

Biotransformation of benzo[a]pyrene
- Analysis, metabolism and adduct formation
in rats and Ahr knockout mice

Dissertation for the degree of Ph.D.

by

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"Dagen i dag er morgendagen du drømte om i går."

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Abbreviations

PAH; polycyclic aromatic hydrocarbon

BP; benzo[*a*]pyrene

Xenobiotic; foreign compound

BPDE; diol epoxide

CYP; cytochrome P450

EH; epoxide hydrolase

AHR; aryl hydrocarbon receptor

HPLC; high performance liquid chromatography

MS; mass spectrometry

ESI; electro spray ionization

LOD; limits of detection

SIR; selected ion reaction

In vitro; outside a living organism

In vivo; inside a living organism

DNA; deoxyribonucleic acid

List of publications

Article I Raymond Olsen, Carlos Sagredo, Steinar Øvrebø, Elsa Lundanes, Tyge Greibrokk and Pål Molander. Determination of benzo[a]pyrene tetrols by column-switching capillary liquid chromatography with fluorescence and micro-electrospray ionization mass spectrometric detection. (*The Analyst* 2005, 130 (6), 941-7.)

Article II Carlos Sagredo, Raymond Olsen, Tyge Greibrokk, Pål Molander and Steinar Øvrebø. Epimerization and stability of two new *cis*-benzo[a]pyrene tetrols by the use of LC-fluorescence and mass spectrometry. (*Chem Res Toxicol.* 2006 Mar;19(3):392-8.)

Article III Carlos Sagredo, Steinar Øvrebø, Aage Haugen, Rita Bæra, Ingrid V. Botnen, and Steen Mollerup. Quantitative analysis of benzo[a]pyrene biotransformation and adduct formation in Ahr knockout mice. (*Toxicol Lett.* 2006 Dec 15;167(3):173-82.)

Article IV Carlos Sagredo, Steen Mollerup, Kathleen J. Cole, David H. Phillips, Heidi Uppstad and Steinar Øvrebø. Biotransformation of benzo[a]pyrene in Ahr knockout mice is dependent on time and route of exposure. (*Chem Res Toxicol.* 2009 Feb 16;22(3):584-591.)

1 Introduction

1.1 General background

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of chemical compounds that consist of fused aromatic rings. PAHs are lipophilic and relatively inert and may be toxic, teratogenic and carcinogenic, depending on the structure and the number of rings in the molecule. Lately 15 individual PAH compounds have been classified as carcinogens by IARC (1).

PAHs are formed during incomplete combustion of organic matter and fossil fuels in both industrial and natural processes, automobile exhaust, cigarette smoke and well-prepared and broiled food (2;3). PAHs are hence ubiquitous environmental contaminants found in air, water, soil and food. PAHs often exist as complex mixtures and they may persist in the environment for a long time. Benzo[*a*]pyrene (BP) is an important member of the PAH family and has served as a model for studies of the metabolic pathway and carcinogenic effects of PAHs (4).

Table 1 gives an overview of the content of BP found in different samples (4-6).

Type of sample	BP concentration
Air (industry, high levels)	> 10 000 ng/m ³
Air (industry, moderate levels)	100 – 1000 ng/m ³
Air (city levels)	1 - 500 ng/m ³
Soil	0.8 ng/kg – 100 mg/kg
Tap water	2.5 – 9 ng/L
Surface water	130 – 500 ng/L
Plants	< 150 000 ng/kg
Food	100 – 20 000 ng/kg

Table 1 Concentrations of BP detected in air, water, plants and food (4-6).

Exposure to PAHs is high in occupational environments like coke production sites, aluminum smelters, foundries and in the manufacture of graphite electrodes and refractory products (5;7). The workers are exposed to PAH mostly through inhalation and skin

uptake. For the population in general the PAH exposure is mainly through consumption of food and air pollution including cigarette smoking (5;8). There are several epidemiological studies showing the association between PAH exposure and different human cancers especially lung cancer, but also bladder, skin, prostate and scrotal cancer (5). Lung cancer is today considered the leading cause of cancer death and continues to increase worldwide (9). A thorough overview of available published data on carcinogenic properties of 60 PAH have been summarized in the latest IARC monograph (1).

1.2 History

Sir Percival Pott reported the first observations of a possible linkage between exposure to coal chimney soot and the occurrence of scrotal cancers among chimney sweeps. The industrial revolution and development introduced new industrial carcinogens, like coal tar, paraffin and mineral oils. The first carcinogenic response study were done on mice in 1915 by Yamagiwa and Ichikawa (10). In the 1920's, Kennaway, concluded that a hydrocarbon in coal tar, was responsible for the observed carcinogenic properties of coal tar. Later Kennaway and Hieger, identified a carcinogenic hydrocarbon as benz[*a,h*]anthracene. Still, it was Cook that finally identified BP as the principal chemical carcinogen in tar, in the 1930 (10).

1.3 The metabolism of BP

PAH are metabolized by different enzymes in a series of steps, where each step forms intermediates that are either detoxified or activated towards reacting with cellular components like DNA and proteins. The aromatic and planar ring of BP has different sites that are suitable for the action by the microsomal P450 enzymes. The enzymatic oxidation inserts one oxygen atom from molecular oxygen into the compound through a NAD(P)H dependent catalytic cycle (11). The epoxide intermediate may then be hydrolyzed by epoxide hydrolase (EH) or undergo isomerization to phenols. Hence, different BP phenols and BP diols are then formed depending on the type of P450 (Cyp) enzyme and the available EH (12;13). The phase II enzymes like UGT and SULT may then further conjugate the phenols and diols. The overall process detoxifies the PAH from the organism

by reducing the lipophilicity and enhancing the clearance and excretion of the compounds from the organism in to bile and urine. (12). See Figure 1.

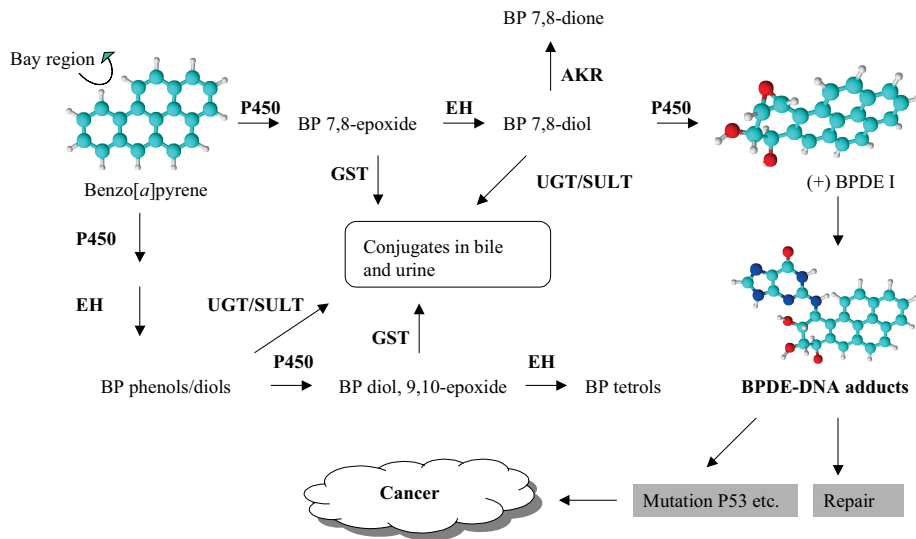


Figure 1 The bioactivation and detoxification of BP by phase I and II enzymes, and the formation of the ultimate carcinogenic diol-epoxides.

The bioactivation of BP includes the formation of mutagenic metabolites, like the BP diol-epoxides (\pm BPDE I), which are strong electrophilic compounds known to be highly reactive towards DNA and proteins (12;14;15). In this metabolic pathway, BP is initially oxidized by cytochrome P450 to form a BP 7,8-epoxide. The epoxide is rapidly hydrolyzed by epoxide hydrolase (EH) to form the proximate carcinogen dihydrodiols, (\pm) BP 7,8 diols, which may further be oxidized by P450 to form the ultimate carcinogenic BP diol-epoxides (16). The P450 produce epoxide groups that are either *cis* or *trans*, while the EH yields diols that are only *trans*. Hence, the result is the formation of different BP diol-epoxide isomers and enantiomers, of which the optically active bay-region (+) 7,8-diol-9,10-epoxide enantiomers, i.e. (+) BPDE I, is considered to be the ultimate carcinogen (12).

In early studies it was believed that the K-region BP 4,5 epoxide was one of the major carcinogenic BP metabolites (17), but gradually it was found that the epoxide was rapidly hydrolyzed by EH leading to the BP 4,5-diol, and other carcinogenic BP metabolites were identified (18). The bay-region theory introduced in the 1970's helps to predict ultimate carcinogenic structures (19). The bay region in the BP molecule is the

sterically hindered region formed by the angular benzo ring. The bay-region diol-epoxides were found to be more chemically reactive and resistant to hydrolysis by EH and hence important in DNA binding and tumorigenesis. (12;16;20). The formation of DNA adducts is the first step in the initiation of PAH induced carcinogenesis (21).

The biotransformation of BP may form BP 9,10 diol, in an analogous way to BP 7,8 diol, leading to the formation of non-bay region diol-epoxides, like 9,10-dihydrodiol 7,8-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, *syn*-BPDE III and *anti*-BPDE III. See Figure 2.

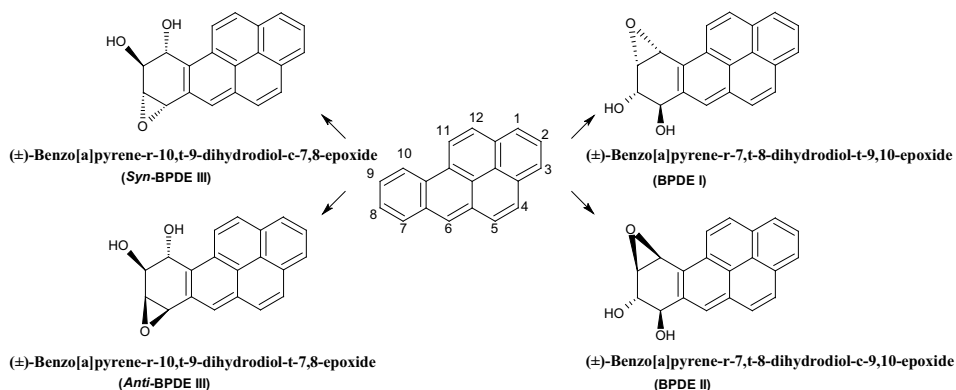


Figure 2 Diol-epoxide formed from BP. The bay region BPDE I is considered the ultimate carcinogen. The non-bay region diol epoxides, BPDE III, are considered to be less mutagenic but highly cytotoxic (22).

The BPDE III diol-epoxides have been shown to form DNA adducts *in-vitro* (23), although their mutagenic activity have been shown to be very low as compared to BPDE I (24). In addition, metabolic studies with BP 9,10 dihydrodiol show primarily a formation of a phenolic compound and only smaller formation of metabolites containing the 7,8,9,10-tetrahydro-BP chromophore. (24). Toxicity studies shows that BPDE III is highly cytotoxic compared to BPDE I (25).

The main pathway of BP activation involves the action of P450 and EH with the formation of diol epoxides as depicted in Figure 3. There are other metabolic routes that form several toxic and reactive molecular species. One pathway involves the formation of radical cations by P450 peroxidases. These radical cations forms unstable depurinating adducts and apurinic sites. The other pathway involves the formation of benzo[a]pyrene-7,8-dione (BP-7,8-dione) catalyzed by human aldo-keto reductases AKR1A1 and

AKR1C1-AKR1C4 (26). The BP-quinones are reactive Michael acceptors and may react with DNA to form either stable bulky adducts or depurinating adducts (27). In addition, the BP-quinones can undergo conjugation reactions with endogenous nucleophiles like GSH, etc., which can lead to a depletion of cellular reducing equivalents and a change in redox state. The ROS that are produced during the redox cycling of BP-7,8-dione can either cause oxidative DNA damage or produce decomposition products of lipid hydroperoxides, which are also reactive towards DNA (28).

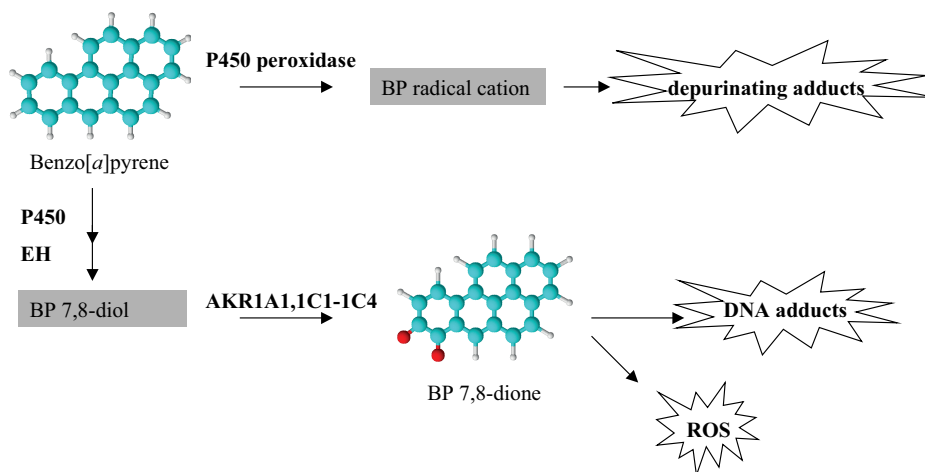


Figure 3 Metabolic activation of BP by P450 and AKR isoforms.

Several BP diones-DNA adducts have been characterized *in vitro* (29), and the metabolic activation of BP by the AKR pathway is believed to add to the initiation of PAH induced carcinogenesis (30).

1.4 The adduct formation

The major sites of attachment for the bay-region diol epoxides in DNA are the exocyclic amino groups of deoxyguanosine, deoxyadenosine and to a very small extent cytosine (31;32). The diol epoxide adducts are formed by both *cis* and *trans* opening of the epoxide at the benzylic position, hence eight dA and eight dG isomeric adducts are possible (33). Theoretical computations along with NMR and X-ray crystal studies have been used to elucidate several structures of different BP-DNA adducts. See Figure 4. The

isomers and enantiomers of the diol epoxides have been shown to have different biological activities, like adduct formation and mutagenic activity, depending on the test system used (12). Several studies using different cell systems have demonstrated that the ultimate carcinogenic BPDE I bind more to DNA and proteins, than BPDE II and BPDE III (12;25;34). The BPDE I shows a high base selectivity towards deoxyguanosine in double stranded DNA that result in mainly BPdG adducts (35). The (+) BPDE I enantiomer has been found to bind to the 2-amino group of deoxyguanosine to a 20-fold greater extent than the (-) BPDE I enantiomer. (36). In the absolute configuration and conformation of the BPdG adduct, the exocyclic amino group is bound to the C10 position of the diol epoxide, in either a *cis* or a *trans* position relative to the hydroxyl group in the C9 position. The C7 and C8 hydroxyl groups are pseudodiaxial in the *cis* product and pseudodiequatorial in the *trans* product (37;38). The studies shows that the adducts are located in a minor groove and directed towards the 5'end or the 3'end of the modified strand (39). Interestingly, although there is flexibility around the C2-N2 bond of guanine and the N2-C10 position between guanine and the diol epoxide, the adducts have strong conformational preferences (40). Both the structure and conformation of the BP-DNA adduct is believed to influence their interactions with the DNA repair enzymes affecting the reparability and removal rate of the BP-DNA adduct, and ultimately the risk of mutation (40;41).

When the DNA adduct remains unrepaired, they can cause a miscoding during the replication by inducing transversion mutations involving both G and A, and hence produce a permanent mutation (42). If the mutation occurs in a critical region of important genes, like oncogenes and tumor suppressor genes, a chemical induced cancer may occur (43).

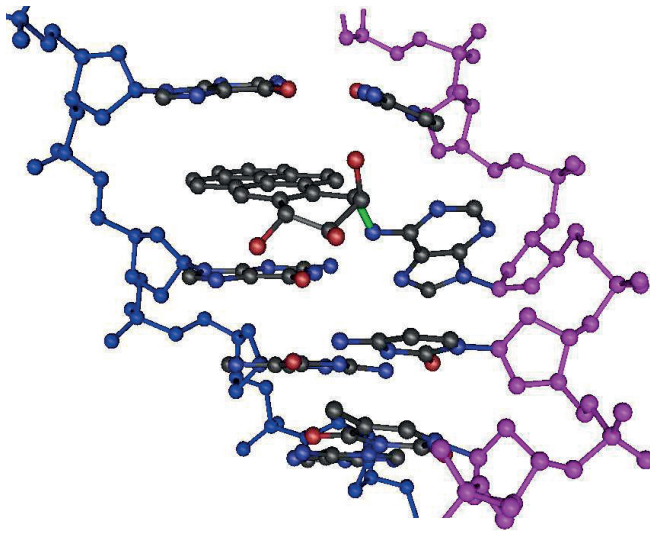


Figure 4 The BP-DNA adduct between (+) BPDE I and the exocyclic N6-amino group of deoxyadenosine. The picture illustrate the BP-DNA adduct inside the major groove of the DNA duplex. Picture adapted from NCBI using Cn3D (33).

Hence, the BP-DNA adducts are well suspected of producing mutations, in specific G to T transversions, within codons of the *p53* tumor suppressor gene and the *K-ras* proto-oncogene in lung cancer (43-45).

1.5 The cytochrome P450

The cytochrome P450 is a group of hem-thiolate mono-oxygenase enzymes, encoded by the superfamily of P450 CYP genes. It was first discovered in rat liver microsomes in the beginning of the 1950's, having a unique spectral absorbance band with a peak around 450 nm (46). Later on, in the 1960's it was characterized as a hemprotein and the catalytical properties were studied. Today over 1000 P450 genes have been identified, cloned and expressed in heterologous expression systems. The P450 genes are found in the genome of virtually all organisms. Yeast has three, *Mycobacterium tuberculosis* has 20, humans have 57 while their number has exploded in plants (47). Although the amino acid sequences are very diverse, the catalytic properties with haem-thiolate binding using cysteine have remained the same throughout evolution. The P450 are involved in a diversity of processes from highly regio- and stereospecific to very diverse reactions like carbon source assimilation, biosynthesis of hormones and fat-soluble

vitamins, and oxidation of endogenous and foreign compounds (48). The most important P450s in the metabolism of PAH in mice and rodent are the Cyp1a1 and Cyp1b1. Historically, the Cyp1a1 has been attributed to the metabolic activation of PAH in mice and rodent models (12;49). Cyp1a1 is constitutive expressed at low levels in liver and different organs, but is highly inducible. Recent studies have shown that Cyp1b1, which is expressed at higher levels in extra-hepatic organs, may also be important in the activation of PAH (50). Other studies has shown that Cyp1b1 is more important than Cyp1a1 in the first activation step of BP and the formation of BP 7,8 diol (51).

The P450 enzymes catalyze about 90 % of the phase I metabolism in humans. The major catalysts of foreign compounds in human liver are CYP3A4, CYP2D6, and CYP2C subfamilies (52). The Cyp1a1, which is the major catalyst of BP in rodent liver, is almost absent in human liver (53). The mice Cyp1a1 activity has been shown to be 40 times higher than any human P450 enzymes in metabolizing BP to BP 7,8 diol and BP diol-epoxides (13). As in rodents, human CYP1B1 are expressed in different tissues. Studies have demonstrated that CYP1B1 is active in the endogenous steroid metabolism, but do also play a role in the metabolism of environmental PAH compounds. In contrast to rodent cyp1b1, human CYP1B1 show lower rates of BP metabolism (54). Hence, human and rodent P450 can display large differences in activities (52). Different studies with isolated human P450 enzymes have shown that several P450 enzymes, i.e. CYP1A1, 1B1, 1A2, 3A4, 2B6, 2C9, 2E1 and 2B6 are capable of oxidizing BP (13;53;55). Some of the enzymes and the metabolic pathways may be detoxifying, like the formation of 3OH-BP by CYP3A4 (55). Although the metabolic pathway may be dependent on the *in vitro* system and the amount of EH, the CYP1A1 seems to have the highest turnovers for the BP metabolism. In addition, CYP1B1, CYP1A2 and CYP2C9 seem to have a high activity for the formation of BP 7,8 diol and BP diol epoxides (13;53;54). The P450s controls the stereoselectivity of the formed BP metabolite, and the balance between activation and detoxification of the PAH compound. Hence, an important aspect of the PAH metabolism by the P450s, includes the different genetic polymorphisms of P450s, that may affect the individual susceptibility to PAH carcinogenesis (56;57).

1.6 Phase II – detoxification

BP is metabolized to various products by the xenobiotic metabolizing enzymes. These enzymes are divided into phase I and phase II enzymes. The microsomal P450 phase I enzymes produce a complex mixture of quinones, phenols, dihydrodiols, triols, pentols but also reactive epoxides (58). The phase II enzymes glutathiontransferase, glucuronosyltransferase and sulfotransferase (SULT) will further act on the oxidized products from the phase I step, and form conjugates of glutathione, glucuronide and sulphate. The formation of highly polar and water-soluble metabolites increases the excretion of BP from the body into urine and bile. Although, the sulphate conjugation is regarded as an important detoxification pathway, there are several compounds that are further activated by forming electrophilic sulphuric ester metabolites (59-61). There are reports of different polymorphisms in SULT1 associated with the risk of lung cancer (62-64). There are also studies showing an additional increase in risk for colorectal cancer with certain combinations of Cyp1b1 and SULT1 polymorphism (65). When the sulphuric ester loses the sulphur group it forms an extremely reactive carbocation. This ion may then react spontaneously with DNA or other cellular components leading to serious toxic lesions and mutations (60;61). A group of compounds that have been shown to be activated both *in vivo* and *in vitro* experiments by sulphotransferase are methyl-substituted PAHs and nitrosamines. Hence, the potential sulfonation of related PAH metabolites like the BP diols and BP tetrols is of interest since such a pathway could reactivate the metabolites (61). Different studies have shown that sulfonation of BP diols *in vitro* is possible although less efficient than with BP phenols. Studies with V79 hamster cells expressing sulfotransferase showed an induction of chromatid exchange after BP tetrol exposure. A different study showed that BP diol and BP tetrol did not exhibit any sulfotransferase dependent mutagenicity nor any DNA adducts formation (66). Nevertheless, it remains unclear whether this pathway contributes to the overall carcinogenicity of BP (66).

1.7 The aryl hydrocarbon receptor

The aryl hydrocarbon receptor (Ahr) is a ligand activated member of the per-arrnt-sim (PAS) family basic-helix-loop transcription factors and is located in the cytosol in association with heat shock HSP(90) and HSP90 accessory protein (67).

The activation of Ahr results in rapid transcriptional activation of numerous genes that control a broad spectrum of cellular functions. Ahr is involved in endogenous physiological and developmental processes but also toxicological processes and oxidative stress (68).

BP and other PAH are primarily activated in the body by the Ahr, which acts as a transcriptional factor of several important xenobiotic genes. BP acts as ligand and binds to the Ahr in the cytoplasm. See figure 5. The liganded Ahr is then translocated to the nucleus where it forms a heterodimer with the Ahr-nuclear translocator (Arnt) (69). The Ahr/Arnt heterodimer recognize and bind to xenobiotic responsive element (XRE) sequences located in the promoter region of several genes such as cytochrome P450 Cyp1a1, Cyp1a2, Cyp1b1, glutathione S-transferases (Gst), UDP-glucuronosyl-transferases (Ugt) and quinine oxidoreductase. The binding results in transcriptional activation of the genes and induction of phase I and phase II metabolizing enzymes as well as phase III transporter proteins (70). The relative expression of these enzymes will determine the degree of bioactivation and detoxification of BP after a given exposure.

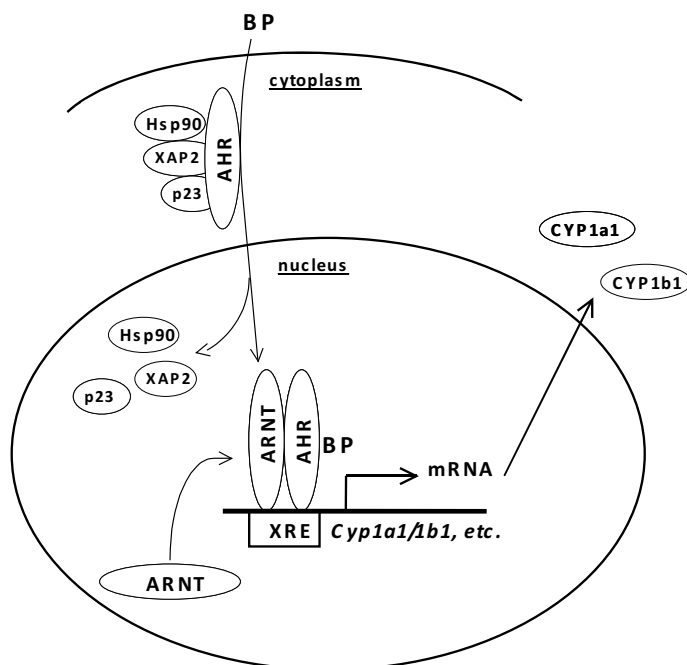


Figure 5 Mechanism of transcriptional activation by Ahr.

The activation of Ahr has also been shown to antagonize other nuclear hormone proteins like the estrogen receptor (ER). Recently, it has been shown that ER- α may interact directly with the Ahr, hence inducing both Cyp1a1 and Cyp1b1 in human bronchial epithelial cells (71). Since ER- α is often more expressed in the lungs of women than of men, this could partly explain the observed gender differences in lung cancer susceptibility (72-74). At the same time TCDD, which is a high affinity ligand for Ahr, has been shown to inhibit estrogen metabolism. The result is an inhibition of the ER-regulated gene expression (75). The inhibitory Ahr-ER cross-talk has been demonstrated in breast cancer cells and may prove valuable for the development of Ahr modulators for treatment of breast cancer (76).

1.8 Mice models

The discovery of the involvement of the cytochrome P450 Cyp1a1 and the Ahr signaling pathways in the metabolism of PAHs has led to several studies with Ah-responsive/non-responsive mice, and Cyp- and Ahr-knockout mice models.

Ah-non-responsive mice exposed to BP by topical application or intraperitoneal (i.p.) injection showed lower risk of mutagenesis and carcinogenesis than Ah-responsive mice (69). When the PAH was administered orally, the Ah-non-responsive mice experienced higher toxicity and tumor formation in organs distal to the site of administration, while the Ah-responsive mice experienced increased toxicity and tumor induction in organs at the site of administration. In addition, the Ah-non responsive showed reduced survival time compared with Ah-responsive mice (69). These results were explained by the first-pass effect (69;77).

Similar observations were made with knockout mice models of Cyp1a1, Cyp1a2 and Cyp1b1. Knockout mice exposed to BP by i.p or oral injection experienced higher levels of toxic lesions and higher mortality rates. In addition, the knockout mice showed a slower clearance of BP and increased levels of BP-DNA adducts than the wild-type mice (77-80). These studies concluded that the Ahr-inducible Cyp1a1 and Cyp1b1 play important and possibly different roles in the activation and the detoxification of BP (80).

In Ahr knockout mice studies by Shimitzu et al. (81), they reported that the Ahr(-/-) mice were more resistant to BP induced toxicity and cancer. The mice received topical

application and subcutaneous injection of the PAH, and only the Ahr wild-type mice developed tumors at the end of the experiment. In a different Ahr knockout mice study, Kondraganti et al. (82) exposed two mice groups, Ahr(-/-) and Ahr(+/+), to a single i.p. dose of BP. They then observed the formation and distribution of DNA adducts in the liver by ³²P-postlabelling. Interestingly, the hepatic DNA adduct levels were different between Ahr(-/-) and Ahr(+/+), but the total sum of hepatic BP-DNA adducts were the same, a result pointing towards an Ahr independent BP activation in the mice liver (82). These studies show that end responses, such as toxicity, lethality and DNA adduct formation, are clearly dependent on both the dose and the route of administration.

1.9 Analytical techniques

Different methods have been used to study DNA and protein adducts, like postlabelling, immunoassay, and HPLC. The BP-DNA adduct formation is usually low; i.e. 1 in 10⁶ to 1 in 10⁷ bases (83). The ³²P-postlabeling has been the most sensitive method to measure BP-DNA adduct (84). The limit of detection (LOD) is as low as 1 adducts/10¹⁰ nucleotides, but due to lack of structural information other methods are implemented (85). GC-MS has successfully been applied for the determination of protein adducts (86). Lately, HPLC coupled with fluorescence or MS, has been used, reducing the sample preparation time and loss of sample due to derivatization (84;87;88). In addition, valuable structural information may be obtained by studying the entire BP conjugates and phase I BP metabolites by HPLC (89;90).

1.10 Epimerization

The hydrolysis of the DNA and protein adduct, releases the corresponding diol-epoxides as four specific BP tetrols (91). Hence, the BP tetrols measurements reflect the formation of specific reactive diol-epoxides and gives valuable information of the metabolites formed in the metabolism of BP (84). Unfortunately, the hydrolysis condition employed to release the BP tetrols, leads to an epimerization between the BP tetrols (92;93). This chemical equilibrium reaction is the result of an isomerisation in particular at the C10 hydroxyl group in the BP tetrol molecule and is very much dependent on the pH of the hydrolysis conditions (94). See Figure 6.

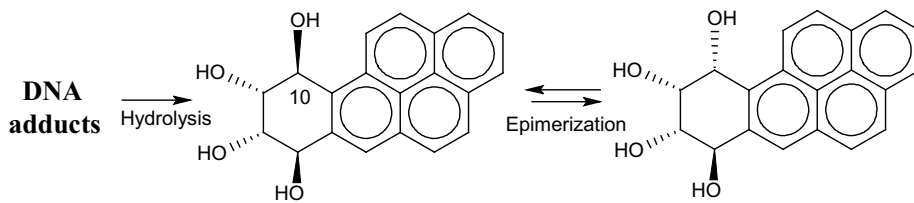


Figure 6 Acid hydrolysis of protein and DNA adducts and the epimerization at C10.

2 Aims of study

- Explore the use of miniaturized LC-MS with large volume injection as a sensitive analytical technique with structural information in the analysis of BP tetrols.
- Identify the mass of two BP protein adducts formed *in vivo* in rats exposed to BP.
- Study the relationship between the Ahr and the metabolism of BP in Ahr knockout mice and Ahr wild type mice.
- Study the effect of time and the administration route on the metabolism of BP in Ahr knockout mice and Ahr wild type mice.

3 Material and methods

3.1 Chromatographic conditions

Solid phase extraction with C₁₈ cartridges was used for sample clean-up and pre-concentration to reduce time as well as consumption of organic solvents.

In Paper I, the LC analysis was performed on a capillary HPLC System with capillary C₁₈ columns, coupled with a UV-diode array detector and a tandem quadrupole mass spectrometer. The mass spectrometer was equipped with a Z-spray atmospheric pressure ionization ion source prepared for electrospray ionization (ESI).

The large volume injection was achieved by coupling column switching with an isocratic capillary pump. In Papers II-IV, the analysis was performed on conventional HPLC attached with a fluorescence detector and where needed a fraction collector. The use of HPLC in sample analysis and sample collection is highly efficient and specific. The automatic fraction collection system was used for adducts purification and metabolites determinations throughout the study.

3.2 Cell and animal models

Human hepatoma cell lines HepG2, obtained from the American Type Culture Collection (Manassas, Virginia, US), were used in paper I.

In Paper II we used male Wistar rats (B & K Universal AS, Norway) weighting about 230 g.

In Papers III and IV we used Ahr heterozygote mice (C57BL6), that were obtained from the Department of Pathology, Graduate School of Medicine, University of Tokyo, Japan. The Ahr (+/-) were interbred to generate Ahr (+/+), Ahr (+/-) and Ahr (-/-) mice. The formation of the knockout offspring did not follow the Mendelian law, since repeatedly only about 10-15 % of the offspring had the Ahr (-/-) genotype. Real-time RT-PCR measurement of AHR expression was carried out on lung tissue samples at the end of the experiments to verify genotypes (81). There were no observed differences in growth rate and appearance between the different genotypes.

3.3 BP exposure

The HepG2 cells were grown to confluence and then exposed to 20 mM BP for 2 days. The medium solution was recovered at the end of the incubation period and aliquots were further taken for sample preparation.

The Wistar rats were treated with a single i.p.dose of BP (100 mg/kg) for identification of the novel BP protein adducts. After 3 days, the animals were anesthetized and drained for blood.

In Paper III, the Ahr mice groups were treated with a single oral dose of BP (100 mg/kg). Internal organs were collected after for 24 hours. In Paper IV, the Ahr wild mice and Ahr knockout mice were exposed to a single dose of BP, by two administration routes; intra peritoneal (i.p) and cutaneous, for 24 hours. In addition, Ahr mice groups were treated with a single oral dose of BP. The animals were placed in cages overnight, with subsequent collection of urine and feces. The mice groups were then sacrificed at different time points.

3.4 BP conjugates

In Paper III we measured both sulphate conjugates and glucuronide conjugates of BP phenols directly using HPLC fluorescence detection. The conjugates were isolated from BP exposed mice urine, and the individual fractions were subjected to enzymatic hydrolysis with glucuronidase/arylsulphatase. In addition, BP sulphates were prepared *in vitro* by conjugating the corresponding BP phenols with arylsulphatase and PAPS (66).

3.5 Cytochrome P450

Gene expression measurements of Cyp1a1 and Cyp1b1 were carried out by quantitative real-time RT-PCR on an ABI PRISM 7900 (Applied Biosystems). The specific gene expression levels were then normalized to the expression of β -actin. (95).

4 Summary of papers

4.1 Paper I

The aim of Paper I was to develop an analytical method with the use of a miniaturized LC system coupled with a column switching system with large volume injection of samples. The detection was performed with coupled on-line ESI-MS and fluorescence detectors. A well-established off-line crude solid phase extraction procedure was first used in order to make the method compatible with several biological matrices. The method was validated over the concentration range 0.1–50 ng/ml benzo[a]pyrene tetrols in a cell culture medium with 100 ml injection volume, fluorescence detection and the first eluting tetrol isomer as model compound. The mass limit of detection (by fluorescence) was 3 pg for all the tetrol isomers, corresponding to a concentration limit of detection of 30 pg/ml cell culture medium. The corresponding mass spectrometric mass limits of detection were 4–10 pg, corresponding to concentration limits of detection of 40–100 pg/ml cell culture medium. The capillary column enhances mass sensitivity due a reduced dilution of the chromatographic band, and further improvements in sensitivity are possible with the use of large volume injections. The method was shown to have good selectivity and sensitivity towards determination of BP protein adducts.

4.2 Paper II

The aim of Paper II was to determine the mass of two novels protein adducts in BP exposed rats. The two protein adducts were isolated from the acid hydrolysis of serum albumin from BP exposed rats, and identified as BP tetrols with the use of a miniaturized LC-ESI-MS system. The retention time and the fragmentation patterns were characteristic of tetrols with formation of the molecular ion and the loss of water molecules. In addition, we observed a fluorescence spectrum for the isolated compounds that where characteristic for the tetrols. The isolated compounds were also each subjected to acid hydrolysis that gave rise to an epimerization between the two unknown tetrols and BPDE II. This epimerization reaction probably involved the OH groups in position C₁₀-OH and C₇-OH of the molecule. The two novel tetrols should then have the C₇-OH and C₈-OH groups in a *cis*

position. The presence of the cis adducts *in vivo* are probably the result of the formation of BPDE III in the metabolism of BP.

4.3 Paper III

The aim of Paper III was to study the relationship between the metabolism of BP i.e. the formation of BP adducts and metabolites and the Ahr genotype in mice. Gene expression measurements on Cyp1a1 and Cyp1b1, showed induction of Cyp1a1 and Cyp1b1 in both Ahr(+/+) and Ahr(+/-) but no induction of the Cyp genes in the Ahr(-/-). There was a significant basal expression of Cyp1b1 in the liver of all genotypes, and this expression was independent of BP exposure. HPLC measurements showed increased levels of protein and DNA adducts, metabolites, conjugates and unmetabolized BP in the internal organs of the Ahr(-/-) as compared to the two other genotypes. These results were indicative of an Ahr independent and/or a slower biotransformation of BP in mice lacking the Ahr.

4.4 Paper IV

The aim of Paper IV was to confirm a slower biotransformation of BP in Ahr knockout mice and also compare the effect of administrations routes. A time course experiment for six months was conducted with animals that received a single oral dose of BP. The Ahr(+/+) mice appeared to have an effective clearance of BP metabolites in the feces, mainly through 3-hydroxybenzo[*a*]pyrene and 9-hydroxybenzo[*a*]pyrene, hence reducing rapidly the levels of DNA and protein adducts in the internal organs. Contrary to this, the Ahr(-/-) mice showed higher levels of DNA and protein adducts in the internal organs during the time course experiment. The Ahr(-/-) mice also experienced a slower excretion of metabolites in the urine and feces compared to the Ahr(+/+) mice. These results confirmed a slower biotransformation of BP in the Ahr(-/-) mice. We also performed an administration route experiment and found that skin-exposed Ahr(+/+) mice showed higher levels of protein adducts, but only in the exposed skin area as compared to the Ahr(-/-). In the non-exposed skin the adduct levels were the same between the two mice groups. These results showed that the distribution and levels of BP and BP-protein adduct in the Ahr(-/-) mice are clearly dependent on the route of exposure. Although not

published, we also found that the Ahr(-/-) mice showed higher levels of metabolites in the examined tissues than the Ahr(+/+) mice. In addition, the conjugate levels were almost the same between the Ahr(-/-) and Ahr(+/+) mice.

5 Discussion

PAH are ubiquitous environmental pollutants found in soil, sediments, water and air. BP is one of the most carcinogenic and genotoxic members of the PAH family. Humans are exposed on a daily basis to PAHs either through the working environment and/or through diverse sources such as polluted air, water, food and cigarette smoking. When assessing the health risk of BP and other PAHs there is a need for both analytical methodologies to detect and measure the BP adducts and BP metabolites, but also for the need of better understanding the metabolism of the compound in the body.

5.1 Analytical aspects and biomarkers

Biomarkers are valuable tools to assess the health risk of exposure to toxic chemicals. BP protein adducts correlate with BP DNA adduct formation and are not subjected to enzymatic repair as DNA (91;96). Hence, the BP protein adduct concentration is generally higher than the BP DNA adduct concentration, which makes the BP protein adducts more sensitive biomarkers to assess and estimate exposure dose (97). In occupational environments, like coke production sites, aluminum smelters, and foundries, the workers are subjected to an exposure for different PAH mixtures, as explained in section 4.1 Therefore it is important to have adequate analytical methods to determine the compounds and their metabolites in body fluids like urine and blood.

Miniaturized analysis schemes are today routinely applied in several applications, and offer some advantages over conventional HPLC like reduced consumption of mobile phases, stationary phases, reduced analysis time and easy coupling with MS (98). On the other hand, the higher LODs and sometimes lack of robustness still challenges the miniaturizing techniques. In Paper I, we implemented a miniaturized HPLC system with packed capillary LC columns and large volume injection, coupled with ESI-MS to determine BP protein adducts. The method involved hydrolysis of BP protein adducts, and the release and measurements of the BP tetrols. The small dimensions of the capillary

columns (0.3x150 mm) offered enhanced mass sensitivity due to reduced dilution of the chromatographic band. In addition, the small 3.5 μm particles of the packed column, offered both an increase in selectivity and efficiency as compared to conventional HPLC packed columns (99). The method was further optimized for large volume injections of samples, with a sample focusing step and a further improvement of the concentration sensitivity. The method also included an off-line SPE step for a crude sample clean up, making the method compatible with different biological matrices. Wang et al had previously demonstrated the use of miniaturized LC-MS for identification and characterization of urinary BP metabolites from mice and rat; however, they did not exploit the potential of large volume injections to improve concentration sensitivity (100;101). In total, the miniaturized LC-ESI-MS technique offer good selectivity and sensitivity and was promising for measurements of low-dose PAH exposure in complex matrices and with limited available samples.

In Paper II, two novel protein adducts were isolated from BP exposed mice, probably formed from a non-bay region diol epoxide. The two adducts were further characterized as BP tetrols by the use of mass spectrometry and fluorescence spectral data. The degree of fragmentation in ESI is soft compared to other ionization techniques in MS. Hence, the most important ions for characterization of the tetrols were the molecular ion and two characteristic water loss fragments. In addition, fluorescence measurements of the two adducts have indicated that the fluorescence excitation and emission spectra were characteristic of the tetrols (92). We performed several acid hydrolysis reactions with the isolated novel BP tetrols, and observed a rapid interconversion between the two novel BP tetrols. The BP 7,8 cis tetrol 2 (X2) epimerizes rapidly in favor of the other novel BP 7,8 cis tetrol 1 (X1). This in pair interconversion has previously been shown for the other four tetrols and is the result of the reversible epimerization of the C10 hydroxyl group during the acid hydrolysis (86;93). When we further increased the temperature of the reaction mixture, we also observed the presence of two other peaks, which were identified as BP-tetrol II-1 and BP-tetrol II-2. A similar pattern was observed when starting the acid hydrolysis with only BP-tetrol II-2. BP-tetrol II-2 reached a rapid and constant equilibrium with BP-tetrol II-1. When increasing the temperature, two other peaks arise, which were identified as BP-7,8 cis tetrol 1 and BP-7,8 cis tetrol 2. Based on our hydrolysis study, and the possible epimerization sites in the BP tetrol molecule (93), we proposed that our observations probably involved an epimerization of the C7 hydroxyl group in the BP

tetrols. The two tetrols should then have the hydroxyl groups in position 7 and 8 in a cis position, and they are most likely formed from a non-bay region diol epoxide, i.e. BPDE III. This epimerization pattern is depicted in Figure 7. In vitro studies in our lab with BPDE-III have confirmed that BPDE III is a precursor to the BP-7,8 cis tetrols (92).

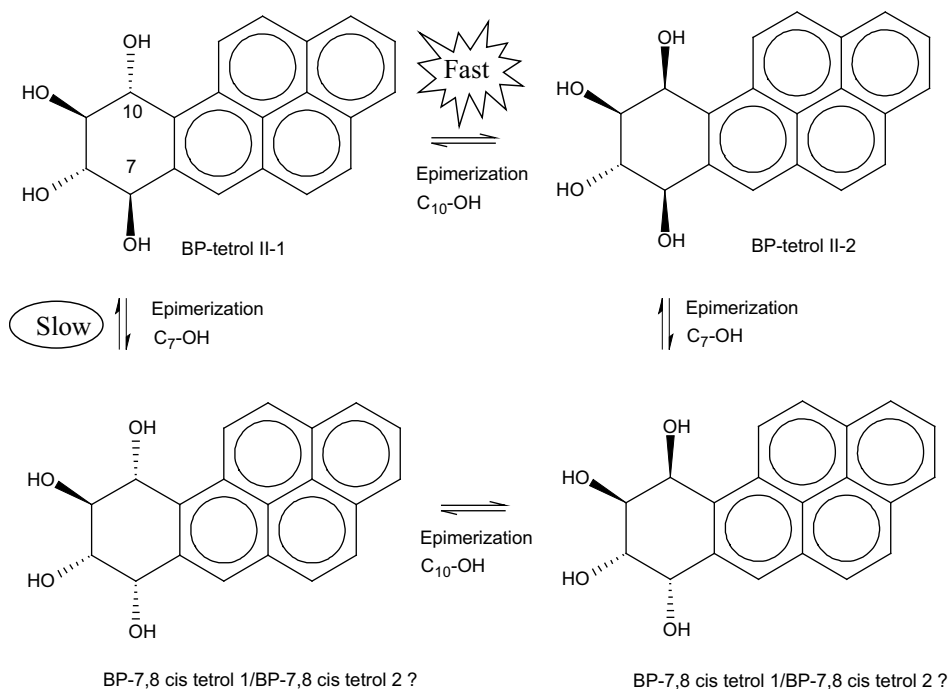


Figure 7 Epimerization at position C7 and C10.

The general attention regarding BP diol epoxide formation has been on the carcinogenic bay-region diol epoxides (BPDE I and BPDE II). The biotransformation of BP forms several reactive diol epoxides, like the non-bay region diol-epoxides (BPDE III). Although, diol epoxides like BPDE III are more easily detoxified by EH, they are still cytotoxic (22;25), and have also been shown to form DNA adducts (102). As we have demonstrated in our study, several BP tetrols are formed when hydrolyzing serum albumin from BP exposed rats. Among these BP tetrols, we have been able to identify two novel BP tetrols. The BP tetrols may serve as important exposure markers for BP, hence we proposed to also include the two BP-7,8 cis tetrols when possible.(94). The biological relevant tetrols should nevertheless, be the sum between BP-tetrol I-1and BP-tetrol I-2 and

between BP-tetrol II-1 and BP-tetrol II-2. In addition it must be necessary to account for the sum of BP-7,8 cis tetrol 1 and BP-7,8 cis tetrol 2.

5.2 Metabolism of BP in Ahr knockout mice

The Ah receptor is central in the induction of the Cyp1a1, hence Ahr knock out mice are valuable animal models to study the metabolism of BP.

In Paper III we investigated the relationship between the Ahr genotype and the biotransformation of BP and the formation of BP adducts in internal organs. In our study, the Ahr mice received only a single oral dose of BP. The Ahr(-/-) mice showed higher levels of BP protein and BP DNA adducts in addition to a high accumulation of unmetabolized BP, than both Ahr (+/-) and Ahr(+/+) mice. Since BP adducts may be used as cancer risk markers it was an interesting result considering that Ahr(-/-) mice have been reported to be more protected against BP induced cancer (81). Nakatsuru et al. (81) observed that Ahr knockout mice had reduced tumor formation compared to the Ahr wild type mice. The mice received BP intraperitoneal or by topical application. Apparently, our results indicated the presence of a slow and Ahr independent biotransformation of BP, possibly involving constitutive Cyp1b1, and other Cyp isoforms, like Cyp2-4.

Kondraganti et al. (37) found that an i.p. dose of BP induced formation of equal amounts of hepatic BP-DNA adducts in both Ahr (+/+) and Ahr (-/-) mice. Although BP did not induce Cyp1a1/1a2 in Ahr (-/-), they showed basal expression of Cyp1a1/1b1 in both Ahr (+/+) and Ahr (-/-) liver. Based on these results they proposed the existence of Ahr independent bioactivation of BP in the knockout mice. Interestingly, Shimada et al. (103) found that Cyp1b1 was expressed at significant levels in different extrahepatic tissues in both Ahr(+/+) and Ahr(-/-) mice. In addition, they proposed that Cyp1b1 may be more important than Cyp1a1 in the first step oxidation of BP and the formation of the BP 7,8 diol (103;104). In our study, we measured constitutive basal levels of Cyp1b1 in both the lung and liver of the Ahr(-/-), that in part can account for the observed metabolism of BP. In other studies, Galvan et al (50;105) have demonstrated that constitutive levels of Cyp1b1 in the bone marrow of Ah non-responsive mice probably affect the metabolism and the toxicity of BP in the bone marrow. Ah non-responsive mice that received a single i.p. dose of BP were found to have a severe bone marrow depletion compared to Ah responsive mice. In early studies by Nebert et al. (69;106;107) they found that Ah

responsive mice survived an oral BP exposure for 6 months and longer, while the Ah non responsive mice died within 3 weeks. The cause of death was identified to be a chemical induced acute depletion of the bone marrow. When administered orally, BP passes through the intestine and enters the bloodstream and the liver. Both the intestine and liver have a substantial and well known metabolizing capacity, resulting in the “first-pass effect” (108), leading to a presystemic elimination of BP. In particular, the induction of Cyp1a1 in the liver of the Ah responsive mice, leads to an efficient metabolism and clearance of BP. Other organs believed to perform a substantial first pass metabolism are the lung and skin (108). The first pass effect protected the animal by reducing the systemic uptake of BP in the Ah responsive mice (69;105;106). In our study, the organs of the Ahr(-/-) mice distal to the stomach (site of administration) like lung and spleen, showed the highest levels of unmetabolized BP. The lack of a first-pass protection in the Ahr(-/-) mice could explain the higher levels of BP accumulated in the internal organs. Similar observations were made with knockout mice models of Cyp1a1, Cyp1a2 and Cyp1b1. Knockout mice exposed to oral doses of BP had higher mortality rates, and showed higher levels of DNA adducts and a slower clearance of BP than the wild type mice (79;80). Apparently, this indicated that the induction of Cyp1a1 was protective and more important in the detoxification than the bioactivation, while the induction of Cyp1b1 was more important in the bioactivation of BP (77). The higher adducts levels in the knockout mice were explained by a reduced phase II conjugation due to a loose coupling between the metabolizing enzymes in the knockout mice and the phase II conjugating enzymes (77). Interestingly, in our study the Ahr(-/-) showed higher levels of BP protein adducts, and of both BP conjugates and BP metabolites, mainly free BP tetrols and BP diols, which may point towards an overall reduced phase II conjugation in Ahr(-/-) mice (109). Hence, the presence of a functional Ah receptor in the Ahr(+/+) mice appeared to be protective, i.e. BP is metabolized and detoxified more efficiently with a reduced formation of BP protein and BP DNA adducts compared to the Ahr(-/-) mice (109). Meanwhile, the Ahr(-/-) mice should have an higher risk in developing genotoxic effects compared to the Ahr(+/+) mice.

In Paper IV we extended the oral exposure regimen and performed a time-course experiment for 6 months with mice that received a single oral dose of BP. The Ahr(-/-) mice were found to have higher levels of BP DNA and BP protein adducts, and unmetabolized BP in all the internal organs. Peak levels of BP DNA adducts in lung were detected after 5-7 days in the Ahr(-/-), whereas the highest levels were observed after 3-5 days in the Ahr(+/+). The results confirmed that Ahr(-/-) mice have a low metabolic

clearance of BP, while the Ahr(+/+) mice have an effective clearance of BP, mainly through 3-OH-BP and 9-OH-BP in the feces in accordance with first pass effect. The Cyp1a1 in different mice studies has been shown to be much more active than any other P450, and the main metabolites formed from BP are BP phenols, mainly 3-OH-BP and 9-OH-BP (13).

The effect of the exposure route on the pharmacokinetics uptake and metabolism of BP has been previously shown on Ah responsive and Ah non responsive mice (106). If BP is given topically, i.p, or subcutaneously, the Ah responsive mice are at greater risk for toxicity and developing tumors at the site of application (106). On the contrary, when BP is given orally, the Ah non responsive mice are at greater risk for bone marrow toxicity, as previously mentioned.

We compared two routes of administration, e.g. topical and i.p. exposure to BP. When the dose was applied topically, the Ahr(+/+) mice showed higher levels of BP protein adducts in the exposed skin area. In addition, in the exposed skin area of the Ahr(+/+) mice, the formation of BP metabolites followed the order BP phenols>BP diols>BP tetrols. In contrast, in the internal organs of the Ahr(-/-) mice the BP protein and BP DNA adduct levels were higher than in the Ahr(+/+) mice. These findings show that the skin of the Ahr(+/+) mice is able to metabolize BP to a much higher extent than the skin of the Ahr(-/-), with a resulting increase in the BP-protein adduct levels locally, and a reduced systemic uptake of BP in the Ahr(+/+). Since BP-protein adducts may be used as cancer risk markers, our results may support the findings of Nakatsuru (36). When a single i.p dose was given, the Ahr(-/-) and Ahr(+/+) mice showed similar levels of BP-protein adducts in the internal organs and the skin. In addition, the BP levels in the internal organs were almost the same between Ahr(-/-) and wild-type mice (+/+), with exception of the lung. The protein adduct measurements are in agreement with the findings of Kondraganti et al. (37), who observed that the total hepatic BP-DNA adduct levels, as measured by ³²P-postlabelling, were equal between Ahr (-/-) and Ahr(+/+) after a single i.p. dose of BP.

In our studies, the Ahr(-/-) mice showed higher levels of BP protein adducts compared to the Ahr(+/+) mice, and of both BP conjugates and BP metabolites, in both administration routes. These observations together with the results from the oral administration study in Paper III shows that the Ahr(-/-) mice seemed to have an overall reduced phase II conjugation compared to the Ahr(+/+) mice.

Thus in the absence of a functional Ahr receptor, and a proper induction of Cyp1a1, there appears to be a lower metabolic clearance of BP resulting in increased levels

of BP protein and BP DNA adducts and metabolites in the Ahr(-/-) mice. There are other oxidative enzymes, like constitutive Cyp1b1 and Cyp2, that may account for the observed BP metabolism in the Ahr(-/-) mice. The constitutive levels of Cyp1b1 appear to be high in the Ahr(-/-) mice as our result shows. Different studies with other mice groups have shown that Cyp1b1 is capable of metabolizing BP to the ultimate carcinogenic diol-epoxide, although at lower rates than Cyp1a1 (49). Our results may indicate that the role of the Cyp metabolism may be more important in detoxification than metabolic activation as other mice model studies also show (110).

6 Concluding remarks

The work presented in this thesis has focused on two subjects related to the PAH science. One part has been the development of analytical methods to measure BP adducts and metabolites in different matrices. The other part has been to understand the relationship between the Ah receptor and the metabolism of BP in mice.

The miniaturized LC-MS system with capillary column switching proved to be an adequate technique in determining BP tetrols in different matrices. One of the major concerns regarding miniaturizing techniques has been the limit in the amount of sample that can be injected into the system. The use of a capillary column switching system with large volume injection circumvented this problem and improved further the sensitivity. Hence, the method has a potential use for measurements of low-dose PAH exposure of workers in different industries. Lately, the number of both users and applications in miniaturized LC has grown in areas where the amount of sample is limited or the analytes of interest are in low concentrations. Another important achievement with the technique was the determination of the molecular mass of two new BP adducts isolated in plasma proteins from BP exposed rats. These new BP adducts were shown to have the same molecular masses as BP tetrols. Epimerization studies gave supporting evidence that these two new BP adducts are in fact BP tetrols, and formed from the less carcinogenic BPDE III. Since these adducts appear to be of biological origin, they should be included when using BP tetrols from plasma proteins as biomarkers of BP exposure. To our knowledge, there are no studies indicating that these adducts have been used as biomarkers.

The Ahr knockout mice model were used to study the metabolism of BP; specifically the relationship between Ahr genotype and bioactivation of BP in internal organs. Interestingly, the lack of a functional Ah receptor results in a slower clearance of BP and higher levels of DNA and protein adducts in the Ahr knockout mice. At the same time, the presence of a functional Ah receptor seems to be beneficial for the detoxification of BP in the Ahr(+/+) mice. Although, the distribution and levels of BP and BP-protein adduct are clearly dependent on the route of exposure. As the skin exposure experiment showed, the Ahr(+/+) mice did actually have higher levels of protein adducts, but only in the exposed skin site. These results indicate that the Ahr(-/-) mice have an Ahr-independent biotransformation of BP that may involve constitutive levels of Cyp1b1 and other oxidative enzymes. Since BP protein adducts may be used as cancer risk markers,

there will be need for further studies to clarify the persistence of BP adducts in the Ahr(-/-) mice, and their relation to cancer.

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Determination of benzo[*a*]pyrene tetrols by column-switching capillary liquid chromatography with fluorescence and micro-electrospray ionization mass spectrometric detection

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The present work displays capillary liquid chromatographic column switching methodology tailored for determination of benzo[*a*]pyrene tetrol isomers in biological matrices using on-line fluorescence and μ -electrospray ionization mass spectrometric detection. A well-established off-line crude solid phase extraction procedure was used in order to make the method compatible with several biological matrices. The solid phase extraction eluates were evaporated to dryness, redissolved in 1.0 ml methanol : water (10 : 90, v/v), loaded onto a 0.32 mm I.D. \times 40 mm 5 μ m Kromasil C₁₈ pre-column for analyte enrichment and back-flushed elution onto a 0.30 mm I.D. \times 150 mm 3.5 μ m Kromasil C₁₈ analytical column. The samples were loaded with a flow rate of 50 μ l min⁻¹ and the tetrols were separated at a flow rate of 4 μ l min⁻¹ with an acetonitrile : 10 mM ammonium acetate gradient from 10 to 90%. A sample loading flow rate up to 50 μ l min⁻¹ was allowed. The fluorescence excitation and emission were set to 342 and 385 nm, respectively, while mass spectrometric detection of the benzo[*a*]pyrene tetrols was obtained by monitoring their [M - H]⁻ molecular ions at *m/z* 319. The method was validated over the concentration range 0.1–50 ng ml⁻¹ benzo[*a*]pyrene tetrols in a cell culture medium with 100 μ l injection volume, fluorescence detection and the first eluting tetrol isomer as model compound, resulting in a correlation coefficient of 0.993. The within-assay (*n* = 6) and between-assay (*n* = 6) precisions were determined to 2.6–8.6% and 3.8–9.6%, respectively, and the recoveries were determined to 97.9–102.4% within the investigated concentration range. The mass limit of detection (by fluorescence) was 3 pg for all the tetrol isomers, corresponding to a concentration limit of detection of 30 pg ml⁻¹ cell culture medium. The corresponding mass spectrometric mass limits of detection were 4–10 pg, corresponding to concentration limits of detection of 40–100 pg ml⁻¹ cell culture medium.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) formed during incomplete combustion of fossil fuels and other organic matter are among the most widespread environmental carcinogens.^{1,2} The highest concentrations of PAHs are found in occupational atmospheres, posing a threat to exposed workers in industries such as coke- and aluminum-plants, iron- and steel-foundries, and rubber- and oil-manufacturing factories.¹ Workers are exposed to PAHs through inhalation, skin contamination and ingestion,¹ and at least 11 PAHs are carcinogenic to experimental animals, based on the International Agency for Research on Cancer (IARC) criteria for carcinogenicity.² Upon metabolism PAHs are converted into electrophiles capable of binding covalently to DNA, RNA and proteins. The formation of DNA-adducts is generally believed to be the initial step in PAH-induced carcinogenesis.^{3,4}

Benzo[*a*]pyrene (B[*a*]P) is the best-studied carcinogenic PAH, and is metabolized in several organs by cytochrome P450 monooxygenases to the ultimate carcinogenic

diol-epoxide *r*-7,*t*-8-dihydrodiol-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-I) and to the less carcinogenic isomer *r*-7,*t*-8-dihydrodiol-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE-II).^{5,6} BPDE-I reacts both with DNA and proteins to form adducts.^{3,4,7} Several studies have employed B[*a*]P tetrols that are liberated upon acid or enzymatic hydrolysis of BPDE DNA— or protein adducts in biological materials isolated from exposed individuals, in order to quantify B[*a*]P adducts.^{7–9} In addition to potential adduct formation in the body, the unstable and reactive BPDEs are further metabolized by epoxide hydrolases to B[*a*]P tetrols (Fig. 1).¹⁰ The bulk part of the excreted B[*a*]P tetrols is likely to originate from hydrolyzed BPDE, but a relation between BPDE adducts and the excreted B[*a*]P tetrols is also plausible.¹¹ Determination of B[*a*]P tetrols in excreta or after liberation from DNA and proteins is thus a promising approach for biomonitoring of PAH exposure, and both conventional gas chromatographic (GC) and liquid chromatographic (LC) methods have been employed for this purpose in combination with a range of sample preparation procedures.^{8,9,11–13} The LC approaches are usually combined with fluorescence detection in order to obtain sufficient

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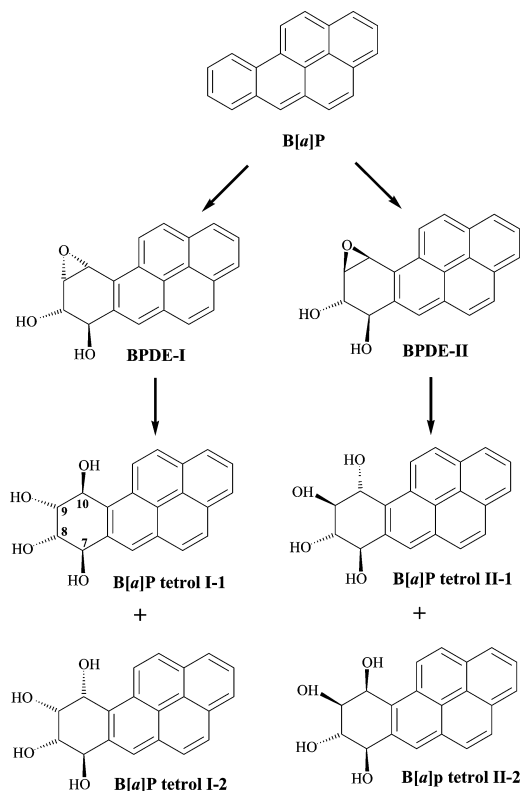


Fig. 1 Metabolism pattern of B[a]P.

sensitivity.^{8,9,14} As the sample amounts in such cases often are low and the concentrations of the B[a]P tetrols also often are correspondingly low, biomonitoring of B[a]P tetrols needs highly sensitive analytical techniques. Miniaturization is often a key word in developing maximum sensitivity with limited sample amounts.

Compared to the use of conventional columns, packed capillary LC with column inner diameters ranging from 500 to 50 μm has offered enhanced mass sensitivity due to reduced dilution of the chromatographic band.^{15,16} Further improvements in concentration sensitivity are accessible with focusing techniques and column switching systems,^{16–21} making time-efficient large volume injections and potentially total sample exploitation available, in addition to on-line sample clean up. Furthermore, the low flow rates used with miniaturized LC columns are especially attractive with regard to electrospray ionization (ESI) mass spectrometry (MS) coupling.²² Wang *et al.* have recently shown the potential of using miniaturized LC-MS for identification and characterization of urinary B[a]P metabolites from highly exposed mice and rat.^{23,24} However, they did not exploit the potential of large volume injections and column switching methodology in order to optimize concentration sensitivity.

The aim of this study was to develop robust and applicable analytical methodology for determination of B[a]P tetrols in various biological matrices by means of a combination of sample enrichment column switching capillary LC with on-line fluorescence and MS detection to obtain an optimized system with regard to separation, sensitivity and structural information.

2. Materials and methods

2.1. Materials and reagents

HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from Rathburn Chemicals (Walkerburn, UK). 96% Ethanol (EtOH) was purchased from Arcus (Oslo, Norway). Water was obtained from an Elgastat Maxima HPLC water purification system (Elga Ltd., Buckinghamshire, UK). Ammonium acetate (NH_4Ac , analytical grade) was provided by Merck (Darmstadt, Germany). The B[a]P tetrols (\pm -benzo[a]pyrene-*r-r*,*t-t*-8,9,*c*-10-tetrahydrotetrol (B[a]P tetrol I-1), (\pm)-benzo[a]pyrene-*r-r*,*t-t*-8,9,10-tetrahydrotetrol (B[a]P tetrol I-2), (\pm)-benzo[a]pyrene-*r-r*,*t-t*-8,*c*-9,*t*-10-tetrahydrotetrol (B[a]P tetrol II-1), (\pm)-benzo[a]pyrene-*r-r*,*t-t*-8,*c*-9,10-tetrahydrotetrol (B[a]P tetrol II-2) were obtained from the National Cancer Institute, Chemical Carcinogen Repository (Kansas City, MO, USA). All fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Nitrogen was obtained from a Whatman nitrogen generator (Haverhill, MA, USA).

Human hepatoma cell lines HepG2 were obtained from American Type Culture Collection (Manassas, Virginia, US), while Dulbecco's modified eagle medium (DMEM), glutamine, penicillin/streptomycin (PS) and foetal bovine serum (FBS) were purchased from Gibco BRL (Paisley, Scotland). Dimethyl sulfoxide (DMSO) and B[a]P were obtained from Fluka (Buchs, Switzerland).

2.2. Chromatographic system

The analytical column (0.3 \times 150 mm, 3.5- μm Kromasil C₁₈) and the enrichment pre-column (0.32 \times 40 mm, 5- μm Kromasil C₁₈) were provided by G&T Septeck (Kolbotn, Norway). A Waters capillary CapLC[®] System (Milford, MA, USA) with a binary gradient pump was used to deliver the mobile phase providing back-flushed desorption from the pre-column and elution on the analytical column. A Model L-7100 (Hitachi Ltd, Tokyo, Japan) isocratic pump was used for sample loading. A Valco Model C4 injection valve or a Rheodyne Model 7125 injection valve (Cotati, CA, USA) (valve1) was used for manual injections of sample volumes of 0.05–1000 μl . A Valco Model CN2 six-port nano-volume valve served as the column-switching valve. The mobile phase gradient initial composition was ACN : 10 mM NH_4Ac (90 : 10, v/v) with a linear gradient to 40% ACN in 26 minutes, followed by a washing step to 90% ACN. The volumetric flow rate was 4 $\mu\text{l min}^{-1}$ throughout the study, while the MeOH : water (10 : 90, v/v) sample loading solvent mixture was delivered at a flow rate of 50 $\mu\text{l min}^{-1}$. The chromatographic system was operated at ambient temperature. The 10 mM NH_4Ac was filtered through a Millex[®]-GS 0.22 μm filter

(Millipore, Billerica, MA, US) daily in order to prevent logging of the analytical column.

An Argos 250B (Flux Instruments, Basel, Switzerland) fluorescence detector equipped with a capillary LC cell with a 20 nl illuminated volume was used for fluorescence detection. The F detector was operated at an excitation wavelength of 342 nm set by an external monochromator and with an emission filter with a nominal cut-off wavelength of 385 nm (50% transmission).

A tandem quadrupole MS equipped with a Z-spray atmospheric pressure ionization ion source prepared for ESI from Micromass (Manchester, UK) was used for MS analysis, with a μ -ESI stainless steel capillary of 26 μ m I.D. Negative mode ESI single ion recording (SIR) of the B[a]P tetrols at $[M - H]^-$ m/z 319 was performed with the applied voltages: capillary voltage: -3.0 kV; sample cone voltage: -25.0 V; and extraction cone voltage: -4.0 V. The nebulizer gas flow was 90 l h⁻¹, the desolvation gas flow was 360 l h⁻¹, the desolvation temperature was 300 °C and the source temperature was 90 °C. The capillary LC system and the tandem quadrupole MS instrument were controlled and data were acquired using MassLynx v3.5 software, and mass spectra were acquired in the m/z range 100–500.

2.3. Standard solutions and sample preparation

Stock solutions of the B[a]P tetrols were prepared in EtOH at a concentration of 1.0 μ g ml⁻¹, from which all working solutions were prepared. In the final method, the sample solvent composition was MeOH : water (10 : 90, v/v), and LC calibration solutions were accordingly prepared in the concentration range 0.1–50 ng ml⁻¹. In order to exploit the method for determination of B[a]P tetrols in several biological matrices such as urine, acid/enzymatic hydrolysis solutions or cell culture media, where the various matrices most probably would influence differently the sample enrichment efficiency, a well-documented, crude off-line solid phase extraction (SPE) procedure was performed on Sep-Pak Plus C₁₈ cartridges (Waters).²⁵ The SPE cartridges were washed with neat water and eluted with neat MeOH, and the eluates were evaporated to dryness under a stream of nitrogen prior to redissolution in 100 μ l MeOH and dilution with water to a total volume of 1.0 ml to obtain the final focusing sample solvent composition.

Validation solutions in the concentration range 0.1–50 ng ml⁻¹ ($n = 5$) were prepared by spiking B[a]P tetrol I-1 to cell culture media, of which replicates of 1.0 ml were subjected to off-line SPE procedure. B[a]P tetrols are sensitive to heat, light, air, and acids, and all solutions were accordingly stored in a freezer at -18 °C prior to analysis.

2.4. Sample

Human hepatoma cell line HepG2 was grown to confluence in 150 mm dishes in DMEM containing 1% glutamine, 1% PS and 10% FBS. All cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere. Cells were exposed to 20 μ M B[a]P (dissolved in DMSO with a 0.05% final concentration) for 2 days. The 10 ml medium volume from the flask was recovered at the end of the incubation period and stored at -20 °C. In this case, an aliquot of 100 μ l of medium was taken

for crude SPE clean-up and subsequent determination of B[a]P tetrols by the developed method.

3. Results and discussion

In industries where workers are exposed to PAHs, the level of exposure is related to their working procedures and tasks. Thus, there is a need for selective and sensitive analytical biomonitoring methods covering a wide concentration range in order to obtain a measure of the exposure to individuals with different working tasks. Especially when DNA adducts are used as biomarkers for exposure the need for sensitivity is often a necessity.²⁶ Furthermore, the non-occupational related exposure to PAHs for non-smokers is minute, even in urban areas, which adds further to the need for analytical sensitivity for biomonitoring of environmental PAH exposure. When keeping in mind that PAH metabolites originating from more than three rings to a large extent are excreted in the faeces,²⁷ it becomes evident that biomonitoring of B[a]P tetrols in biological fluids would benefit from methods with high sensitivity, such as miniaturized LC.

3.1. Optimization of separation

LC mobile phase optimization with ESI-MS detection is usually more challenging than with UV or fluorescence detectors, due to the fact that the MS signal is strongly dependent on the ionization efficiency of the solutes, depending on mobile phase properties. Since the B[a]P tetrols are sensitive to acids,^{7,28} commonly used LC-MS mobile phase additives like acetic and formic acid are best avoided. Thus, only NH₄Ac at concentrations of 0, 5, 10 and 20 mM was evaluated in the present study in combination with isocratic mobile phase compositions of MeOH : water (40 : 60, v/v) or ACN : water (40 : 60, v/v), in order to determine the mobile phase composition providing the best MS signal-to-noise (S/N) ratio. The isomer B[a]P tetrol I-1 (Fig. 1) was employed for this purpose, since B[a]P tetrol I-1 is the isomer found in highest concentrations in biological samples.^{29,30} Solutions containing 0.2 μ g μ l⁻¹ B[a]P tetrol I-1 dissolved in the various mobile phase compositions were introduced to the MS by direct infusion at a flow rate of 4 μ l min⁻¹ and mass spectra were acquired over 1 min. A NH₄Ac concentration of 10 mM provided maximum signal intensity both in combination with ACN or MeOH as organic modifiers. However, the signal intensity with the ACN containing mobile phase was approximately two orders of magnitude higher than with the MeOH containing mobile phase. Thus, ACN in combination with 10 mM NH₄Ac was chosen as organic modifier in further work.

Based on the high bonding density and experiences from previous work,¹⁹ 3.5 μ m Kromasil C₁₈ was employed as the stationary phase material on the analytical column. A mobile phase gradient with an initial composition of ACN : 10 mM NH₄Ac (90 : 10, v/v) followed by a linear gradient to 40% ACN in 26 minutes provided optimized conditions for the separation of the B[a]P tetrols at flow rate of 4 μ l min⁻¹ on the 0.3 \times 150 mm column when using an injection volume of 50 nl. An additional washing step to 90% ACN was included in order to elute more retained compounds, such as other PAHs or metabolites.

3.2. Optimization of detection

With regard to fluorescence detection optimization, excitation at various UV maximum wavelengths was evaluated, and excitation at the highest UV maximum at 342 nm resulted in the highest signal-to-noise ratio in combination with an emission filter with a nominal cut-off wavelength of 385 nm (50% transmission). The fluorescence detector was coupled in-line between the analytical column and the MS by fused silica capillaries of 50 μm I.D. of less than 50 cm length in order to minimize extra-column volumes potentially resulting in reduced MS sensitivity.

In μ -ESI-MS the needle orifice diameter is reduced as compared to conventional ESI, resulting in the emission of much smaller primary droplets that undergo subsequent droplet fissions and evaporation, finally resulting in ion emission. The miniaturized ESI sources have several advantages compared to conventional ESI sources, such as enhanced ionization efficiency and higher tolerance toward salt concentration, which can be of importance when working with biological matrices. In the present study a stainless steel capillary of 26 μm I.D. was employed, especially compatible with flow rates in the low $\mu\text{l min}^{-1}$ range. The needle tip in μ -ESI is typically located closer to the MS inlet lenses as compared to conventional bore ESI capillaries operated with higher flow rates, often resulting in improved ion extraction.²²

The ESI capabilities of the B[a]P tetrol isomers were investigated individually by dissolving the isomers in ACN : 10 mM NH_4Ac (40 : 60, v/v) followed by direct infusion experiments. Ionization in both positive and negative mode was initially explored. However, positive ESI was excluded due to very low ionization as compared to negative mode. These results are in contrast to the studies by Wang *et al.*,^{23,24} where positive ESI were employed. Maximum $[\text{M} - \text{H}]^-$ (m/z 319) MS signal intensities for the various B[a]P tetrol isomers were obtained at partly different operating ESI conditions, illustrated by increased in-source fragmentation of one of the isomers at cone voltages not capable of ionizing other isomers sufficiently. Due to the narrow separation window of the isomers, MS signal optimizing was conducted with special emphasis on obtaining sufficient signal intensities at m/z 319 for all isomers. In general, a stable signal was obtained with increasing capillary voltages up to -3.0 kV, while in-source fragmentation was best avoided at cone voltages below -25 V, when using an optimized extractor cone voltage of -4.0 V and ion source and desolvation temperatures of 90 and 300 $^\circ\text{C}$, respectively. Multiple reaction monitoring (MRM) experiments after fragmentation to m/z 301 ($-\text{H}_2\text{O}$) or 283 ($-2 \text{H}_2\text{O}$) resulted in an overall reduced sensitivity as compared to SIR at m/z 319. Thus, SIR at m/z 319 was used in the final MS method.

3.3 Large volume injection

The main advantage of using miniaturized LC is the increased mass sensitivity, which is beneficial when only limited sample amounts are available. This advantage is, however, only of practical value if a substantial part of the limited sample is introduced to the chromatographic system, often implying enrichment of sample volumes that are larger than the 50 nl

injection volumes which are convenient with capillary columns of ~ 0.3 mm I.D. Furthermore, low flow rates are beneficial only with concentration sensitive detectors, implying that miniaturized LC is not beneficial with regard to sensitivity when using mass-flow sensitive detectors. Fortunately, both ESI-MS at low rates and fluorescence detectors operate according to concentration sensitive principles.²²

The relative high hydrophobicity of the B[a]P tetrols requires the presence of an organic modifier in the sample focusing solution in order to avoid precipitation of the solutes. This requirement imposes that a stationary phase material providing high retention of the B[a]P tetrols is preferred in order to obtain sufficient sample enrichment capabilities particularly with high sample loading flow rates. 5- μm Kromasil C_{18} particles provide a compromise between sample capacity and column backpressure during sample loading at elevated flow rates. Despite the fact that similar alkyl ligands were used on both the analytical column and the pre-column, the use of 5- μm pre-column particles has a potential of providing reconcentration of the solutes on the analytical column containing 3.5 μm particles, and was thus used as the pre-column stationary phase material. The 0.32 mm I.D. pre-column was of a length of 40 mm in order to be easily mounted in the switching valve.

Methanol as an organic modifier in the focusing sample solution resulted in slightly higher retention of the B[a]P tetrols on the pre-column as compared to ACN, while still providing similar solubility. A standard mixture of the four B[a]P tetrol isomers was dissolved in aqueous solvent mixtures with MeOH content of 5, 10, 15 and 20%, and an expanded injection volume of 100 μl of the sample solutions was injected using the total column switching system and a flow rate of 4 $\mu\text{l min}^{-1}$. The peak widths at half peak height and the peak resolutions were measured with the different sample focusing compositions. For this purpose a fluorescence detector was employed. The peak widths were constant within the 5–20% MeOH content interval, while the baseline separation between B[a]P tetrol II-2 and B[a]P tetrol II-1 was not preserved when increasing the MeOH content from 10 to 15%. The peak resolution declined further when the MeOH content was increased to 20% (Fig. 2). Thus, a composition of MeOH : water (10 : 90, v/v) was used as sample focusing solution in the final method. Peak area measurements when comparing 100 μl injection volumes using this sample focusing composition with 50 nl injections with equal absolute mass, dissolved in neat MeOH, yielded identical peak areas, confirming complete dissolution of the B[a]P tetrols in this final sample focusing composition and no break-through.

Injection of large sample volumes using a loading flow rate of 4 $\mu\text{l min}^{-1}$ is a time consuming process. However, due to the low column backpressure on the pre-column, higher flow rates were applicable within the pressure limits of the chromatographic system. Elevated loading flow rates might, however, destroy the sample focusing process, potentially resulting in band broadening or sample break-through. A loading flow rate of 50 $\mu\text{l min}^{-1}$ was applicable with the final sample focusing composition used in the present study without exceeding the pressure limits of the system. Identical peak shapes, peak resolutions and peak areas were obtained when injecting 100 μl using a loading flow rate of 50 $\mu\text{l min}^{-1}$ as

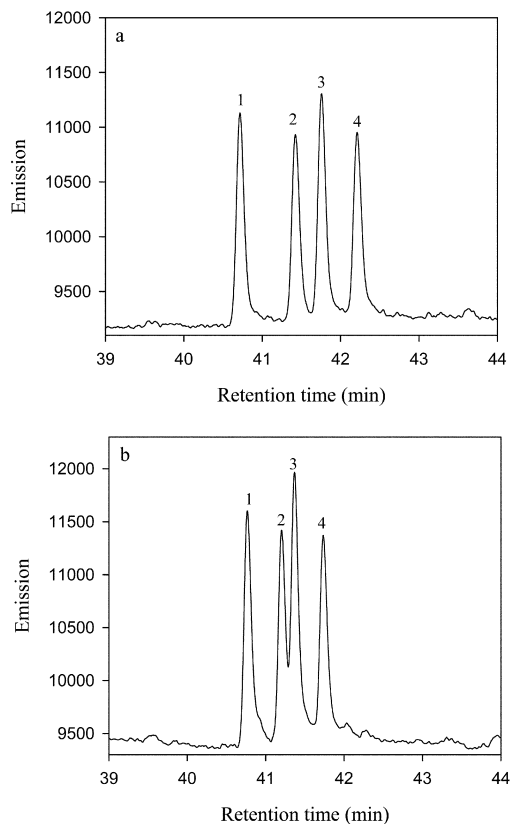


Fig. 2 Chromatographic profiles of the B[a]P tetrols using an injection volume of 100 μl and a focusing sample solution composition of water : MeOH (90 : 10, v/v) (a) and water : MeOH (80 : 20, v/v) (b) (fluorescence detection). Elution order: B[a]P tetrol I-1 (1), I-2 (2), II-2 (3) and II-1 (4).

compared to when loading at $4 \mu\text{l min}^{-1}$. Thus, a loading flow rate of $50 \mu\text{l min}^{-1}$ was used in the final method, reducing the loading time from 25 to 2 minutes. Fig. 3 shows the chromatographic profiles of a standard mixture of the B[a]P tetrols when using the final column switching methodology with fluorescence and MS detection.

In order to examine if injection of volumes larger than 100 μl were applicable with the final column switching methodology, a sample volume of 1 ml was loaded at $50 \mu\text{l min}^{-1}$. The peak shapes and peak resolutions were comparable to the 100 μl injections, and the peak areas were 10 times larger, supporting the fact that injection of sample volumes up to at least 1 ml is applicable with this method for further improvement of concentration sensitivity.

3.4. Method validation

As pointed out earlier, B[a]P tetrols can be present in various biological matrices, mainly in excreta or after liberation from DNA and proteins. A well-documented, crude off-line reversed

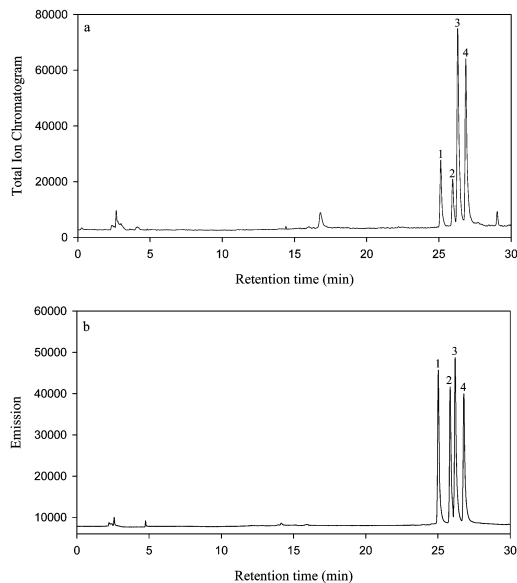


Fig. 3 Column-switching capillary LC-fluorescence (a) and LC-MS (b) profiles of the B[a]P tetrols using 100 μl injection volume. Elution order: B[a]P tetrol I-1 (1), I-2 (2), II-2 (3) and II-1 (4).

phase SPE method has in a number of studies been employed for off-line sample pre-treatment of B[a]P tetrols from various matrices.²⁵ As biological matrices might differently influence the pre-column sample enrichment, this off-line SPE method was used in the present study as well, in order to obtain a sensitive analytical methodology suitable for a maximum number of applications. In the method validation, aqueous cell culture media solutions served as a model matrix, to which exact amounts of B[a]P tetrols were spiked. After SPE elution, the eluates were evaporated to dryness and redissolved in 1.0 ml of the final sample focusing solution, whereof 100 μl was injected with a loading flow rate of $50 \mu\text{l min}^{-1}$. The first eluting isomer, B[a]P tetrol I-1, served as a model compound in the precision and recovery experiments, with fluorescence detection.

The total method was validated in the concentration range $0.1\text{--}50 \text{ ng ml}^{-1}$ B[a]P tetrol I-1 in cell culture medium. The method was linear within the investigated concentration range with a coefficient of correlation of 0.993.

The within- and between-assay precision were established by injecting six sets of samples at three spike concentration levels (0.1, 25 and 50 ng ml^{-1}) within one and six days, respectively, by the same analyst. The within-assay ($n = 6$) and between-assay ($n = 6$) precision was in the range 2.6–8.6 and 3.8–9.6%, respectively, as summarized in Table 1.

The recoveries of B[a]P tetrol I-1 were established by comparing the resulting peak areas from 100 μl column-switching injections of validation solutions with 100 μl injections of the LC calibration solutions directly onto the analytical column ($n = 3$). The recoveries at the different concentration levels were in the range 97.9–102.4%, as summarized in Table 1.

Table 1 Within- and between-assay precision and recoveries of the total method

Analyte	Concentration level/ng ml ⁻¹	Within-assay	Between-assay	Recovery (%)
		precision (% RSD, n = 6)	precision (% RSD, n = 6)	
B[a]P tetrol I-1	0.1	8.6	9.6	98.4
	12.5			97.9
	25	3.8	4.0	99.7
	37.5			98.6
	50	2.6	3.8	102.4

The mass limit of detection (mLOD) of the total method was 3 pg (S/N = 3) for all the B[a]P tetrol isomers when using fluorescence detection, corresponding to a concentration limit of detection (cLOD) of 30 pg ml⁻¹ cell culture medium when using a 100 µl injection volume and 1.0 ml cell culture initial volume. Since the method is capable of introducing at least 1 ml sample volumes, the cLOD can potentially be improved by a factor of 10. The mLODs when using ESI-MS detection were 7, 5, 10 and 4 pg for B[a]P tetrols I-1, I-2, II-1 and II-2, respectively, corresponding to cLODs of 70, 50, 100 and 40 pg ml⁻¹ cell culture medium with 100 µl injection volume and 1.0 ml cell culture initial volume. The LC-ESI-MS mLODs are improved by a factor of about 10 as compared to the study by Wang *et al.* who recently reported negative ESI LC-MS mLODs of B[a]P metabolites in the range 40–60 pg.²³

The within- and between-assay precision of retention times were below 1% RSD, and the column efficiency remained invariant throughout the study. The same pre-column and analytical column was used throughout the study, and identical chromatographic performance was observed when the pre-column and the analytical column were replaced with columns prepared identically. All calibration, validation and pre-treated real sample solutions were stable for at least two months when stored in the dark at -18 °C.

3.5. Determination of B[a]P tetrols in cell culture medium

Cell culture is a frequently used model system to study biotransformation and molecular effects of carcinogenic compounds like PAHs.³⁰ In order to monitor the time dependence of biotransformation reactions, analytical methodology based on full sample exploitation enables the capture of small cell medium volumes from on-going biotransformation reactions without disturbing growth by large reduction of the cell culture volumes. A 100-µl aliquot of the 10-ml total cell culture medium volume was incubated for two days, in order to illustrate the applicability of the method, and Fig. 4 shows the resulting LC-MS chromatogram. The determined amounts of the B[a]P tetrols in the samples when injecting 100 µl of the total 1.0 ml redissolved SPE eluate were 179, 8, 53 and 35 pg of B[a]P tetrol I-1, I-2, II-1 and II-2, respectively, corresponding to a cell medium concentration of 17.9, 0.8, 5.3 and 3.5 ng mL⁻¹, respectively.

4. Conclusion

This paper describes a sensitive and selective miniaturized LC method for determination of B[a]P tetrols using robust sample enrichment methodology. The implemented well-documented

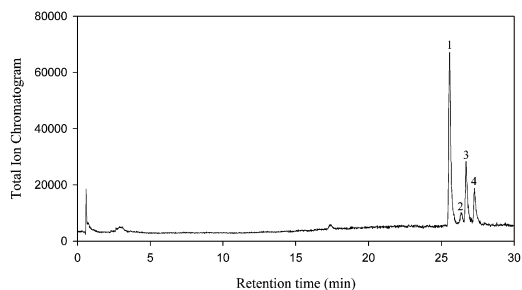


Fig. 4 Column-switching capillary LC-MS profiles of the formed B[a]P tetrol metabolites from cell culture medium exposed to B[a]P. 100 µl cell culture medium of a total volume of 10 ml was subjected to initial SPE clean-up, whereof 100 µl of the total 1.0 ml volume of the evaporated and redissolved SPE eluate was injected. Elution order: B[a]P tetrol I-1 (1), I-2 (2), II-2 (3) and II-1 (4).

off-line SPE procedure offers crude sample clean up, making the method compatible with most biological matrices of various nature, although the demonstration of the applicability of the method in the present study is limited to cell culture medium matrices only. The column switching method can easily be automated for high throughput analysis of large sample series and subsequent assessment of PAH exposure in groups of individuals of particular interest. The developed column switching system might also be modified to include the determination of other B[a]P metabolites in various matrices. In such cases, application of larger sample volumes can potentially improve the cLODs of the method. The method is to be used for measurement of low-dose PAH exposure of workers from relevant industries.

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