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Optimization and Validation Study of HPLC with Fluorescence Detection Method for Analysis of Benzo[C]Fluorine

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ABSTRACT

Optimization and method validation of HPLC with fluorescence detector for the determination of benzo[c]fluorene were studied. The results showed that benzo[c]fluorene had a maximum excitation wavelength at 309 nm and a maximum emission wavelength at 354 nm. The mixture of acetonitrile-water was used as a mobile phase at a ratio of 85:15. The standard curve was linear over the concentration range of 0.5-50 ng/ml. The limit of detection and the limit of quantification were 0.27 ng/ml and 0.90 ng/ml, respectively. The relative standard deviation of precision for intra-day and inter- days were less than 5% for both low (5 ng/ml) and high (50 ng/ml) concentration of benzo[c]fluorene. The method was validated and showed satisfactory results of linearity, precision, limit of detection, and limit of quantification. Accuracy in terms of percent recovery strongly depends on sample matrix. Therefore, further studies are needed to investigate the accuracy when the method is applied in the real sample such as environmental and food samples.

Keywords: Benzo[c]fluorine; Fluorescence detector; HPLC; Method validation; Optimization

1. Introduction

Benzo[c]fluorene (BcF) is a 4-ring polycyclic aromatic hydrocarbon as shown in fig.1. Even though the International Agency for Research on Cancer (IARC) has classified this compound as group 3 i.e., the agent is not classifiable as to its carcinogenicity to humans [1], the genotoxic effect of BcF has been shown in the

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literatures[2-5]. The well-known carcinogenic polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP), was not the only compound responsible for genotoxicity of coal tar exposure in animals. BcF was found to produce DNA adduct in lung tissue of mice fed with coal tar as well [2]. Tumorigenic potential of BcF was studied through both oral and intraperitoneal routes of exposure in mice [5]. Intraperitoneal exposure to BcF and BaP showed comparable results on tumor induction, whereas tumor incidence of oral exposure to BcF was more than BaP.

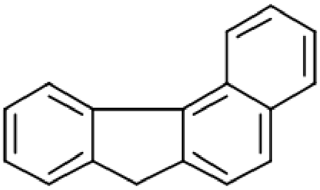


Fig. 1. Chemical Structure of Benzo[c]fluorene

The Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) has recommended evaluation of BcF in food [6]. The JECFA's recommendation led to the suggestion of BcF analysis in food in the European Union [7]. Although the regulation limit of BcF in the environment or in food has not been set, the public have been concerned about the toxicity and contamination of this compound in the environment and in food. A recent study in Japan [8] showed that BcF was found in both the gas and particulate phase in atmospheric samples. In addition, the results demonstrated that the contribution of BcF to carcinogenic risk in mice from polycyclic aromatic hydrocarbons in the atmosphere was 5-7 times that of BaP.

Analysis of BcF has been usually reported as one of the polycyclic aromatic hydrocarbons mixture contaminated in the environmental and food samples. HPLC with fluorescence detection has been normally used [9-13]. However, the validation parameters have not been clearly reported. Gas chromatography-mass spectrometry (GC-MS) has been used in the analysis of polycyclic aromatic hydrocarbons including BcF in food as well [8, 14, 15, 16]. Recently HPLC with fluorescence detection method was applied to the analysis of BcF in atmospheric samples and showed a lower limit of detection (LOD) as compared to the GC-MS method [17]. Since genotoxicity of BcF has been proved, analysis of this compound alone would be useful for a risk assessment study. Therefore, the aim of this study is to optimize and validate the HPLC with fluorescence detection method for analysis of BcF. Validation parameters in terms of linearity, precision, LOD and limit of quantification (LOQ) are shown and discussed.

2. Materials and Methods

2.1 Materials and reagents

BcF was purchased from Supelco (Bellefonte, PA, U.S.A.). Acetonitrile HPLC grade was obtained from Kanto chemical (Tokyo, Japan). A stock solution of 50 μg/ml BcF was prepared. Working solution was prepared by dilution of stock solution with the mobile phase. Solutions of 0.1 ng/ml, 0.5 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml and 50 ng/ml were prepared for setting up a standard curve. The 5 ng/ml and 50 ng/ml solutions were used for precision study.

2.2 Instrumentation

The HPLC system consisted of two pumps (LC-10A, Shimadzu, Kyoto, Japan), a fluorescence detector (RF-10A, Shimadzu), a system controller (SCL-10A, Shimadzu), an integrator (Chromatopac C-R7Ae, Shimadzu), a degasser (DGU-14A, Shimadzu), an autosampler (SIL-10A, Shimadzu), a column oven (CTO-10AS, Shimadzu), and an analytical column (Mightysil RP-18 GP II 150 mm x 4.6 mm, 5 µm, Kanto Chemical, Tokyo, Japan).

2.3 Optimization of mobile phase

Different ratios of acetonitrile to water, 75:25, 80:20, 85:15, 90:10 and 95:5 , were pumped at a flow rate of 1 ml/min to analyze 1 ng/ml of BcF. The fluorescence detector was set at excitation wavelength (λex) 309 nm and emission wavelength (λem) 359 nm. The injection volume was 20 µl. The oven temperature was 30°C.

2.4 Method validation

Repeatability (intra-day) and inter-day precision were studied using two concentrations of BcF (5 ng/ml and 50 ng/ml) and six determinations per concentration. The relative standard deviation (% RSD) for six determinations of each concentration was calculated for repeatability and inter-day precision. A standard curve consisted of six concentrations of BcF (0.1 ng/ml – 50 ng/ml). The concentrations were chosen based on a low expected value of BcF in the real samples. The standard curve was generated from the peak area. LOD and LOQ were calculated from the standard curves.

3.Results and discussion

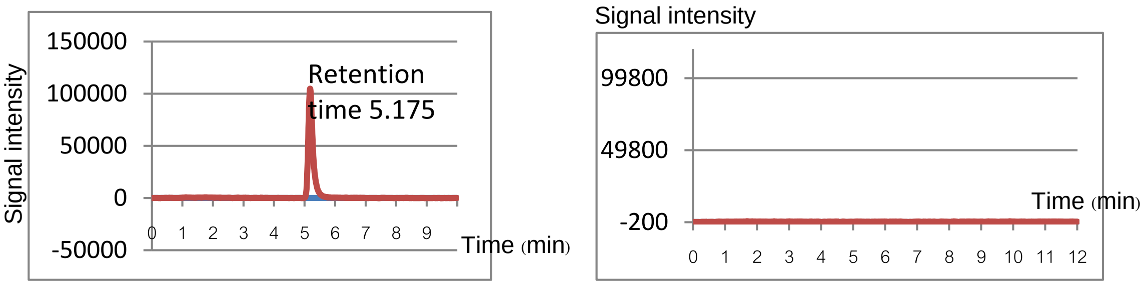
3.1 Optimization of HPLC condition

A fluorescence scan of BcF was performed using a Waters 2475 multi wavelength fluorescence detector (Waters Corporation, Millford, MA, USA). The results showed that BcF had a maximum λ_{ex} at 309 nm and a maximum λ_{em} at 354 nm. The results were comparable to the other studies. Brasseur et al. used λ_{ex} at 304 nm and λ_{em} at 353 nm for analyzing BcF in smoked flavorings [9]. Purcaro et al. used λ_{ex} at 300 nm and λ_{em} 354 nm for analyzing BcF in olive oils [13]. However, the notably different λ_{ex} and λ_{em} have been reported. λ_{ex} at 270 nm and λ_{em} at 376 nm were set for analyzing BcF in drinking water [11] and λ_{ex} at 240 nm and λ_{em} at 355 nm were used for analyzing BcF in food supplements [12]. Although λ_{ex} at 270 nm and λ_{em} at 376 nm were tried in this study to analyze 1 ng/ml of BcF, there was no peak detected.

Optimization of the mobile phase was performed with different ratios of acetonitrile to water. Table 1 shows the results of retention time, peak area and peak height in response to different ratios of acetonitrile to water. In addition to the data in the table 1, ratios of 60:40 acetonitrile to water and 70:30 acetonitrile to water were tried. However, a run time longer than 20 minutes was needed with 60:40 acetonitrile to water. The 70:30 acetonitrile to water showed retention time longer than 11 minutes, and smaller peak area and peak height compared to the higher ratio of acetonitrile to water.

Table 1. Mean value (n=3) of retention time, peak area and peak height in response to **different ratio of acetonitrile to water to analyze 1 ng/ml BcF**

Acetonitrile : Water	**Retention time (minute)**	**Peak area**	**Peak height**
75:25	**8.531**	**178,190.33**	**12,258.00**
80:20	**6.583**	**180,593.00**	**14,594.67**
85:15	**5.165**	**185,435.67**	**17,138.67**
90:10	**4.139**	**187,374.00**	**20,063.00**



(a) chromatogram of 1 ng/ml BcF (b) chromatogram of the blank mobile phase

Fig. 2. A chromatogram of 1 ng/ml BcF (A) and a chromatogram of the blank mobile phase (B)

Since the retention time and peak area between 85:15 and 90:10 acetonitrile:water were not considerably different, the ratio 85:15 was used throughout the study.

3.2 Final condition of HPLC for method validation

Mobile phase : 85:15 acetonitrile:water
λex - λem : 309-354
Flow rate : 1 ml/min
Injection volume : 20 µl
Oven temperature : 30°C
Run time: 10 minute

The final HPLC condition was capable of differentiating BcF from the blank mobile phase. Fig. 2 shows a typical chromatogram of standard BcF 1 ng/ml (a) and the blank mobile phase (b).

3.3 Method validation

Repeatability (intra-day) and inter-day precision were studied using two concentrations of BcF (5 ng/ml and 50 ng/ml) and six determinations per concentration. Repeatability and inter-day precision were expressed as % RSD for retention time and peak area as shown in tables 2 and 3, respectively. Percent RSD was calculated according to this formula %RSD= [Standard deviation (SD)/ mean] x 100. Both repeatability and inter-day precision showed less than 5 % of RSD which was satisfactory at ng/ml (parts per billion, ppb) level. Repeatability of the HPLC-fluorescence detection method in analysis of mixed polycyclic aromatic hydrocarbons including BcF in food showed comparable %RSD range from 2-8% [9, 11, 12, 13]. Inter-day precision in analysis of the food sample ranged from 4-8% [9,13]

Table 2. Repeatability (intra-day) of the method (retention time, peak area and peak height)

Determination	Concentration of BcF (ng/ml)			
	5		50	
	Retention time (minute)	Peak area	Retention time (minute)	Peak area
1	5.175	1131146	5.192	11561621
2	5.173	1118209	5.192	11577627
3	5.173	1118151	5.200	11592541
4	5.174	1117338	5.192	11575140
5	5.173	1098896	5.183	11194574
6	5.176	1111767	5.184	11392947
mean	5.174	1115918	5.190	11482408
SD	0.001	10498.79	0.006	159324.5
%RSD	0.02	0.94	0.12	1.39

Table 3. Inter-day precision of the method (retention time and peak area are expressed in mean value of 6 determinations per day)

Day	Concentration of BcF (ng/ml)			
	5		50	
	Retention time (minute)	Peak area	Retention time (minute)	Peak area
1	5.174	1115918	5.190	11482408
2	5.161	1126378	5.177	11427701
3	5.155	1182287	5.158	10655592
mean	5.163	1141528	5.175	11188567
SD	0.010	35683.96	0.016	462379.69
%RSD	0.19	3.12	0.31	4.13

A standard curve consisted of six concentrations of BcF (0.1 ng/ml – 50 ng/ml). The concentrations were chosen based on a low expected value of BcF in the real samples. Six determinations were performed for each concentration. A standard curve was generated from peak area (table 4, fig.3). LOD and LOQ were calculated from the standard curve according to the following formula: LOD = 3 x [standard deviation of the response/slope of the standard curve] and LOQ = 10 x [standard deviation of the response/slope of the standard curve]. LOD and LOQ of this study were 0.27 and 0.90 ng/ml, respectively. LOD for HPLC with fluorescence detection method in the analyses of mixed polycyclic aromatic hydrocarbons including BcF in food were 0.06-1.2 µg/kg [10,12,13]. The results showed linearity of quantification within at least two orders of magnitude (0.5-50 ng/ml). R-squared (R2) value for the standard curve was 0.9986, which means the regression line fit the data well.

Table 4. Concentrations of BcF and peak area response (n=6 for each concentration)

	Concentration of BcF (ng/ml)						
Determination	0.5	1	2.5	5	10	25	50
1	85193	217526	615348	1139941	2470048	5328198	11312067
2	89799	218900	623070	1146347	2487999	5223114	11387093
3	88976	213971	630754	1123349	2464578	5346982	11337321
4	87314	219623	629750	1113709	2430789	5308054	11383576
5	88787	222717	621564	1115525	2436004	5329300	11313168
6	85590	216202	612673	1119397	2455431	5412464	11339624
mean	87609.83	218156.5	622193.17	1126378	2457474.83	5324685.33	11345474.83
SD	1900.15	3007.91	7335.50	13556.67	21529.38	61366.45	32997.89

Average SD : 20241.99
Slope of standard curve : 224523
LOD : 0.27 ng/ml
LOQ : 0.90 ng/ml

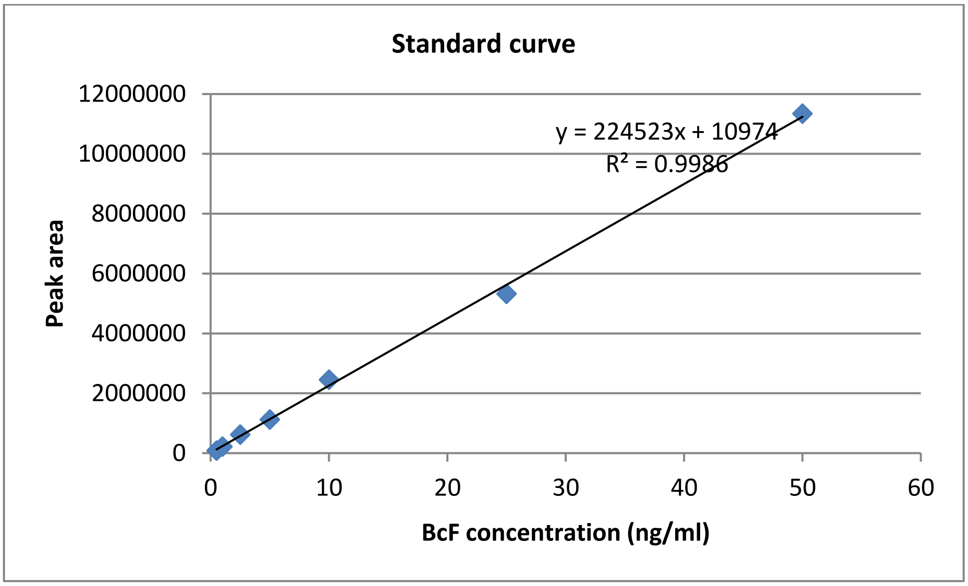


Fig. 3. Standard curve of BcF generated from peak area

4. Conclusions

The validated method showed satisfactory results of linearity, precision, LOD and LOQ. The application of this validated method should be further studied, in the environment or in food samples, to investigate the accuracy in terms of percent recovery. Since percent recovery mainly depends on sample matrix, further study should also be carried out in different matrices. To reduce the matrix effect, using an internal standard to improve recovery rate as can be seen in the study of Morisaki et al. [17] should be considered. BcF has never been mentioned in Thailand before. This study can provide useful guidance for any researchers who would like to determine BcF in the environment and food samples.

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