three 10 ml urine samples from an individual not involved in the char-broiled food study (equivalent to 0.15 pmol/ml when compared to the human samples). Several laboratory personnel were screened to obtain a urine sample that was below the fluorescence detection limit of the assay. Radiomatic analysis (Beckman LS581 liquid scintillation counter, Beckman Instruments Inc., Fullerton, CA) showed that it was possible to recover >30% of [^{3}H]-BP-7,10/8,9-tetrol using a combination of immunoaffinity chromatography and HPLC (510 ± 64 fmol, mean ± SD; 34%) (Table I). These data were further corroborated by fluorescence analyses (485 ± 70 fmol; 32%) of the urine sample containing the radiolabelled standard. Recovery from immunoaffinity chromatography alone was in excess of 90%, most losses occurred during HPLC (data not shown).

A synchronous fluorescence spectrum obtained from an immunoaffinity chromatography/HPLC isolate of human urine is shown in Figure 1. These and other fluorescence spectral data demonstrate the presence in human urine samples of BP-7,10/8,9-tetrol. Levels of this tetrol were quantitated by comparison with a standard curve that was generated from the emission maximum (379 nm) of synchronous fluorescence spectra of authentic BP-7,10/8,9-tetrol samples. Emission peaks observed at 379 nm for appropriate HPLC fractions were compared to the fluorescent intensities generated from known amounts of authentic standard materials in order to determine BP-7,10/8,9-tetrol levels in human urine. In this pilot study four human urine samples have been assayed, and levels of BP-7,10/8,9-tetrol ranged from 0.24 to 3.12 pmol BP-7,10/8,9-tetrol/ml of urine (individual values: 0.24, 0.40, 0.76 and 3.12). The lower limit of assay sensitivity was determined to be 0.01 pmol/ml for a 10 ml sample of urine. These data were generated for four individuals who had consumed a diet containing relatively large quantities of char-broiled meat for 7 days (6).

A criterion for identification of BP-7,10/8,9-tetrol requires fluorescent materials to elute with a retention time equal to that of authentic reference material (19.0 min in the HPLC system described). For one individual a second peak of fluorescence was observed with a retention time of 21.5 min (Figure 2). SFS indicated the presence of the pyrene fluorophore (i.e. a PAH or metabolite containing a pyrene ring structure), and from the immunoaffinity chromatography it can be deduced that a compound related to BP-7,10/8,9-tetrol was present in these fractions (9,10). It was shown by HPLC of an authentic sample of *r*-7,*t*-8,*t*-9,*t*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[α]-pyrene (BP-7/8,9,10-tetrol) that the retention time for this isomer (the minor hydrolysis product of BPDE I, (11)) was also 21.0–21.5 min. These data indicate the excretion in human urine of materials that when hydrolysed can be shown to be derived from the highly carcinogenic BPDE I. However, the data provide no evidence for variation in stereoselectivity of metabolism of PAHs in humans. However, it has been previously reported in experiments where human tissues (skin) were treated with PAHs *in vitro* that stereochemically distinct metabolic pathways are possible (12,13). The data presented here provide no evidence for the formation in human tissues *in vivo* of diol-epoxides of BP other than the carcinogenic BPDE I.

Since BP is an ubiquitous environmental contaminant BP-tetrol levels will be compared with known exposures. Urine samples are being analyzed from lung cancer cases and controls (14), coal-tar treated psoriasis patients, healthy volunteers consuming char-

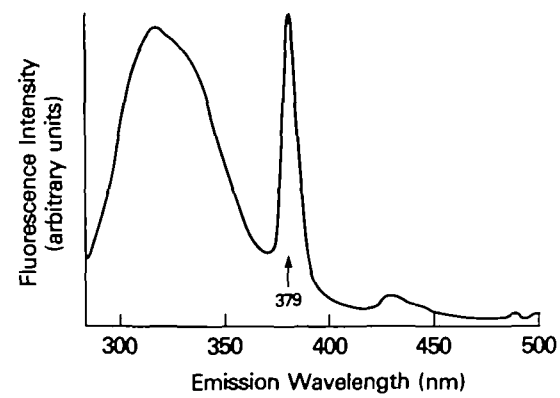

Fig. 1. Synchronous fluorescence spectrum of an isolate of human urine prepared by acid hydrolysis, octadecasilane column chromatography, immunoaffinity chromatography and HPLC. An intense signal (379 nm) specific for the pyrene fluorophore was found in HPLC fractions eluting with the same retention time as the BP-7,10/8,9-tetrol.

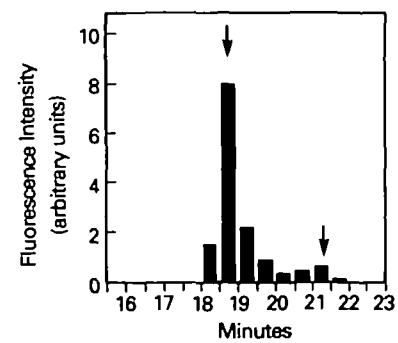

Fig. 2. Relative fluorescence intensities (SFSΔλ34 nm, emission peak 379 nm, see Figure 1) for fractions (30 s) eluting from the high performance liquid chromatograph. The retention time for the major hydrolysis product of the *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene-(BP-7,10/8,9-tetrol) is 19 min, whereas the minor product of this reaction BP-7/8,9,10-tetrol elutes at 21.5 min. The major (BP-7,9,10/8-tetrol) and minor (BP-7,9/8,10-tetrol) hydrolysis products of the *r*-7,*t*-8-dihydroxy-*c*-9,10 epoxy-7,8,9,10-tetrahydro-benzo[α]pyrene share a retention time of 20.5 min and were not detected in this analysis.

Table I. Recovery of BP-7,10/8,9-tetrol standard from human urine to which was added 1500 fmol of a tritium labelled BP-7,10/8,9-tetrol standard

Assay no.	Quantity recovered, detection by SFS (fmol/10 ml)	Quantity recovered, detection by [^{3}H] label (fmol/10 ml)
1	454	437
2	565	534
3	436	559
Mean ± SD	485 ± 70	510 ± 64
% Recovery	32	34

broiled hamburgers where PAH-adduct levels have been measured in lymphocytes by ELISA and other individuals potentially exposed to PAHs (6). Positive samples are also being screened by fluorescence excitation-emission matrix analysis to detect other potential carcinogens or their metabolites in the same samples (8). Benzo[*a*]pyrene tetrols that have been found in hydrolysates of human urine probably result from multiple metabolic pathways. These may include: exfoliated BP–DNA adducts, glutathione and glucuronide conjugates and protein adducts (15). Therefore, the origins of the BP-7,10/8,9-tetrol deserved further investigation.

A more sophisticated determination of the ratio of the level of parent hydrocarbon to the level of BP-7,10/8,9-tetrol metabolite might allow for determination of a meaningful metabolic phenotype. Currently, there is no simple *in vivo* phenotyping assay for CYP1A1, that is the isozyme thought to be primarily responsible for BP-hydroxylation. Caffeine demethylation has been used as an indication of CYP1A1 metabolism, however, this metabolic pathway is complex and has been shown to be more specific for CYP1A2 (16). The results of these studies may be highly complementary to a DNA–restriction fragment length polymorphism assay for CYP1A1 and glutathione-S-transferase which are currently being examined in a lung cancer case-control study (17). With regard to measures of cancer prevention, these studies may assist in the evaluation of human cancer risk associated with exposure to these compounds.

A protocol has been developed to isolate, identify and quantitate tetrols of BP (specifically BP-7,10/8,9-tetrol and BP-7/8,9,10-tetrol) in human urine. The procedure described for a 10 ml urine sample gives a consistent yield of >30% when 1500 fmol of BP-7,10/8,9-tetrol standard is present. Levels of BP-7,10/8,9-tetrol of between 0.24 and 3.12 pmol/ml were measured in samples donated by four human subjects.

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References

1. Fazio,T. and Howard,J.W. (1983) Polycyclic aromatic hydrocarbons in foods. In Bjorseth,A. (ed.), *Handbook of Polycyclic Aromatic Hydrocarbons*. Marcel Decker, New York, pp. 461–505.
2. Swallow,W.H. (1976) Survey of polycyclic aromatic hydrocarbons in selected foods and food additives available in New Zealand. *New Zealand J. Sci.*, **19**, 407–412.
3. International Agency for Research on Cancer (1973) *IARC Monographs on the Evaluation of Carcinogenic Risk of the Chemical to Man: Certain Polycyclic Aromatic Hydrocarbons and Heterocyclic Compounds. Vol. 3*. IARC, Lyon.
4. Haugen,A., Becher,G., Benestad,C., Vahakangas,K., Trivers,G.E., Newman,M.J. and Harris,C.C. (1986) Determination of polycyclic aromatic hydrocarbons in the urine, benzo(*a*)pyrene diol epoxide–DNA adducts in lymphocyte DNA, and antibodies to the adducts in sera from coke oven workers exposed to measured amounts of polycyclic aromatic hydrocarbons in the work atmosphere. *Cancer Res.*, **46**, 4178–4183.
5. Ames,B.N., McCann,J. and Yamasaki,E. (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat. Res.*, **31**, 347–364.
6. Rothman,N., Poirier,M.C., Baser,M.E., Hansen,J.A., Gentile,C., Bowman,E.D. and Strickland,P.T. (1990) Formation of polycyclic aromatic hydrocarbon–DNA adducts in peripheral white blood cells during consumption of charcoal-broiled beef. *Carcinogenesis*, **11**, 1241–1243.
7. Santella,R.M., Lin,C.D., Cleveland,W.L. and Weinstein,I.B. (1984) Monoclonal antibodies to DNA modified by a benzo[*a*]pyrene diol epoxide. *Carcinogenesis*, **5**, 373–377.
8. Weston,A. and Bowman,E.D. (1991) Fluorescence detection of benzo[*a*]pyrene–DNA adducts in human lung. *Carcinogenesis*, **12**, 1445–1449.
9. Santella,R.M., Weston,A., Perera,F.P., Trivers,G.E., Harris,C.C., Young, T.L., Nguyen,D., Lee,B.M. and Poirier,M.C. (1988) Interlaboratory comparison of antisera and immunoassays for benzo[*a*]pyrene-diol-epoxide-I modified DNA. *Carcinogenesis*, **9**, 1265–1269.
10. Vahakangas,K., Haugen,A. and Harris,C.C. (1985) An applied synchronous fluorescence spectrophotometric assay to study benzo[*a*]pyrene-diolepoxide–DNA adducts. *Carcinogenesis*, **6**, 1109–1115.
11. Yang,S.K., Deutsch,J. and Gelboin,H.V. (1978) Benzo[*a*]pyrene metabolism: activation and detoxification. In Gelboin,H.V. and Yang,S.K. (eds), *Polycyclic Hydrocarbons and Cancer*. Academic Press, New York, pp. 205–231.
12. Weston,A., Hodgson,R.M., Hewer,A.J., Kuroda,R. and Grover,P.L. (1985) Comparative studies of the metabolic activation of chrysene in rodent and human skin. *Chem. Biol. Interactions*, **54**, 223–242.
13. Hall,M. and Glover,P.L. (1990) Polycyclic aromatic hydrocarbons: metabolism, activation and tumor initiation. In Cooper,C.S. and Grover,P.L. (eds), *Chemical Carcinogenesis and Mutagenesis II*. Springer, New York, pp. 327–359.
14. Caporaso,N.E., Tucker,M.A., Hoover,R., Hayes,R.B., Pickle,L.W., Issaq,H., Muschik,G., Green-Gallo,L., Buivys,D., Aisner,S., Resau,J., Trump,B.F., Tollerud,D., Weston,A. and Harris,C.C. (1990) Lung cancer and the debrisoquine metabolic phenotype. *J. Natl Cancer Inst.*, **85**, 1264–1272.
15. Osborne,M.R. and Crosby,N.T. (1987) Benzopyrenes. In *Cambridge Monographs on Cancer Research*. Cambridge University Press, New York, pp. 73–164.
16. Butler,M.A., Iwasaki,M., Guengerich,F.P. and Kadlubar,F.F. (1989) Human cytochrome $P\text{-}450_{PA}$ (P-450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. *Proc. Natl Acad. Sci. USA*, **86**, 7696–7700.
17. Shields,P.G., Sugimura,H., Caporaso,N.E., Petruzzelli,S.F., Bowman,E.D., Trump,B.F., Weston,A. and Harris,C.C. (1992) polycyclic aromatic hydrocarbon–DNA adducts in human lung. *Environ. Health Perspect.*, **38**, 191–194.

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