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Full Length Research Paper

Phase 1 metabolites of polynuclear aromatic hydrocarbons in blood samples of exposed subjects

Chimezie Anyakora[1], Funke Babalogbon[1], Ugochukwu Obiakor[1], Ibukun Afolami[2] and Herbert Coker[1]

[1]Department of Pharmaceutical Chemistry, University of Lagos, Nigeria.
[2]Department of Biochemistry, University of Lagos, Nigeria.

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Automobile mechanics are known to have a high occupational exposure to toxic substances, which include polynuclear aromatic hydrocarbons (PAHs) found in refined petroleum products which are the main source of contamination for these subjects. This paper describes a qualitative investigation of phase I metabolites of PAHs in the blood samples of exposed subjects. In the study, thirty-one healthy adult volunteers residing in Lagos were screened using a gas chromatograph, coupled to a mass spectrometer. Several phase I metabolites such as 1-phenanthrene carboxylic acid, 1,4-naphthalene dione, 2-naphthalene methanol and 9,10-anthacenedione were detected. These are mainly the metabolites of low molecular weight polynuclear aromatic hydrocarbons i.e. anthracene, phenanthrene and naphthalene. No correlation was established between the presence of these metabolites and the years of exposure of the subjects, which can be attributed to the complex and varied metabolism of PAHs in different individuals.

Key words: Phase I metabolites, GC/MS, occupational exposure, PAHs.

INTRODUCTION

Automechanics, as a result of their occupation, are exposed to complex mixture of polynuclear aromatic hydrocarbons (PAHs) which have been implicated in vast number of toxicological manifestations in humans (Liu and Korenga, 2001; Alonge, 1988; Nousianinen et al., 1984; Legraverend et al., 1983). PAHs are a class of compounds composed of two or more aromatic rings. They are a component of crude and refined petroleum and coal. Over a hundred PAHs have been identified and these usually are found as complex mixtures (Mottier et al., 2000). PAHs are a major class of environmentally hazardous organic compounds due to their known or suspected carcinogenicity (Simko, 2002; Martson et al., 2001). PAHs are known to be toxic (Nousianinen et al., 1984; Legraverend et al., 1983; US EPA, 1989; ATSDR, 1995; IPSC, 1998). They are acutely lethal in concentrations of a few ppm and chronically lethal in sublethal concentrations in ppb (Anyakora, 2005). Toxic effects observed due to PAHs include decreased body weight, enlarged liver with cell oedema and congestion of the liver liver parenchyma and inflammation of kidney cells (Kawai, 1979). PAHs have been found to cause reproductive toxicity. Administration of benzo(a)pyrene to mice reduced the number of pregnant female reaching parturition and reduced fertility of offspring (Mackenzie and Angevine, 1981). PAHs can affect female fertility by the destruction of oocytes. Developmental toxicity such as embryolethality, reduced fetal weight and malformations have been reported in response to benz(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthraxcene and naphthalene (IPCS, 1998). A series of studies have been conducted in several countries on the reproductive and developmental toxicity in human (Gladen et al., 2000; Vassileve et al., 2001; Whyatt et al., 1998, Dejmek et al., 2001).

PAHs enter the body through a variety of ways. They enter through the lungs, when they are breathed in air that contains them (usually stuck to particles or dust). Cigarette smoke, wood smoke and smoke from any industrial site may contain PAHs. Drinking water and swallowing food, soil or dust particles that contain PAHs are other routes for these chemicals to enter the body, but absorption is generally slow when PAHs are swallowed. PAHs can enter the body if the skin comes in contact with soil that contains high levels of PAHs or with products

*Corresponding author. E-mail: chimezie@email.com

that contain high level of PAHs such as crude oil. The rate at which PAHs can enter the body can be influenced by the presence of other compounds. PAHs can enter all the tissues of the body that contain fat. They tend to be stored mostly in the kidney, liver and fats. Smaller amount are stored in the spleen, adrenal gland and ovaries. PAHs are changed by the body into different substances some of which are more harmful than the original PAHs.

The metabolism of polynuclear aromatic hydrocarbons is complex and it can occur in all the tissues and extensive studies have been carried out on this, both *in vitro* and *in vivo* (Fung et al., 1999; Thakker et al., 1985 Thompson et al., 1989). It can be divided into two phases; phase I and II reactions. Phase I reaction involves alteration of the structure of the compound to increase the polarity and include reactions such as oxidation, reduction and hydrolysis. Phase I reactions make the compound more electrophilic resulting in increased reactivity. Phase II reactions involve the addition of polar groups thereby increasing the bulkiness and aqueous solubility. Phase II reactions include reactions such as conjugation and synthesis. Usually phase II reactions are preceded by phase I reaction.

In general, the metabolic process of PAHs involves, firstly, the epoxidation of double bonds, a reaction catalyzed by cytochrome P-450-dependent monooxygenase leading to the formation of several arene oxides which are usually transitional and may follow several possible pathways such as spontaneous rearrangement to phenols, and hydration to the corresponding trans-dihydrodiols. Other possible metabolic pathways for PAHs is the formation of phenols by direct oxygen insertion under the influence of P-450 system to form a hydroxyl derivative which may be further oxidized either spontaneously or metabolically to quinones (ATSDR, 1995).

In essence, phase I metabolites of PAHs include all the products of oxidation, reduction and hydrolysis, which include, quinones, phenols, carboxylic acids etc. In this study, with the aid of GC/MS, blood samples of exposed subjects were screened for the presence of PAH metabolites. This was achieved by mass spectral match against those of NIST library. Many metabolites were detected and these include 1-phenanthrene carboxylic acid, 1,4-naphthalene dione, 2-naphthalene methanol, 9,10-anthracenedione and 9,10-methanoanthracene-1-ol.

EXPERIMENTAL

Subjects

The study was a qualitative screening of 31 healthy adult volunteers resident in the city of Lagos in Nigeria. The volunteers were of two categories. The first was made up of subjects thought to have little exposure to PAH contamination. These were students of the University of Lagos, whose ages ranged from 20 to 26 years. They were 11 in number made up of five males and six females. The second category was made up of 20 subjects thought to have greater exposure to PAHs as a result of their occupation. They mainly work as automobile mechanics or related profession, and have a lot of contact with petroleum products which are a good source of PAHs. They were all adult men of ages ranging from 17 to 55 years. Their years in the occupation and hence exposure to PAHs ranged from 1 to 30 years.

Blood collection, processing and storage

3 ml of blood was taken from an antecubital vein of each of the volunteers using 5 ml syringes and were transferred into labeled heparinised sample bottles. The sample bottles were placed into a covered ice container immediately after collection to protect the samples from heat and ambient light. The blood samples were then centrifuged at 10,000 rpm for 30 min on RP-1000 Centrifuge to obtain the plasma. The plasma from each sample was then separated from the whole blood and stored in tightly capped vials at - 4^{o}C until ready for use.

Chemicals

All solvents used were of HPLC grade or reagent grade. HPLC grade Dichloromethane was obtained from Sigma-Aldrich laboratories GmbH, Seeize, Germany. Heparin 2500 I.U. was obtained from Choongwae Pharmaceuticals, China. Sodium Chloride I.V. Infusion B.P. was obtained from Unique Pharmaceuticals, Lagos. Methylated Spirit B.P. was obtained from Nino Pharmaceuticals and Chemical Company Ltd, Lagos.

Sample preparation

The plasma samples were thawed at room temperature. PAHs were extracted from the plasma by the means of liquid-liquid extraction using high purity Dichloromethane. The apparatus for this consisted of a 25 ml volume separating funnel mounted on a retort stand. The separating funnel was thoroughly washed and dried over night in a muffle furnace at an elevated temperature. Prior to use the funnel was rinsed vigorously with dichloromethane for several minutes. This was removed and allowed to drain and dry completely in fume cupboard. The plasma sample (of less than 3 ml) was mixed with 5 ml of dichloromethane. This was shaken vigorously for 2 min and allowed to separate and settle. After 10 min the organic layer was removed and the process repeated with the aqueous layer twice. The three portions of the organic phase were combined and evaporated to 1 ml volume using a rotary evaporator.

Chromatographic instrumentation and condition

The analysis was carried out on a Hewlet Packard Gas Chromatograph coupled to a mass spectrometer with an autosampler and a 30 m 0.33 id DB-5 MS fused silica capillary column. Chromatographic analysis was run and interpreted with Chemstation for GC/MS for Agilent Technologies running on a Del computer with Windows NT. Helium was used as the carrier gas and the column head pressure was maintained at 10 psi to give an approximate flow rate of 1 ml/min. The injector and transfer line were maintained at 290 and 250^{o}C, respectively. All injection volumes were 1 µl in the splitless mode. The column temperature was initially held at 70^{o}C for 4 min, ramped to 300^{o}C at a rate of 10^{o}C/min, and then temperature was held at 300^{o}C for 10 min. The mass spectrometer was used in electron ionization mode and all spectra were acquired using a mass range of m/z 50 – 400 and automatic gain control (AGC).

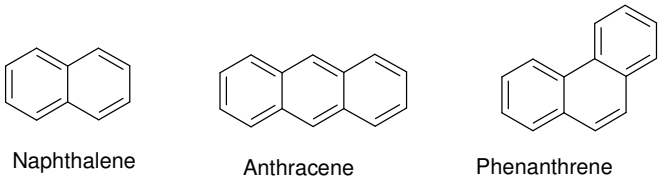


Figure 1. The chemical structure of naphthalene, anthracene and phenanthrene.

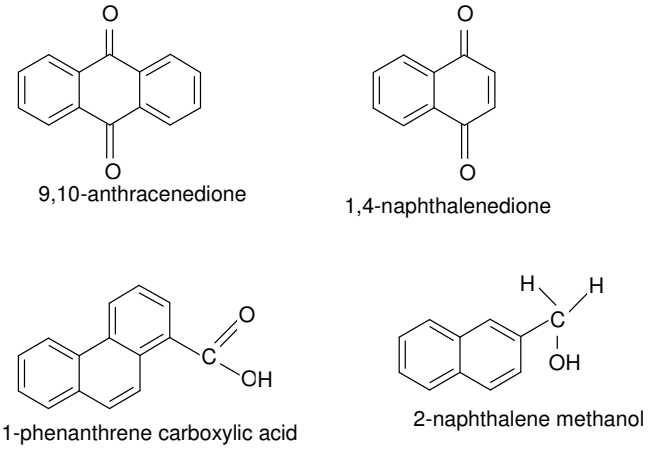


Figure 2. The chemical structures of the detected PAH metabolites.

RESULTS AND DISCUSSION

A number of phase I metabolites of PAHs were identified in this study. These metabolites are mainly the metabolites of low molecular weight polynuclear aromatic hydrocarbons namely: naphthalene, phenanthrene and anthracene (Figure 1) and their metabolites detected are 1-phenanthrene carboxylic acid, 1,4-naphthalene dione, 2-naphthalene methanol and 9,10-anthacenedione (Figure 2)

As can be seen from the structures of the metabolites, some of the parent compounds are alkylated PAHs. This is logical, as several studies have indicated that PAHs occur in groups made up of both alkylated and non-alkylated PAHs. Figures 3 - 6 attempt to trace the possible metabolic pathways of the four metabolites, basing the pathway on established probable route of PAH degradation in the body (ATSDR, 1995). This study could identify only the metabolites of low molecular weight PAHs probably because of the fact that these compounds are easier to degrade (Yun et al., 2003). They also occur at much higher concentration than the high molecular weight compounds especially in refined petroleum products which are the probable sources of PAH contamination to the subjects.

As shown in Figure 3, the most probable parent compound for 9,10-anthracenedione is anthracene.

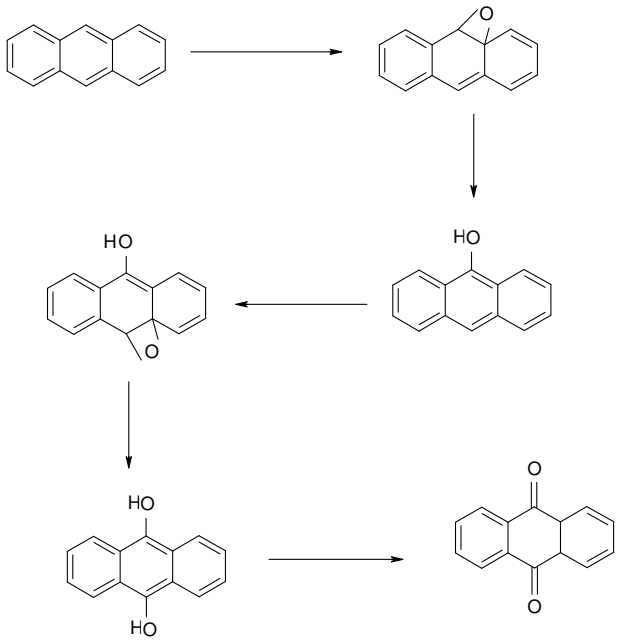


Figure 3. Conversion pathway of anthracene to 9,10-anthracenedione.

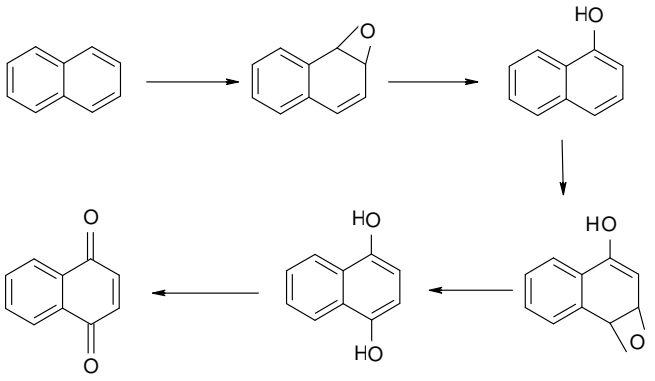


Figure 4. Conversion pathway of naphthalene to 1, 4-naphthalendione.

Anthracene is an alternant polynuclear aromatic hydrocarbon. Like many alternant PAHs, it is metabolized by micro somal cytochrome P-450 systems to several possible arene oxides but most probably at carbon atom 9. This arene oxide formed is transitional and may follow two possible pathways. First is a spontaneous rearrangement to a phenol. The second possibility is hydration to corresponding trans dihydrodiol in a reaction catalyzed by microsomal epoxide hydrolase (Lau and Baird, 1994). Based on the metabolite determined in this study the first possibility is a probable path followed in the metabolism. Thermodynamically, a phenol is more stable than a dihydrodiol of anthracene. There is also a possibility of formation of phenol by direct oxygen insertion catalyzed by

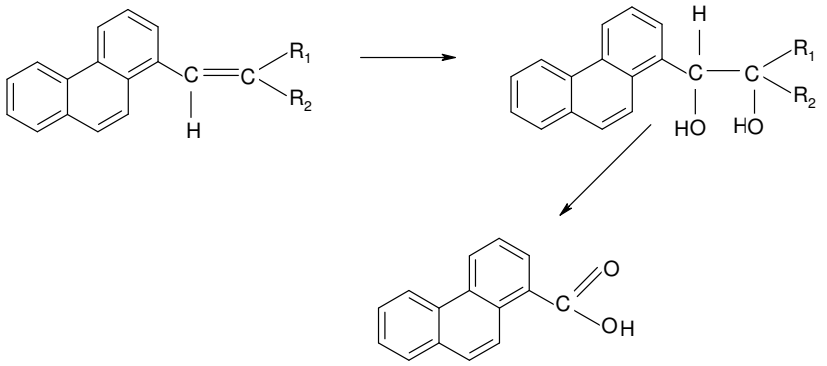


Figure 5. Conversion pathway of an alkylated phenanthrene to 1-phenanthrene carboxylic acid.

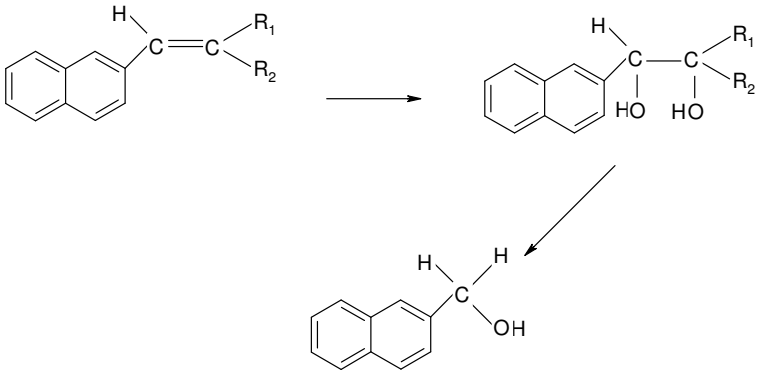


Figure 6. Conversion to 2-naphthalene methanol.

by cytochrom P-450 system.

With the formation of 9-hydroxyanthracene, the carbon 10 position which is equally as reactive as carbon at position 9 can also undergo the same reaction to give a 9,10-dihydroxyanthracene. This compound is further oxidized to give 9, 10-anthracenedione. A number of *in vitro* studies on some alternant PAHs especially benzo(a)pyrene corroborated with mechanism and pathways described above (Phillip and Groover, 1994; Kulkami, 1986; IARC, 1983; Martson et al., 2001; Mellikian et al., 1999).

As in the case of anthracene, naphthalene being an alternant PAH followed a pathway of epoxidation, rearrangement to a phenol at carbon 1 position. Then a repeat reaction at position 4 to give 1,4-dihydroxynaphthalene which is further oxidized to 1,4-naphthalenedione. This compound is most stable of all the possible quinones of naphthalene.

The metabolite, 1-phenanthrene carboxylic acid, indicates that here the metabolism, unlike the previous two involved only reactions 'extra nuclear', or outside the aromatic system of the compound. This suggests that the starting compound is an alkylated phenanthrene with a reactive carbon to carbon double bond. This being more reactive than the aromatic system will be preferentially oxidized by the cytochrome P-450 system to give a glycol which on further oxidation gives a carboxylic acid.

In the case of 2-naphthalene methanol too, there seem to be an 'extra nuclear' reaction which also suggest an alkylated compound. There are numerous possible pathways to the eventual metabolite (2-naphthalene methanol) but a simplest of them is a conversion to a glycol and then a reduction, catalyzed by the corresponding enzyme. The reason for reduction instead of oxidation as in the case of phenanthrene is not very clear but metabolism in the body is very complicated with competing pathways (ATSDR, 1995). The focus of the study is more of the determination of phase I metabolite.

Conclusion

The presence of these metabolites clearly shows that these compounds are absorbed by these subjects. This corroborate with earlier studies which determined the presence of unmetabolized PAHs in the blood sample of these subjects. Metabolism of PAHs is very complex and varies among individuals of the same specie. Therefore we were not able to establish any correlation between years of exposure and presence of these metabolites.

Metabolites of high molecular weight PAHs were not identified in these samples, probably for two reasons. These compounds occur less in refined petroleum products which are mainly the source of PAHs contamination for these subjects. Secondly high molecular weight PAHs are more difficult to degrade and so when present, their metabolites may occur in less detectable concentration. Phase II reactions may also have occurred leading to the conjugation of the electrophilic, polar phase I metabolites leading to more complex conjugated structures which are not the focus of this study. The study has given some insight and corroborated with existing study on the metabolism of PAHs in the body.

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