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Incorporation of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) into hair of mice

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The incorporation of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) into hair of newborn mice was investigated in order to contribute to the validation of PhIP in hair as a suitable biomarker for human dietary exposure. Black mice (C57BL/6J; 7–9 days old) were given graded doses of [^{3}H]-PhIP subcutaneously during the start of the hair growth period. The distribution of [^{3}H]-PhIP and incorporation into hair were investigated by tape-section autoradiography. Almost all the radioactivity in hair represented PhIP as shown by high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS). A dose–response proportionality of incorporation into hair was found when incorporation was determined by liquid scintillation counting. Autoradiography showed that PhIP was rapidly cleared from the skin, but remained for at least 28 days in the part of the hair shafts which was formed during the exposure period. The present results obtained using the mouse as a model, further support the suggestion that PhIP in hair may be a suitable biomarker for human exposure to dietary PhIP.

Keywords: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), biomarker, hair, mouse, liquid scintillation counting, autoradiography.

Abbreviations: HAA, heterocyclic aromatic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; GC–MS, gas chromatography–mass spectrometry; SIM, selected ion monitoring; HREI, high resolution electron impact; HPLC, high performance liquid chromatography.

Introduction

Heterocyclic aromatic amines (HAAs) are formed in meat during regular cooking (Knize *et al.* 1997, Skog *et al.* 1997). These compounds are potent mutagens, carcinogenic in laboratory animals, and are suspected to play a role in the causation of human cancers. The formation of HAAs varies, depending on cooking method, from undetectable levels to 270 ng g^{-1} in grilled and fried meat (Knize *et al.* 1997). Since intake of meat, cooking methods and HAA levels in cooked food vary greatly, it is difficult to obtain a valid measure of individual HAA intake using a questionnaire. More reliable measures of dietary HAA intake might be obtained by suitable biomarkers.

The levels of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) and MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), the most abundant of

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the heterocyclic amines in cooked meat, have been investigated in human blood and urine by several groups (Murray *et al*. 1989, Ushiyama *et al*. 1991, Lynch *et al*. 1992, Ji *et al*. 1994, Turesky *et al*. 1994, Poirier 1997, Reistad *et al*. 1997). However, due to the rapid excretion of HAAs, the urine levels only reflect a very recent intake. Furthermore, the levels of serum protein adducts formed from PhIP and MeIQx are very low and therefore not suited as biomarkers (Lynch *et al*. 1993, Reistad *et al*. 1994).

The concentrations of several drugs and toxicants in hair have been shown to be powerful indicators of long/medium-term exposure (Uematsu *et al*. 1992, DuPont and Baumgartner 1995, Nakahara *et al*. 1995). Since several HAAs show affinity to melanin, we are currently investigating whether PhIP in hair could be a biomarker for a longer time period of exposure. Recently, we found that PhIP accumulated in hair from humans consuming a regular diet containing cooked meat (Reistad *et al*. 1999).

The objective of the present study was to evaluate the dose–response relationship of PhIP incorporation in hair by the use of a mouse model. Furthermore, we studied the time course of incorporation and retention of [^{3}H]-PhIP into mouse hair by autoradiography.

Materials and methods

Chemicals

[^{3}H]PhIP was purchased from Chemsyn Science Laboratories, Lexena, Kansas. [$^2H_3$]PhIP was a gift from Dr Spiros Grivas, Swedish University of Agricultural Sciences, Uppsala, Sweden. Sodium dodecyl sulphate (SDS) was purchased from Fluka Chemie AG, Buchs, Switzerland and Aquasafe 300 from Zinsser Analyte, Maidenhead Berks, UK. Other chemicals were of HPLC or analytical grade quality.

Animals

Black mice (C57/bl) purchased from The Jackson Laboratory (USA) were bred at the National Institute of Public Health, Oslo, Norway. They were given the breeding diet SDS RM3(E) (Special Diet Services Ltd, Essex, UK) and water *ad libitum*. The mice were kept in plastic cages in a room with 12/12h light/dark cycle, controlled humidity and temperature. At 7–9 days after birth (≈5 g), when hair growth of the naked pups could be observed, they were injected subcutaneously (s.c.) once daily for 4 days. For the purpose of PhIP determination in hair, groups of mice were given totally 0.25, 1, 10 or 25 μCi [^{3}H]-PhIP/mouse corresponding to 30, 120, 1170 or 2920 ng per mouse. [^{3}H]-PhIP was dissolved in 0.9 % saline. The mice were killed by gaseous CO_2 30 days after [^{3}H]-PhIP administration. The hair was shaved from the backs of the mice and prepared for analysis as described below. For the purpose of autoradiography, each mouse was given a total dose of 50 μCi [^{3}H]-PhIP, corresponding to 5840 ng. The mice were killed by gaseous CO_2 at day 1, 3, 7, 14, 21 and 28 after [^{3}H]-PhIP exposure, and frozen on dry ice.

Treatment and analysis of hair samples

The hair samples were weighed, washed with 0.1 % SDS (10 ml/100 mg hair) and rinsed three times in water (10 ml/100 mg hair), using whirlimixing for 1 min, ultrasonication for 1 min and collected by centrifugation, followed by decanting of the liquid. Care was taken to ensure minimal loss of sample (< 1 % w/w, totally) upon decanting. To the dried hair sample 1 N NaOH (3 or 15 ml/100 mg hair) was added and kept at 100 °C for 1 h. Duplicate samples of 100–200 μl of the hair suspensions were mixed with 10 ml Aquasafe 300 and counted for 5 min, using a Packard Liquid Scintillation Analyzer. Aliquots of 0.1–4.5 ml of the solubilized hair sample from each dose level were further treated by solid phase extraction using a Bond Elut ENV+ column (IST Isolute, UK). The column was preconditioned successively with 5 ml 50 % ethyl acetate/50 % methanol–ammonia (v/v), 5 ml methanol and 5 ml 20 mM ammonium acetate buffer (pH 5.3). Methanol–ammonia was a mixture of methanol and concentrated ammonia (33 %), 50:1, (v/v). After loading the sample, the column was washed with 10 ml 20 mM ammonium acetate buffer followed by 5 ml methanol. The analytes were eluted with 5 ml 50 % ethyl acetate/50 % methanol–ammonia (v/v). The sample was taken to dryness by nitrogen gas and dissolved in 100 μl of methanol. The HPLC equipment consisted of the following components: a Perkin-Elmer

series 4 HPLC pump, a Hewlett Packard 1040A photodiode array detector and a 7125-075 Rheodyne injector with a 100 µl sample loop. Column: Nova Pak C_{18}, 100 × 8 mm (Waters Chromatography Division/Millipore Corporation, Milford, MA, USA). The following gradient was used with a flow rate of 1 ml min^{-1}: 5 min isocratic elution with 81 % water/19 % methanol (v/v) containing 0.1 % diethylamine (pH 7.0), followed by a 20 min linear gradient to 100 % methanol with 0.1 % diethylamine and finally 10 min isocratic elution. Injection volume was 20 µl, and the effluent from the column was collected in 1 ml fractions. Aquasafe 300 was added, and the radioactivity was recorded in a Packard Liquid Scintillation Analyzer. For GC–MS analyses, 5 ng of internal standard, [$^2H_3$]PhIP, dissolved in methanol, was added to 2 ml of the solubilized hair (dose level, 2920 ng) and purified by solid phase extraction (Bond Elut, ENV+).The analytes were eluted with 50 % ethyl acetate/50 % methanol–ammonia (v/v), and the sample was brought to dryness by nitrogen gas and derivatized for GC–MS by acylation and methylation (Reistad *et al*. 1997).

Gas chromatography–mass spectrometry

The mass spectrometers were operated in the high-resolution electron impact (HREI) mode with selected ion monitoring (SIM). Good selectivity and sensitivity were obtained by monitoring the main fragment $(M–C_3F_7)^+$. An HP 5890 Ser. 2 GC coupled to a VG AutoSpec high-resolution MS instrument was used, with a column of HP 5 MS, 60 m, i.d. 0.25 mm, film thickness 0.1 µm. Temperature programme: 100 °C, 1 min, raised to 300 °C at 30 °C min^{-1}, held at 300 °C for 5 min. Other MS conditions were: resolution 8000, source temp. 265 °C, and electron energy 42 eV. The base peak $(M–C_3F_7)^+$ was recorded. Selected ions were PhIP (*m/z* 265.1089) and [$^2H_3$]PhIP (*m/z* 268.1277).

Tape-section autoradiography

The frozen animals were embedded in a gel of carboxymethylcellulose (1%) and immersed in a bath of hexane and solid carbon dioxide (–75 °C). Sagittal whole-body sections (30 µm) from different levels of the body were collected at –20 °C on tape (No. 821, 3M Co., St. Paul, MN, USA) in a PMV cryomicrotome (PMV 450 MP, Palmstierna Mekaniska Verkstad, Stockholm, Sweden) according to the method of Ullberg (1977). The tissue sections were freeze-dried at –20 °C for 48 h. All sections were apposed to Hyperfilm 3H (Amersham, UK). The films were developed after an exposure time of 3 weeks.

Statistical analysis

The dose–response correlation was calculated by the non-parametric Spearman rank order correlation test (SigmaStat software, Jandel Scientific, Germany). A *P*-value of <0.05 was regarded as significant.

Results

Quantification of PhIP incorporation into hair

At 7–8 days of age, the hair growth of the mice could be observed visually. The hair grew uniformly and rapidly during the dosing period. About 4 weeks after the administration the hair was long enough for the fur to be shaved off. The concentrations of [^{3}H]-PhIP in the hair of mice correlated significantly with the given doses; $r = 0.960$ (figure 1). Significant differences were seen between the levels of all dosing groups when compared with each other ($P < 0.05$), showing that higher doses resulted in higher concentrations of [^{3}H]-PhIP in hair. The total hair samples contained approximately 10 % of the dose at all dose levels. By visual judgement, about three-fifths of the total weight of fur had been shaved off, hence roughly 15 % of the dose was incorporated into hair. In order to verify that the radioactivity in the hair samples was due to PhIP and not a metabolite, an aliquot of a solubilized hair sample from each dose group was further purified using an ENV+ column and analysed by HPLC. During the extraction procedure, fractions of each step were collected, and radioactivity measured. Most of the radioactivity (70–80 %) was recovered in the HAA eluting fraction. As shown in figure 2 the radioactive peak in the hair sample had the same retention time as that of the PhIP

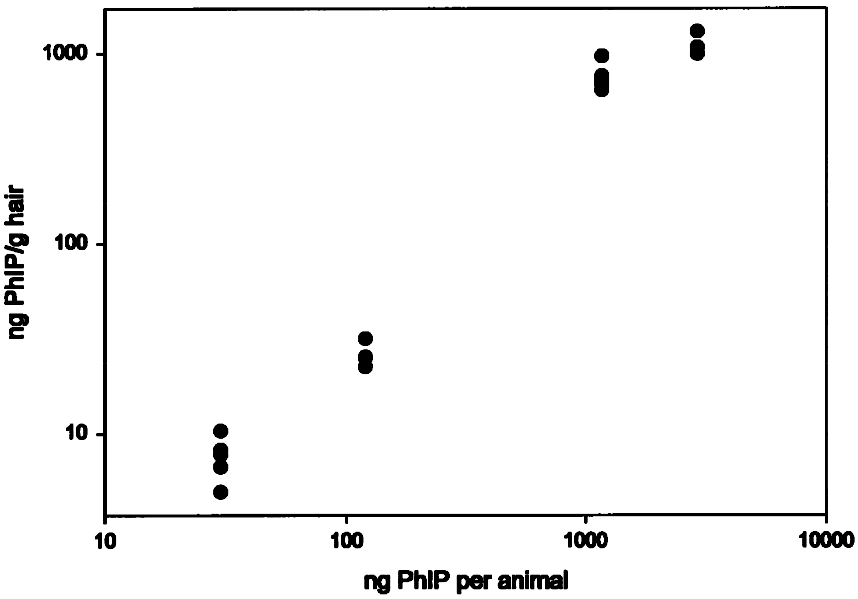

Figure 1. Relationship between the given doses of PhIP (ng per animal) and PhIP (ng g^{-1} hair) in the hair as measured by liquid scintillation counting. Newborn pigmented mice (C57BL/6J) exposed to [^{3}H]-PhIP subcutaneously once daily for 4 days (corresponding to 30, 120, 1170, 2920 ng per mouse). The concentration of PhIP was determined in the hair 30 days after the last PhIP exposure.

standard. In order to further confirm the identity, three aliquots of the hair samples at the highest dose level (2920 ng) were extracted and prepared for GC–MS analysis. PhIP was found at a concentration of 804 ng g^{-1} hair, in comparison with the determination based on radioactivity which gave 1075 ng g^{-1} hair. PhIP was not detected in the hair of an untreated mouse.

Tape-section autoradiography

The time course pattern of incorporation and retention of radioactivity into hair was studied by tape-section autoradiography. At 24 h after the last injection, the autoradiograms of the [^{3}H]-PhIP-treated mice showed accumulation and retention of radioactivity mainly in the hair roots whereas little could be seen in other parts of the skin (figure 3(A)). At day 3 most of the radioactivity had disappeared from the hair roots, and appeared in the hair shafts (figure 3(B)). At day 7 all the radioactivity was cleared from the hair roots and was present only in the hair shafts (figure 3(C)). Fourteen, 21 and 28 days after administration, the radioactivity still remained in the hair shafts. The autoradiograms at day 28 showed that the radioactivity was absent from the newly formed segments of the hair shafts, but remained in the part of the hair shafts formed during the exposure period (figure 4).

Discussion

In a previous study we found that the food mutagen PhIP can be detected and quantified in hair from humans consuming a regular diet. PhIP was found in 12 out

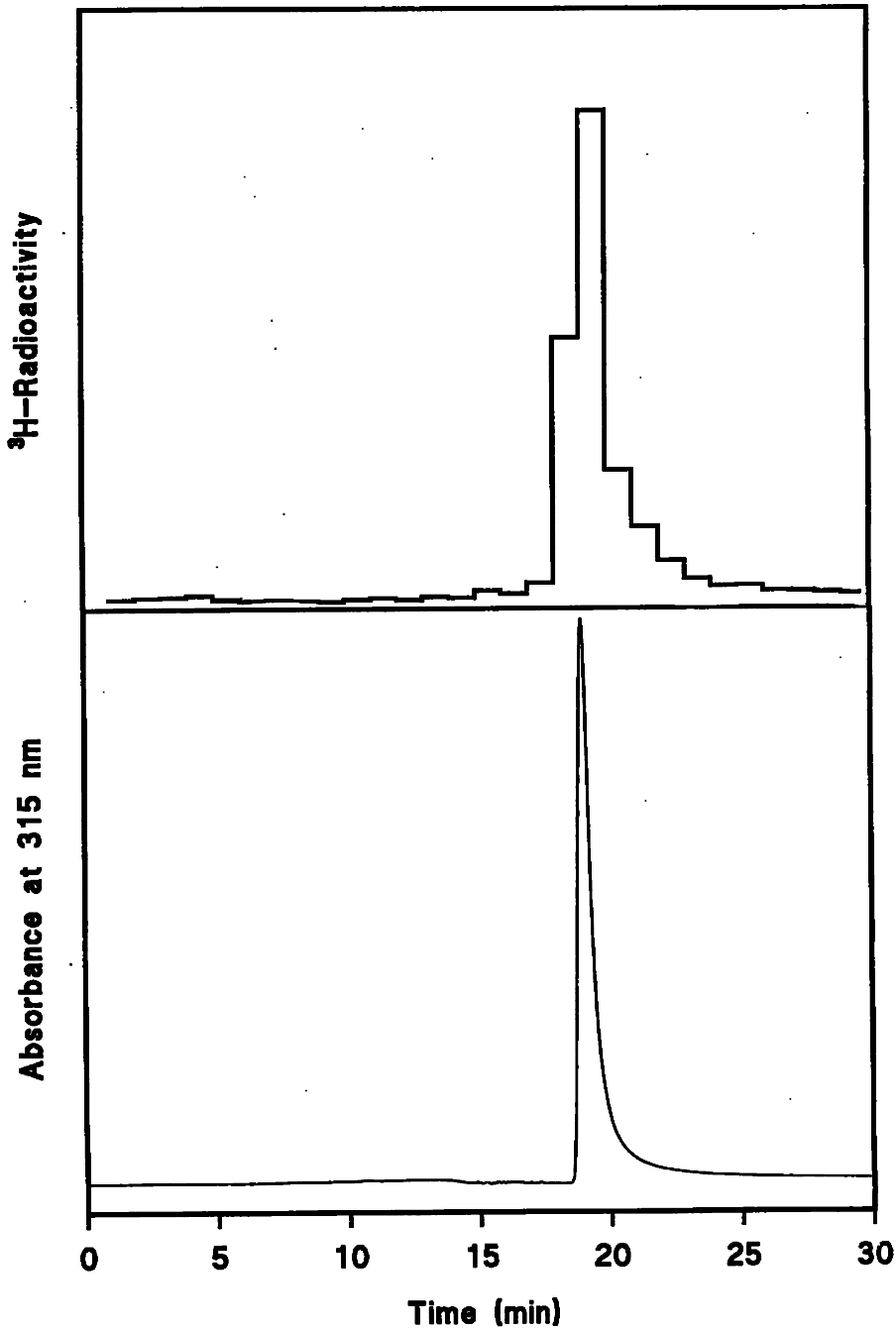

Figure 2. Reversed phase HPLC separation of PhIP (A) [^{3}H] radiochromatogram of PhIP (146 μg kg^{-1} body weight), (B) $UV_{315\ nm}$ chromatogram of a standard of PhIP.

of 14 hair samples, in the range of 50–5000 pg g^{-1} hair (Reistad *et al.* 1999). The intake of PhIP could, however, not be accurately determined using a questionnaire, and it was not possible in this study to demonstrate a dose-related incorporation of PhIP into hair. However, in the present study using an animal model we could

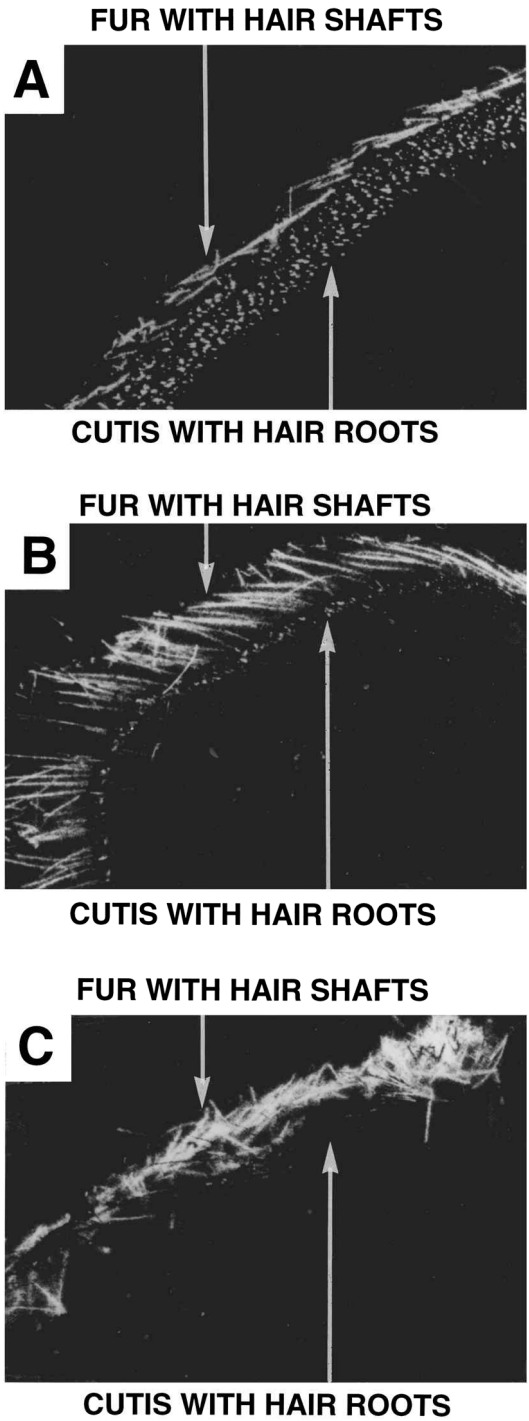

Figure 3. Autoradiograms showing dorsal cutis of pigmented mice (C57BL/6J) after subcutaneous administration of [^{3}H]-PhIP. A: 1 day after the last administration. B: 3 days after the last administration. C: 7 days after the last administration. White areas correspond to high levels of radiolabelled compound. Note the time-dependent decline in radioactivity of the hair roots.

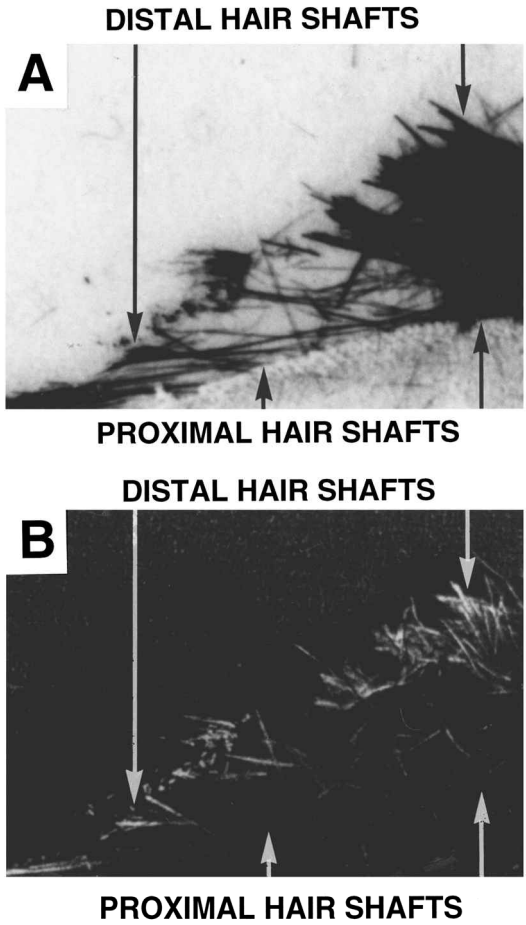

Figure 4. Photopositive of section (A) and corresponding autoradiogram (B) showing dorsal cutis of a pigmented mouse (C57BL/6J) 28 days after the last subcutaneous administration of [^{3}H]-PhIP. White areas of the autoradiogram (B) correspond to high levels of radiolabelled compound. Note that radioactivity is mainly present in the distal parts of the hair shafts.

control the PhIP exposure and study the time course incorporation of PhIP in hair. In particular, using new-born mice, a rapid and uniform hair-growth was secured. In this model our results clearly show that [^{3}H]-PhIP binds to and accumulates in the hair during its formation. Furthermore, chemical analysis confirmed that PhIP, and not a metabolite, is the dominating radiolabelled compound accumulating. This is supported by previous observations. Brittebo *et al*. (1992) found a high level of radioactivity in the skin following [^{14}C]-PhIP exposure. They also showed that PhIP bound firmly to synthetic melanin pigment *in vitro*. Furthermore, Tjøtta *et al*. (1992) demonstrated the presence of radioactivity in the pigmented part of the skin of hooded rats, 6 days after the administration of a related heterocyclic amine [^{14}C]-MeIQx.

In the present study we could also evaluate the dose-related incorporation of PhIP into hair. Since oral administration at 1 week of age is very difficult, [^{3}H]-PhIP was administered subcutaneously. Although we used this route of administration, the mechanism of incorporation of PhIP from the bloodstream

into hair would be the same. Also, in the case of subcutaneous administration PhIP is rapidly distributed to the liver for biotransformation. The study clearly demonstrates that PhIP incorporation into the hair of mice increases in a dose-dependent way. The positive correlation between exposure to PhIP and its incorporation in hair accords very well with previous reports describing a similar relationship for several drugs, medications and other xenobiotics such as methyl mercury (WHO 1990, Uematsu *et al*. 1992, DuPont and Baumgartner 1995, Nakahara *et al*. 1995, Green and Wilson 1996).

Furthermore, our autoradiographic study showed that PhIP was rapidly cleared from the hair roots, but still remained in the hair shafts after 28–30 days. This shows that incorporation of PhIP into hair is an irreversible process, which is in accordance with previous studies of various drugs. (Uematsu *et al*. 1991, 1992, DuPont and Baumgartner 1995, Gygi *et al*. 1995).

Although the exposure levels of PhIP in the present study are several times higher than the human exposure to these compounds via food (Knize *et al*. 1997, Reistad *et al*. 1997), the dose–response proportionality indicates that this would also be valid at lower doses, making PhIP in hair a promising biomarker of exposure in humans also.

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