Sensitive Monoclonal Antibody Method Based ELISA on Filter for Detection of Benzo[a]pyrene in Air

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Abstract There are several hundred PAHs; the best known is benzo[a]pyrene -B(a)P. Most measurements have been made on B(a)P, with different sampling and analytical procedures requiring prolonged treatment of the sample and subsequent chromatographic analysis. We proposed the present method for initial screening to check for B(a)P, developing a rapid and inexpensive immunoassay method, based on antibodies use, that allows the revealing of benzo(a)pyrene in air environmental samples.

Keywords Benzo(a)pyrene, ELISA, Exposure assessment methodology

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a family of hydrocarbons, of over 100 different chemicals, similar to benzene rings, which contain many rings joined together through at least one pair of carbon atoms. The PAHs pollutants have high molecular-mass (4 and more condensed aromatic rings) are considered to be more dangerous than two and three rings PAHs in view of their potential. There are several hundred PAHs, but the US Environmental Protection Agency (EPA) introduced a priority pollutant list of 16 PAHs (EPA 610) in 1982, the best known is Benz[a]pyrene (B(a)P). This chemical agent is considered the most dangerous of PAHs because of its chemical and physical characteristics and its ease of accumulation in the food chain [1, 2] B(a)P was the first PAH to be identified as a carcinogen and has therefore been studied most often. The International Agency for Research on Cancer (IARC) and the Environmental Protection Agency (EPA) has classified probably carcinogenic to humans. B(a)P is not only a carcinogenic compound, but recent studies demonstrated its involvement in the development of cardiovascular diseases [3]. Because it is a lipophilic compounds, readily crosses blood-brain barrier, and it has also been shown to be accumulated and metabolized in the brain; there have been some studies that show neurotoxic effects in vivo [4, 5, 6]. The analytical methods traditionally used to verify the presence of PAHs in atmospheric particles, are those suggested by international organizations such as US-EPA and NIOSH providing high volume sampling, solvent extraction, purification and analysis by gas chromatography/mass spectrometry (GC/MS) or High performance liquid chromatography/fluorescence detection (HPLC/FID). These methods are time consuming, mainly due to complex procedures involved in the preparation of samples, which usually includes removal of disturbing matrix compounds and concentration of the target analytes.

The objective of our project is to develop a rapid immunoassay which can be applied to screening of a large number of samples saving the time of the analysis in chromatography. Recently it is reported on the generation of a very specific monoclonal antibodies for PAHs and its application for the determination of these chemicals in soil and water extracts [7]. The objective is to develop a rapid and inexpensive immunoassay method based on specific antibody use to B(a)P, that allows its detection in air environmental samples.

ELISA (enzyme-linked immunosorbent assay) is the most commonly used immunoassay technique for environmental analysis. In fact there are several commercial ELISA kits for the assessment of BTX and PAHs, which allow the quantization in samples of sediment or other environmental matrices [8].

These techniques still require previous treatment of the environmental standard, and are not typically used for air samples, which are usually collected on a filter.

We developed ELISA technique directly on the filter sampling. Since immunoassay methods can provide quantitative data when configured with a series of reference standards that are analyzed and used to construct a standard curve, we tested the optimal conditions for ELISA on filter, from a solution of B(a)P neat in toluene, commercially...
ELISA tests were performed on PTFE filters as used for PAHs sampling, using a BSA blocking agent for nonspecific sites. Between different types of commercially antibodies, we selected for our ELISA – screening assay, the primary mouse antibody BAP-13 (monoclonal) and rabbit antimouse BaP13 HPR, which exhibited properties suitable for intended purposes. Ten different tests were carried out with the conditions specified, and each test was performed in triplicate. The repeatability of these measures is around 75%. The results show that the best conditions obtained with PTFE filter, were BSA blocking buffer and relationship 1:2000 (antibody I) and 1:5000 (antibody II). To select the best organic solvent, three different solvent were tested and the sensitivity of the method on low concentrations, was estimated to preparing decreasing concentrations in CH2Cl2/n.Hexan (1:1) solvent, from a standard B(a)P.

2. Methods

2.1. Selection of Organic Solvent

The organic solvent for dilution of stock solution of B(a)P, was selected using a concentration of 10 ul/ml of B(a)P on PTFE filters in three different organic solvent: toluene, CH2Cl2, hexane/CH2Cl2 (1:1, v/v).

2.2. Preparation of Benzo (a) Pyrene Standard

For construction of the calibration curve, we used a B[a]P stock solution (10 mg/ml in toluene). This solution was diluted with hexane/CH2Cl2 (1:1, v/v) to obtain standard solutions which cover the concentration range 1.0–0.0001 mg/ml; for the white sample: sample without B(a)P, disks were inoculated only with the solvent.

2.3. Selection of Blocking Buffer

To choose the best blocking buffer for ELISA testing of filter were made of 1:10 and 1:100 dilutions of the stock solution of B(a)P, has started the test using both blocking buffers: 0.14 M NaCl, 0.01 M PBS and 0.003 M KCl, pH 7.6, and 1% BSA, w/v; 200 ul/well) for 1 h at room temperature by agitation on a horizontal shaker. Plates were then washed with PBS-Tween (0.14 M NaCl, 0.01 M PBS and 0.003 M KCl, pH 7.6, and 0.05% Tween 20) using an automatic plate washer (Multiwash III); after washing with PBS-Tween, primary antibody, mouse monoclonal Polynuclear Aromatic Hydrocarbons antibody [BAP-13] was added (1:2000 in blocking buffer; 100 ul /well) and incubated at room temperature with agitation for 1 h. The plate was washed as before. After washing, Secondary antibodies Rabbit polyclonal to Mouse IgG-HRP (1:5000 in blocking buffer; 100ul/well) and incubated at room temperature with agitation for 1 h. The plate was washed as before. The TMB substrate solution read to use (100 ul/well) was added and the plates were shaken for about 45 min for colour development. Finally, the TMB reaction was stopped with sulphuric acid (25 ul/well) and the absorbance was read at 450 nm in a microplate spectrophotometer (ELX808, Lonza). All determinations were made at least in triplicate.

3. Results and Discussion

The solvent that gave the best response, with the concentrations of antibodies used in ELISA tests, is the hexane/CH2Cl2 (1:1, v / v), as shown in Figure 1.
The optimal dilution of primary antibody BAP-13 (AbCam Ab768) and secondary antibody Rabbit to mouse-HPR (AbCam Ab6728) was determined by checkerboard titration.

The dilution of BAP-13 antibody was tested in the range of 1:2000 to 1:8000. The optimal dilution for this antibody is 1:2000. Secondary antibody Rabbit to mouse-HPR was examined at dilutions of 1:5000 and 1:10000, and it was found that dilution of 1:5000 was suitable. The optimal dilutions for these antibodies were given in figure 3.

After having determined the concentration of primary and secondary antibody and the solvent, were carried out several tests in order to evaluate the sensitivity of the method, as shown in Figure 4.

The standard used for the tests is a solution of B(a)P in toluene; then it was necessary to prepare the dilutions in an appropriate solvent, considered the chemical characteristics of the filter used, and the interaction of the solvent with the antibodies used for the ELISA. Figure 1 show that the best solvent is n-hexane/CH₂Cl₂, since, during the process of drying of the filter evaporates without leaving traces that may interfere with the antigen-antibody binding. In fact, at the same concentration of B(a)P, the toluene has a response 10-fold lower, while the use of CH₂Cl₂ does not allow the antibody to bind to benzo[a]pyrene present on the filter. The decision to use a high concentration of benzo[a]pyrene (10 ug/ml) was made because we wanted to be sure that the negative response depended only on the solvent used, not on the sensitivity of the method.

Simultaneously with the choice of solvent, we tested two different blocking buffers to choose the one that gave the best solution to our method of filter. In the case of blocking the selection has been forced, because the milk binds too strongly to the filter, blocking both the aspecific sites and the specific ones, in fact the value of Abs450 found is very similar to the respective blank, as shown in Figure 2.

**Figure 2.** Blocking Buffer used for the preparation of the filter and the antibodies solution for ELISA assay. Each value represents the mean of three replicates

**Figure 3.** Combinations of primary antibody and secondary antibody respectively. Each point represents the mean of three replicates
To find the optimal dilution of primary and secondary antibody to be used, have been carried out the tests, crossing three different dilutions of primary antibody with two dilutions of secondary antibody conjugate. Figure 3 shows that with the primary antibody diluted 1:2000 and secondary antibodies 1:5000 we have the best response of the filter. The test was carried out to test the interference of both the solvent, that the matrix (filter), in the binding between the antigen and primary antibody and between primary and secondary antibody, since in the literature there are no examples of ELISA on filter for benzo[a]pyrene.

After completion of all in the preliminary tests referred to above, we have carried out tests for dilution of benzoin (a) preens to see the sensitivity of the method. Figure 4 shows the concentration of BaP (mg/ml) in relation to the value of Abs at 450 nm detected with the reading system; each point is the result of the average of at least ten measurements. Was included in the graph the trendline and the index of regression; these indicate that the data obtained are in line with those expected, whereas the value of R2 is 0.9957, therefore very close to one.

It is evident that we used high concentrations of benzo[a]pyrene for our tests, so our method is certainly less sensitive the official methods for the detection of B(a)P in the workplace (HPLC-FID, GC-MS, etc.). However, this method is not intended to replace the official technical, but wants to be a preliminary screening of that filters have sampled many liters of air, concentrating greatly the benzo[a]pyrene which may be present in specific work environments. The advantage of using this preliminary method is in the complete absence of extraction from the filter used for the sampling, limiting the use of solvents, and especially in the rapidity of execution of the test. Furthermore, the value obtained can help to select the most appropriate dilution of the sample to be used in the official methods.

4. Conclusions

Benz[a]pyrene B(a)P is a human carcinogen and a major carcinogenic component of multiple human exposures involving polycyclic aromatic hydrocarbons (PAHs) [10, 11]. PAHs released into the environment may originate from many sources, including gasoline and diesel fuel combustion and tobacco smoke, for example. PAHs are detected in air, soil and sediment, surface water, groundwater, and road runoff; are dispersed from the atmosphere to vegetation; and contaminate foods. Anthropogenic and natural sources of PAHs in combination with global transport phenomena result in their worldwide distribution [12].

It is known that the risk of exposure to B(a)P in the workplace and strongly related to the type of work, in fact individual sources of PAH are characterised by combustion processes and by particular industrial processes which utilise PAH-containing compounds, e.g. processing of coal, crude oil, creosote, coal-tar and bitumen.

The International Agency for Research on Cancer [13] reported that workers from industrial settings where airborne PAH levels are high, such as gas works, coke works, and the primary aluminium industry, show excess rates of cancers. Aluminium production, coke production, coal gasification and coal tar pitches/coal tar fumes are carcinogenic to humans according to IARC classifications. PAH are genotoxic carcinogens, inducing chromosomal effects in exposed individuals. The most toxic effects on humans, other than cancer, arising from PAH-containing coal tars and creosote oils concern the skin and eyes. An overview of effects is given in the ACGIH’s TLV documentation of coal
tar pitch volatiles and benzo(a)pyrene [14]. Techniques officially used for the monitoring of PAH and of benzo(a)pyrene in particular are HPLC-UV and GC-MS, even if the ELISA assay, is used for the detection of benzo(a)pyrene or its metabolites, in environmental samples such as water and sediments [15], which in biological samples such as blood and urine [16, 17]. In some cases are used commercial tests, Such as BTEX RaPID Assay ® and c-PAHs RaPID Assay ®, while in other cases, as for the determination of benzo(a)pyrene in potable water an indirect competitive ELISA assay was developed. The study of risk at the workplace is also based on the analysis of air. The study of PAHs and benzo(a)pyrene in particular, in this matrix requires environmental sampling of the filter or of the column, the subsequent extraction and analysis with the techniques mentioned previously, our method is proposed as an initial screening for the evaluation of the presence of benzo(a)pyrene. In conclusion, fairly selective and reproducible ELISA assay of high sensitivity has been developed and optimized for the detection of B(a)P on filter. It has the advantages over conventional chromatographic methods of low cost, rapidity, and ease of performance and is a feasible screening method for B(a)P contamination of air in workplace.

REFERENCES


