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CHLORINATED POLYCYCLIC AROMATIC HYDROCARBONS ASSOCIATED WITH DRINKING WATER CHLORINATION - PREPARATION, QUANTIFICATION AND GENOTOXICITY CHARACTERIZATION

CHLOROWANE POLICYKLICZNE WĘGLOWODORY AROMATYCZNE ZWIĄZANE Z CHLOROWANIEM WODY PITNEJ - POWSTAWANIE, OZNACZANIE I CHARAKTERYSTYKA GENOTOKSYCZNA

Summary: Polycyclic aromatic hydrocarbons (PAHs) are among the most persistent and toxic organic micropollutants present in water and several of them are mutagens and carcinogens. Furthermore, recent studies suggest that PAHs and their metabolites might be endocrine disrupters. Although it has been shown that chlorinated derivatives of PAHs (Cl-PAHs) are formed during the water chlorination procedure, little is known about their potential genotoxic and carcinogenic effects. Considering that drinking water quality is of essential public health concern, the identification of Cl-PAHs formed as chlorination by-products and the investigation of their potential hazard to humans, seems to be highly relevant. The objectives of this work were to prepare and characterize the major chlorinated derivative of benzo[a]pyrene (BaP), to develop analytical methodologies for its quantification in water samples and to evaluate its genotoxic and potentially carcinogenic effects. Chlorinated standards from BaP were first prepared by adaptation of Mulder et al method using copper dichloride in carbon tetrachloride and by a newly developed two phase method (water/*n*-hexane), in acidic medium, using aliquot 336 as the phase transfer catalyst. 6-chlorobenzo[a]pyrene (6-Cl-BaP) was obtained by both methods, as the major product of chlorination of BaP. All products obtained were isolated (by semi-preparative HPLC) and characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The chemical stability was studied at 18ºC and 37ºC, using HPLC with fluorescence detection (FLD), NMR and gas chromatography with MS detection. A SPE-HPLC-FLD methodology for the quantification of 6-Cl-BaP in water samples was developed and validated, with a limit of detection of 0.0032 μg/dm^3. The genotoxic effect of 6-Cl-BaP was characterized in comparison to that of equimolar concentrations of BaP, using the comet assay in a human liver-derived cell line. Preliminary data show that 6-Cl-BaP is genotoxic and that it is able to induce higher levels of DNA damage than BaP, suggestive of a more potent genotoxic effect.

Keywords: polycyclic aromatic hydrocarbons (PAHs), water, chlorination by-products, preparation, spectroscopic characterization, 6-chlorobenzo[a]pyrene, analytical methodologies, genotoxicity, human health

Polycyclic aromatic hydrocarbons (PAHs) represent an important class of chemical carcinogens that are widespread in the environment due to fossil fuel combustion, transportation, industry, cigarette smoke, and exposure in specific occupations as well as smoked and grilled foods [1-3]. These compounds are also classified as endocrine disruptors because they interact with estrogen receptors ERα and ERβ [4, 5].

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Chlorinated PAHs (Cl-PAHs) are PAHs derivatives detected in some environmental samples such as urbain air, road tunnel air [6, 7] and tap water, formed as chlorination by-products [7-9]. The few toxicological studies presented in the literature indicate that Cl-PAHs display greater mutagenicity than the corresponding parent PAHs [10, 11]. Considering that drinking water quality is of essential public health concern, the identification of Cl-PAHs formed as chlorination by-products and the investigation of their potential hazard to humans, seems to be highly relevant.

The objectives of this work were to prepare and characterize the major chlorinated derivative of benzo[a]pyrene (BaP), to develop analytical methodologies for its quantification in water samples and to evaluate its cytotoxic, genotoxic and potentially carcinogenic effects in a human hepatoma cell line.

Methods

Chlorinated standards from BaP were first prepared by adaptation of Mulder et al method, using copper dichloride in carbon tetrachloride [12]. 6-chlorobenzo[a]pyrene (6-Cl-BaP) was isolated, by semi-preparative reverse phase high pressure liquid chromatography with ultraviolet/visible detection (HPLC-UV/Vis), as the major product in 82% yield. The product was obtained as a yellow solid and characterized by nuclear magnetic resonance (NMR), mass spectrometry (MS) and fluorescence excitation (FExS) and emission spectra (FEmS): ^{1}H-NMR (400 MHz, CD_3CN) δ (ppm): 9.23÷9.16 (2H, m, H10 + H11), 8.87÷8.84 (1H, m, H7), 8.55 (1H, d, J = 9.5, H5), 8.44 (1H, d, J = 9.0, H12), 8.37 (1H, d, J = 7.4, H1), 8.24 (1H, d, J = 7.2, H3), 8.17 (1H, d, J = 9.6, H4), 8.09÷8.06 (1H, m, H2), 7.98÷7.97 (2H, m, H9 + H8); GC-MS (EI): m/z (%): 286 (100) [M^+], 250 (30) [M^+-HCl], 143 (7), 125 (16); FExS (λ_{max}, nm): 242, 266, 288, 306, 323, 340, 359, 381; FEmS (λ_{max}, nm): 343, 359, 417, 441, 481. 6-Cl-BaP was also prepared by a newly developed two phase method (water/*n*-hexane), in acidic medium, using aliquot 336 as the phase transfer catalyst: a suspension of aliquot 336 (3.5 mg, 8.56 μmol), NaOCl (800 mm^3), water (4 cm^3) and HCl 0.1 M solution (800 mm^3) was added to a solution of BaP (21.6 mg, 0.856 μmol) in *n*-hexane (10 cm^3) and the resulting mixture was shaken for 10 minutes in a separatory funnel. The organic phase was extracted and evaporated to dryness in a rotating evaporator. The residue obtained was dissolved of acetonitrile (4 cm^3) and purified by semi-preparative HPLC-UV/Vis. 6-Cl-BaP was obtained, as the only product of chlorination of BaP, as a yellow solid (21.6 mg, 96%).

A methodology for the determination of 6-Cl-BaP in water samples, was developed using a solid phase extraction technique (SPE) followed by a reverse high pressure liquid chromatography-fluorescence detection (HPLC-FLD) methodology ($\lambda_{excitation}$ = 295 nm and $\lambda_{emission}$ = 440 nm). A SPE vacuum manifold, twelve positions was used. The C_{18} cartridges (500 mg, 6 cm^3) were previously conditioned with 7 cm^3 of acetonitrile and 14 cm^3 of ultra-pure water. Aqueous samples (250 cm^3) were passed through the cartridge at the maximum rate allowed. The cartridge was dried by blowing N_2 for 20 minutes. The adsorbed 6-Cl-BaP was eluted with 10 cm^3 n-hexane and the cartridge was then washed with 2 cm^3 *n*-hexane. The solutions were concentrated to 1.0 cm^3, with N_2 blowing, after the addition of 2 cm^3 acetonitrile. The separation was performed with a C_{18} HPLC column and elution temperature was maintained at 20ºC, during 20 minutes running with 100% acetonitrile. The flow rate was 2 cm^3/min. 6-Cl-BaP was quantified by peak area using the

external standard method. Ten calibration solutions were injected directly in the HPLC system, using eleven replicates of the end points.

The human HepG2 cells (ATCC, Rockville, MD) were used for genotoxicity testing. Cells were cultured in DMEM/F12 medium (Invitrogen) containing 15% FBS. $5 \cdot 10^5$ cells were plated in complete medium for 24 h and were then exposed to BaP or to 6-Cl-BaP (0.1 to 125 μM) dissolved in dimethylsulfoxide (DMSO) and acetonitrile, respectively. Positive (ethyl methanesulfonate, EMS, 20 mM, 1 h) and solvent controls were also tested. After 24 h exposure, the culture medium was discarded and cells were washed twice with saline. Cells were trypsinized, counted and viability was assessed by the trypan blue exclusion technique. The alkaline comet assay was performed as described by Singh et al (1988), with minor modifications [13]. Two slides were prepared for each treatment condition and one hundred cells were analysed from replicated slides using the CometImager software (MetaSystems, GmbH). The percentage of DNA in tail was evaluated as a measure of DNA damage. Cytotoxicity was determined by the neutral red assay.

Discussion

The two phase procedure developed for the preparation of 6-Cl-BaP is a very easy and fast method, leading to the formation of the product in high yield. Moreover, it has the advantage of avoiding carcinogenic solvents such as carbon tetrachloride, which was used in already existing methods [12].

The spectroscopic data presented for the major product, obtained in both reactions, is characteristic of 6-Cl-BaP (Fig. 1). When compared with BaP, the most noticeable differences observed in 6-Cl-BaP ^{1}H-NMR spectrum (both recorded at 18ºC) are the disappearance of the singlet (8.82 ppm) from H6 and the downfield shift of the signal from H7. These observations are entirely consistent with the presence of a chlorine atom in the 6th position of the polycyclic aromatic structure. The assignment of all signals was based on correlations observed in the COSY spectrum. The mass spectrum (EI) shows the presence of the molecular ion (M^+) at m/z 286, which indicates the existence of a chlorine atom, and a fragment ion occurring at m/z 250, representing the loss of hydrogen chloride (HCl) from the molecular ion. In the mass spectrum it is also visible the presence of the molecular ion (M^+) at m/z 288, with one third of the intensity of the m/z 286 peak, characteristic of ^{37}Cl-BaP. The spectroscopic information was similar to the data presented by other authors [12, 14].

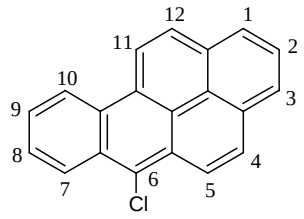

Fig. 1. Structure of 6-Cl-BaP

The thermal stability of 6-Cl-BaP was studied using HPLC-FLD, NMR and gas chromatography with MS detection (GC-MS). For the study using HPLC-FLD, a solution

of 6-Cl-BaP was prepared in acetonitrile (1 mg/dm^3), and the stability was studied at 18ºC and 37ºC for approximately 8 and 4 hours, respectively (Fig. 2). For the NMR study, a solution of 6-Cl-BaP was prepared in acetonitrile-d_3 (1 mg/dm^3), and the stability was studied at 37ºC for approximately 4 hours, where spectra was recorded every 10 minutes. In the GC-MS study, the stability was studied at 37ºC for approximately 4 hours using a solution of 6-Cl-BaP in ethyl acetate (10 mg/dm^3).

From Figure 2 it can be seen that 6-Cl-BaP is stable at 18ºC but at 37ºC it is noticeable some instability, since the value of the peak area suffers effective changes, although reversible, during the experiment. Unexpectedly, the GC-MS study did not show significant changes in the chromatogram during the 4 h experiment. The ^{1}H-NMR spectra recorded at 37ºC did not show an effective change in the structure, since no extra signals are observed and the shift and integration area of all signals seem to be unchanged during the 4 h experiment. Nevertheless, the slight changes in the shape of some signals (for example H7) over the experiment time, can evidence that at this temperature some modification occurs in the molecule, due to conformational or electronic changes. Some experiments are now in progress in order to better understand this modification.

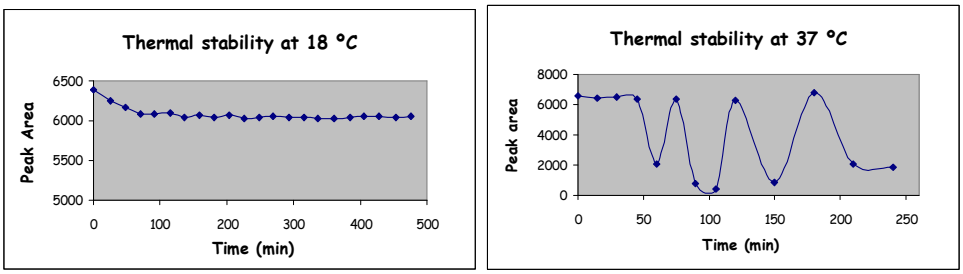

Fig. 2. Thermal stability of 6-Cl-BaP at 18 and 37ºC (HPLC-FLD)

In the SPE procedure, the associated recovery of 6-Cl-BaP was studied for a 10 cm^3 *n*-hexane elution volume. The average recovery (R) and relative standard deviation (RSD) obtained were, respectively, 84.4 and 12.3%. The high recovery obtained can be justified by the low water solubility and high hydrophobicity of the compound, which is probably similar to the parent BaP and to other PAHs. These compounds have low Henry's constant values (K_H) and high octanol-water partition coefficients (K_{OW}), which explains their high affinity to the organic fraction.

The analytical parameters evaluated to validate the SPE-HPLC-FLD methodology were: linearity and limits of detection (LOD) and quantification (LOQ). The linearity of the curve was studied following two steps: 1) adjustment of the working range for a 99% confidence interval, using the F-test for homogeneity of the variances; 2) calculation of the linearity, applying the International Standard ISO 8466-1. The results are presented in Table 1.

Table 1

Linearity parameters for the analytical methodology

Instrument linearity [µg/dm^3]	**m**	**b**	**R^2**	**RSD [%]**
1-10	12.26	–2.68	0.9909	5.6

A good linear correlation between concentration and peak areas was obtained, with an estimation coefficient (R^2) of 0.9909 and RSD values less than 6%.

LOD and LOQ were calculated using two different criterions. The first criterion is based on the determination of the residual standard deviation (s_y). The other uses the standard deviation (s_0) of fortified solutions at a sub nanogram per dm^3 concentration, in ultra-pure water. The solutions underwent the entire methodology, and the concentrations were calculated using the calibration curve. The results are presented in Table 2.

Table 2

LOD and LOQ of the analytical methodology

First criterion		**Secon criterion (n = 12)**		**LOQ Test (n = 12)**
LOD* [μg/dm^3]	LOQ [μg/dm^3]	LOD** [μg/dm^3]	LOQ [μg/dm^3]	RSD [%]
0.0048	0.0145	0.0032	0.0097	3.7

* LOD = $[(3.3 \cdot s_y/m)/250]/0.844$; m - slope of the calibration curve; n - number of replicates
** LOD = $AC_0 + 3.3 \cdot s_0$ (AC_0 - average concentration of the fortified solutions)

The LOD obtained was 0.0048 μg/dm^3, using the first criterion, and 0.0032 μg/dm^3, using the second criterion. The second criterion is much stricter, since it considers the entire methodology. The LOQ was later experimentally confirmed, and the RSD value obtained was less than 5%, indicating that the limit is accurately determined. The limit of detection obtained allows the determination of 6-Cl-BaP at the levels required by the Portuguese legislation for the quantification of BaP.

To determine the trueness, repeatability and intermediate precision of this methodology, samples are being prepared at a 0.03 μg/dm^3 concentration level (ten replicates) in three different matrices, ultra-pure, tap water and surface water, by three analysts. The analytical recoveries (R) will be used to evaluate the trueness of the method. The repeatability will be calculated as within-day RSD of analyte concentration and intermediate precision evaluated as RSD of analyte concentration, obtained in consecutive days by three analysts, in the different matrices.

The cytotoxic and the DNA damaging activities of 6-Cl-BaP, in comparison with BaP, were assessed using HepG2 cells. This cell line has the advantage of retaining some of the liver metabolic capacity, which is essential to transform BaP and, presumably, 6-Cl-BaP in their reactive forms. On the other hand, the alkaline comet assay is increasingly used for evaluation of the genotoxic potential of chemicals because is rapid, simple to perform, requires minimal amounts of test substances and presents high sensitivity and specificity [15]. Preliminary data from the comet assay showed that 6-Cl-BaP, as well as BaP, are not able to significantly induce DNA damage at a low dose-range (0.1 to 20.0 μM), in comparison with solvent controls (Fig. 3A).

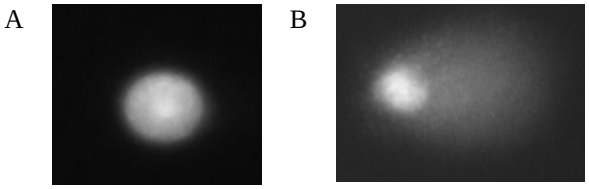

Fig. 3. Comet images representative of non-damaged (A) and damaged cells (B)

At higher concentrations (100 and 125 μM), however, 6-Cl-BaP was able to induce a significantly higher level of DNA damage than BaP ($P = 0.022$ and $P = 0.005$, respectively). As expected, treatment with EMS (positive control), yielded a significantly increased percentage of DNA in tail, in agreement with its known DNA damaging activity (Fig. 3B). Data from the cytotoxicity assay showed that both compounds, BaP and its derivative, have similar cytotoxic effects.

Conclusions

A new simple, fast and selective two phase procedure was developed for the preparation of 6-Cl-BaP, leading to the formation of the product in high yield. This technique is now being applied to other PAHs, for the preparation of their chloride derivatives and can also be applied to other aromatic compounds.

The methodology developed for the quantification of 6-Cl-BaP in water samples, enables its quantification in real samples at subnanogram per dm^3 concentration, allowing the assessment of human exposure to Cl-PAHs-contaminated water.

The present data from the comet assay show that 6-Cl-BaP is genotoxic and that, at the equimolar doses of 100 and 125 μM, 6-Cl-BaP is able to induce a significantly higher level of DNA damage than BaP, in HepG2 cells. Further studies are underway, to provide new insights into the DNA damaging potential of 6-Cl-BaP.

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CHLOROWANE POLICYKLICZNE WĘGLOWODORY AROMATYCZNE ZWIĄZANE Z CHLOROWANIEM WODY PITNEJ - POWSTAWANIE, OZNACZANIE I CHARAKTERYSTYKA GENOTOKSYCZNA

Streszczenie: Wielopierścieniowe węglowodory aromatyczne (PAH) są jednymi z najczęściej występujących i najbardziej toksycznych organicznych mikrozanieczyszczeń obecnych w wodzie, mają także działanie mutagenne i rakotwórcze. Co więcej, ostatnie badania sugerują, że PAH i ich metabolity mogą zakłócać pracę układu wydzielania wewnętrznego. Chociaż wykazano, że chloropochodne PAH (Cl-PAH) powstają w czasie chlorowania wody, to niewiele wiadomo o ich potencjalnym działaniu genotoksycznym i rakotwórczym. Biorąc pod uwagę podstawowe znaczenie wpływu jakości wody pitnej na zdrowie publiczne, identyfikacja Cl-PAH powstających jako produkt uboczny w czasie chlorowania wody i zbadanie pojawiających się wskutek tego potencjalnych zagrożeń jest zadaniem o dużym znaczeniu. Celem pracy było zsyntezowanie i scharakteryzowanie głównych chloropochodnych benzo[a]pirenu (BaP), opracowanie metod analitycznych do oznaczania go w próbkach wody i ocena jego działania genotoksycznego i rakotwórczego. Zsyntezowane próbki 6-chlorobenzeno[a]pirenu scharakteryzowano za pomocą jądrowego rezonansu magnetycznego i spektroskopii masowej. Za pomocą HPLC, NMR i chromatografii gazowej zbadano trwałość chemiczną otrzymanego związku w temp. 18ºC i 37ºC. Opracowano metodę oznaczania 6-Cl-BaP w wodzie za pomocą SPE-HPLC-FLD. W metodzie tej granica wykrywalności wynosiła 0,0032 μg/dm^3. Porównano genotoksyczność 6-Cl-BaP i BaP. Otrzymane wyniki wstępnych badań sugerują wyższą genotoksyczność 6-Cl-BaP w porównaniu do BaP.

Słowa kluczowe: wielopierścieniowe węglowodory aromatyczne (PAH), woda, produkty uboczne chlorowania, synteza, charakterystyka spektroskopowa, 6-chlorobenzo[a]piren, metody analityczne, genotoksyczność, zdrowie ludzkie