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Development and validation of a HPLC method for the determination of benzo(a)pyrene in human breast milk

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Abstract A simple analytical procedure was developed for the quantitation of benzo(a)pyrene in human breast milk using solid phase extraction (SPE) combined with high performance liquid chromatography. Before the chromatographic process, SPE, including C18 functional groups in silicagel cartridges, was conducted for sample preparation. A C18 column (100×4.6 mm id, 3 μm particle size) was used with acetonitrile:water (80:20) as the mobile phase at a flow rate 1 mL/min at 30°C. Fluorimetric detection was performed for excitation and emission at 290 and 406 nm, respectively. It was observed that the calibration curve was linear over the range of 0.5–80 ng/mL. The limit of detection and limit of quantitation were found to be 0.5 and 1.07 ng/mL, respectively. Intraday and interday relative standard deviation values were less than 5.15%. Moreover, the newly developed method provides a fast, simple, cost effective, and sensitive assay to detect an important carcinogen substance, benzo(a)pyrene, in human breast milk.

Keywords: benzo(a)pyrene, breast milk, fluorimetric detection, high pressure liquid chromatography, solid phase extraction

Introduction

Benzo(a)pyrene (BAP), also called as 3,4-benzopyrene, is a hydrocarbon comprising five fused benzene rings and is formed during the combustion of organic substances. BAP mainly exists in gasoline and diesel exhaust; cigarette and soot smoke; coal tar and coal tar pitch; charcoal-broiled foods and certain other foods; amino acids, fatty acids, and carbohydrate pyrolysis products; creosote and shale oils; and petroleum asphalt. Inhalation of BAP causes respiratory tract irritation, exposure to BAP may destroy the reproductive organs and cause cancer, ingestion leads to gastrointestinal damage, and skin contact with BAP may cause irritation. Therefore, it should only be used for research purposes. In the natural environment, BAP occurs as part of a mixture of polycyclicaromatic hydrocarbons. Exposure to polycyclic aromatic hydrocarbons (PAHs) and BAP for long duration may cause cancer. The International Agency for Research on Cancer (IARC) has mentioned BAP as a probable carcinogen agent and has designated it to be a group 1 human carcinogen (1).

BAP is used as a marker to detect carcinogenic polycyclic aromatic hydrocarbon levels in environmental matrices (2,3). In the human body, it is absorbed from the intestinal tract and accumulates mainly in body fat and fatty tissues. Its half-life in blood and liver is less than 5 and 10 min, respectively (4). BAP stimulates its own metabolism by inducing microsomal cytochrome P-450 mono-oxygenases and epoxide hydrolases. The induction of isozymes belonging to the cytochrome P-450IA subfamily (CYPIA1 and CYPIA2) is mediated by binding to a cytosolic receptor protein (5). BAP is firstly oxidized to several arene oxides and phenols. Moreover, it can easily adhere to human skin, muscles, and fat tissues from the environment or chemical exposure and cause carcinogenesis (3,6). It is excreted from breast milk and urine (7). The quantitation of BAP in breast milk is an important and required process to determine the quality of milk and to protect the health of infants. According to Regulation (European Union) No 835/2011, the allowed maximum level of BAP in formulas for infants and follow-on formulas, including infant milk and follow-on milk, is 1.0 μg/kg (8).

To date, there are several methods developed to determine BAP in urine, serum, meat products, water, and soil samples using different techniques such as high performance liquid chromatography (HPLC) (9), gas chromatography with a flame ionization detector (GC–FID) and a mass spectrometer (GC–MS) (10-13), fluorimetry (14), and electrochemistry (15). However, no studies have been reported on the detection of BAP in human breast milk. Therefore, this study is novel due to the fact that it is specific for BAP in breast milk. The total polyaromatic hydrocarbon amount in breast milk can be determined by GC–MS (16); however, there is no method specific to detect BAP, as a polyaromatic hydrocarbon.

In this study, an HPLC technique was developed to analyze BAP in

ng/mL level in human breast milk using fluorimetric detection. BAP provides sensitive fluorimetric detection due to its condensed aromatic structure. Solid phase extraction (SPE) is performed as a sample pretreatment procedure. SPE with reduced solvent consumption and easy and fast process is simpler than liquid–liquid extraction (LLE).

Material and Methods

Apparatus Fluorescence spectra and measurements were obtained using a Shimadzu spectrofluorimeter model RF-1501 (Shimadzu, Kyoto, Japan), including a xenon lamp and quartz cells with 1 cm pathway. Excitation and emission wavelengths were observed at 290 and 406 nm. pH measurements were conducted using a Wissenschaftlich-Technische-Werkstätten pH meter (pH 526 digital WTW; Weilheim, Germany).

The SPE SiliaPrep cartridge (SiliCycle Inc.-Canada, Ville de Québec, Canada) (500 mg/3 mL) is composed of silicagel containing C18 functional groups. HPLC trials were conducted on a Shimadzu LC 20 liquid chromatograph equipped with a LC-20AT pump, SIL AT-HT autosampler part, and SPD-20A HT fluorimetric detector, which was set at 290 nm for excitation and 406 nm for emission and column oven temperature was at 30°C (Shimadzu; Kyoto, Japan).

Chemicals and solutions BAP was purchased from Sigma (St. Louis, MO, USA), acetonitrile, (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was purified by a Human Water Purification Systems (Tokyo, Japan).

A stock solution of BAP (0.1 mg/mL) was prepared in acetonitrile and diluted to obtain standard solutions of various concentrations from 0.5 to 100 ng/mL.

Sample preparation and general procedure Breast milk samples were collected from a 36-year old volunteer (informed consent document was taken according to the Bezmialem Vakif University Clinical Trials Ethical Commitee approval) into polyethylene storage packs. Different amounts (0.5–80 nm/mL) of BAP were spiked with the milk samples. The milk samples were stored at −20°C. To isolate BAP from the milk samples, before use, each cartridge was conditioned with 2 mL acetonitrile and 2 mL water. After loading the cartridges with the breast milk sample (1 mL per cartridge, flow rate 1 mL/min, and pressure 85 mmHg), the cartridges were washed with 1 mL acetonitrile, dried for 1 min by leaving under vacuum pressure in the SPE chamber (1 min was considered as the optimum time for drying using the trials). The extract 10 μL was directly injected to HPLC system. The chromatographic system suitability parameters, such as capacity factor, resolution, asymmetry factor, tailing factor, and height equivalent to a theoretical plate, were calculated to reveal the quality of the separation process.

Result and Discussion

Pretreatment process The aim of a pretreatment procedure is to change a complicated matrix into a sample suitable for analysis. This procedure depends on the chemical and physical features of the analytes and other matrix constituents. In general, for biological matrices protein precipitation, LLE and SPE techniques have been used. In this study, firstly, LLE was trialed with various organic solutions, such as chloroform, dichloromethane, and hexane, with different volumes. It was noted that the recoveries were not high enough (approximately between 65 and 80%) and the procedure was time consuming. The protein precipitation was also tested; however, it was not efficient for breast milk samples due to very low recoveries (at about 60%) and interferences caused by the matrix in the chromatogram. Based on the trials, SPE was chosen because of high recoveries of the analyte and complete removal of interferences.

Chromatographic process Different mobile phase and column type and size combinations were trialed with different flow rates and column temperatures. Reverse phase HPLC was preferred due to the non-polar structure of BAP, and a C18 (ODS) column (100×4.6 mm id, 3 μm particle size) was used with acetonitrile:water (80:20) as the mobile phase with a flow rate of 1 mL/min at 30°C. Depending on the particle size of the stationary phase, higher pressure was applied at about 7,000 psi by the chromatographic system. The peaks of BAP were observed at approximately 7.8 min using fluorimetric detection. Excitation and emission were measured as 290 and 406 nm, respectively. The chromatograms for blank and 50 ng/mL BAP in breast milk are showed in Fig. 1 and the system suitability parameters of the chromatograms are listed in Table 1.

Preparation of the calibration curve A calibration curve was constructed using the assays of standard BAP solutions with various concentrations between 0.5 and 100 ng/mL. However, the linearity was observed between 0.5 and 80 ng/mL. According to linear least-squares regression analysis, the linear concentration ranges (each concentration was studied as five replicates) of the method were determined and the equation of the calibration curve was calculated as $y=ax+b$, wherein y showed the peak areas and x indicated the concentrations of BAP in ng/mL.

Validation of the method Validation of the method was conducted depending on the requirements of the International Conference on Harmonization (ICH) (17).

Accuracy and precision Accuracy and precision were determined by quantitation of the quality control (QC) samples at three different concentration levels: 5, 30, and 80 ng/mL as low, medium, and high. The accuracy was expressed as recovery and the precision by intraday

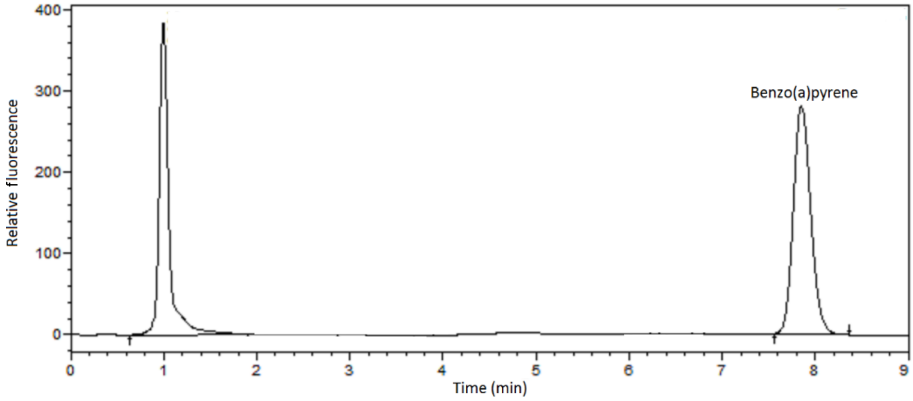

Fig. 1. Chromatogram of human breast milk containing 30 ng/mL B(a)P

Table 1. System suitability parameters

Capacity factor*	Height equivalent to a theoretical plate*	Tailing factor*	Asymmetry factor*
6.9	18615	1.7	1.3

[1)]*mean values of the parameters from the chromatograms of quality control (QC) samples are mentioned.

and interday assays, and relative standard deviation (RSD) values. The accuracy of the method was determined using a standard addition method by spiking the QC samples of standard BAP solutions with milk containing 30 ng/mL of BAP. Recovery values were calculated using the formula: Recovery %=[(Ct−Cu)]/Ca]×100, where Ct was the total concentration of the analyte found, Cu was the concentration of the analyte in the sample matrix, and Ca was the concentration of the added standard analyte. Accordingly, the recovery value was found as 101.18% and the RSD of these results was 4.32. Table 2 shows the recovery and RSD values.

To evaluate the precision of the proposed method, intraday and interday repeatability values were investigated by calculating RSD. Five replicates of samples at each concentration (QC samples) were determined on the same day for intraday and on 3 different days for interday precision. The RSD values of both intraday and interday assays were less than 5.15%. Therefore, it can be noted that the results, as shown in Table 2, of the method show good precision and accuracy.

Linearity Calibration curve was obtained using linear least-squares regression analysis by plotting the peak areas versus the corresponding BAP concentrations. The equation of the calibration curve (*n*=5) obtained from 7 points was y=1.19392x−3.2204 (correlation coefficient=0.9996), where y represented peak area of BAP and x represented the concentration of BAP. The method was linear over the range of 0.5–80 ng/mL. Table 3 summarizes the parameters about linearity study.

Sensitivity The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the formula: LOD or LOQ=kSDa/b, where k=3 for LOD and 10 for LOQ, SDa was the standard deviation of the intercept, and b was the slope. As mentioned in Table 3, LOD was 0.5 and LOQ was 1.07 ng/mL.

Robustness Robustness was evaluated by slightly modification of the column oven temperature, flow rate, and acetonitrile and water phase ratios of the mobile phase. The mobile phase ratios were changed from 80:20 (v/v) to 85:15 and 75:25; the column temperature was changed from 30 to 50°C; and the flow rate was changed from 0.8 to 1.8 mL/min. These changes had no significant effect on the peak areas and chromatographic system suitability parameters. Table 4 shows the robustness results.

Stability The freezing and thawing effects on BAP were studied by spiking milk with QC samples of BAP. The stability of BAP was determined as 24 h at room temperature and 4 weeks at −20°C.

The presented method is a combination of SPE and HPLC, which can be used for the determination of carcinogenic substance BAP in breast milk. No methods have been reported in the literature that

Table 2. Accuracy/precision of the method

Spiked concentration (ng/ mL)	Found concentration (ng/mL) (Mean±standard deviation[1)])	recovery (%)	RSD of recovery (%)	RSD of recovery of intraday variation (%)	RSD of recovery of interday variation (%)
5.00	4.98±0.04	97.14	3.66	3.34	2.45
30.00	30.30±0.82	101.18	4.32	4.75	1.19
80.00	77.60±1.15	97.12	5.15	5.14	0.05
	Mean relative recovery=98.48				

Table 3. Analytical parameters of the method

Parameters	Method
Concentration range[1)] (ng/mL)	0.5–80
Regression equation[2)]	y=1.19392x–3.2204
Intercept±standard deviation	3.2204±0.873
Slope±standard deviation	1.1939±0.741
Correlation coefficient (r^2)	0.9996
LOD (ng/mL)	0.5
LOQ (ng/mL)	1.07

[1)]Average of five determinations
[2)]$y=xC$+b where C is the concentration in ng/mL and y is the peak area

Table 4. Robustness of the LC conditions

Condition	Value	Recovery %	RSD %
Flow rate mL/min	0.8	112.21	5.43
	1.8	107.88	5.21
Column temperature	30	96.32	2.44
	50	103.24	4.36
Mobile phase ratio	75:15	98.56	2.15
(acetonitrile:aqueous phase)	85:15	100.42	3.22

N=3 for all the QC sample levels

can analyze BAP solely in human breast milk. In a previous GC–MS method (16), the amount of BAP was investigated simultaneously with 12 other PAHs and the validation studies were conducted in bovine milk samples. The advantage of the present study is that MS detection is not required, thereby reducing the cost. Moreover, HPLC with fluorimetric detection in human milk samples is relatively simple. Previously, different matrices such as water, meat, and soil were used to quantitate BAP, which are different than that used in the proposed method (9–15). In a previous study (10), pressurized liquid extraction was performed that was more time consuming, complicated, are required mixing, drying, and cooling procedures compared to the proposed SPE procedure; moreover, temperature was an important parameter for the process. In another method (12), the up-and-down shaker-assisted dispersive liquid–liquid micro-extraction technique was applied to water samples prior to GC–MS. The presented SPE is applied to a different matrix with good recovery. Moreover, SPE provides a fast and efficient pretreatment procedure, whereas HPLC provides a fast and improved separation procedure. Fluorimetric detection provides analysis in the ng/mL level with this method. The quantitation of BAP in biological fluids in a simple way is a required process. Breast milk is an important matrix for BAP assays in terms of infant and mother's health also it is important to measure the exposure. It is possible to use the proposed method for routine analysis of BAP.

Disclosure The authors declare no conflict of interest.

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