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Determination of bisphenol AF (BPAF) in tissues, serum, urine and feces of orally dosed rats by ultra-high-pressure liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

As a homologue of bisphenol A (BPA), there is concern about the potential reproductive and developmental toxicity of bisphenol AF (BPAF) based on *in vitro* tests. In this study, a simple and universal analytical method was developed for the determination of trace BPAF in various tissues and excreta of rats after they were orally dosed. The samples were hydrolyzed with glucuronidase/arylsulfatase followed by ultrasonic extraction with acetonitrile. The crude extract was purified with a mixed-mode anion exchange (Oasis MAX) solid-phase extraction (SPE) cartridge. Separation and quantification was then conducted by ultra-high-pressure liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) in negative ionization mode. The recoveries at three fortification levels in different biological samples were from 71.0% to 102.3% with relative standard deviations no more than 13.2% ($n = 6$). The quantification limits of the method were from 0.5 μg/kg to 3 μg/kg depending on the matrix. This method was successfully applied to the determination of BPAF in tissues, serum, urine and feces of orally dosed rats.

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1. Introduction

Bisphenol AF (1,1,1,3,3,3-hexafluoro-2,2-bis (4-hydroxyphenyl) propane, BPAF) has a structure of two phenolic rings joined together through a carbon bridge. It is a homologue of bisphenol A (BPA) in which the methyl groups are perfluorinated (Fig. 1). BPAF has broad applications in areas such as food processing equipment, electronic devices and optical fibers and especially in fluoroelastomers as the vulcanizer due to its excellent stability and hot tear strength [1]. Although industrial production of BPAF is increasing considerably, no data are available on the annual production or the occurrence of BPAF in the environment [2].

As the fluorinated homologue of BPA, a proven endocrine disrupting compound, there is concern that BPAF is potentially more harmful to human health because its CF_3 moiety may be much more electronegative and reactive than the CH_3 of BPA. The acute oral toxicity of BPAF in laboratory animals is low [3], but recent research indicates that this chemical may pose high potentiality as an endocrine disruptor for humans and wildlife *via* binding with hormone receptors. *In vitro* assays indicate that BPAF binds to estrogen receptor-alpha approximately 20 times more effectively than BPA and to estrogen receptor-beta almost 50 times more effectively. BPAF appears to shift endocrine action toward greater toxicity [2]. Another study found that BPAF exhibited both high estrogenic and anti-androgenic activities [3]. These potential risks have prompted the US National Institute of Environmental Health Science to nominate BPAF for comprehensive toxicological characterization [4].

Currently, the limited study of BPAF is mainly concentrated on the mechanism of its endocrine disrupting effect *in vitro*, and there are no published reports of an analytical method for the determination of BPAF. In order to assess the exposure level of BPAF in organisms and conduct further BPAF toxicological studies, a reliable analytical method for BPAF in bio-matrices was needed. The objective of this paper was to develop a fast and universal method for the determination of BPAF in bio-matrices after orally dosed exposure.

2. Experimental

2.1. Chemicals and reagents

BPAF (98% purity) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). HPLC-grade acetonitrile and methanol were

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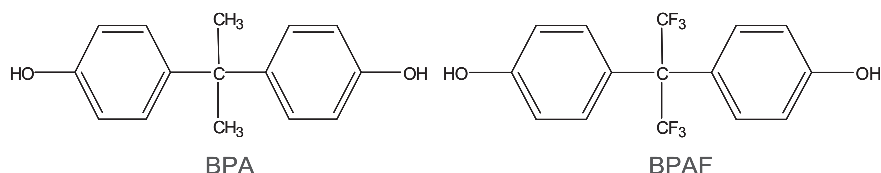

Fig. 1. Structure of BPA and BPAF.

supplied by Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was obtained by using an in-house Milli-Q Ultrapure water system (Millipore, Bedford, MA, USA). Formic acid (99%) was purchased from Acros Organics (New Jersey, NY, USA). Corn oil was supplied by Sigma–Aldrich. Oasis MAX SPE cartridges (60 mg, 3 mL) were purchased from Waters (Milford, MA, USA).

2.2. Collection of murine specimens

Male Sprague Dawley (SD) rats of 8–9 weeks old (250–300 g) were obtained from the experimental animal center at the Academy of Military Medical Sciences, China. All experiments were carried out in compliance with the guidelines of the experimental animal center of the Beijing Center for Disease Control and Prevention. The animals were housed in a temperature-controlled room (25 °C) under a 12 h/12 h light/dark cycle with free access to standard food and water. Four rats were caged individually in steel metabolism cages to collect the urine and feces that was used for blanks. After the collection of excreta, whole blood samples were obtained by femoral artery puncture, and the rats were euthanized by cervical dislocation. The following tissues were dissected and used as blanks: kidney, liver, testis, adipose and muscle. Serum was obtained by centrifugation (15 min, 3000 rpm, 4 °C) and stored at −20 °C. Tissues were immediately placed on ice and then stored at −80 °C until analyzed.

To investigate the applicability of this method in rats and the distribution of BPAF in different tissues, 4 SD male rats were allowed to acclimate 1 week before treatment. A 10 mg/kg dose of BPAF dissolved in corn oil (a non-toxic dose, approximately 350-fold lower than the published acute LD50 data in rats) was given to rats by oral administration for 2 consecutive weeks. Immediately after the administration, the rats were returned to the metabolism cage, and urine and feces samples were collected. At the end of the exposure, animals were euthanized for necropsy. The obtained serum was stored at −20 °C. Tissues (kidneys, liver, testis, adipose and muscle) were immediately placed on ice and then stored at −80 °C until analyzed. The concentration of BPAF in the biological matrices was determined by processing the samples as described, followed by detection using LC–MS/MS.

2.3. Analytical procedure

2.3.1. Instruments and conditions

BPAF identification and quantification were performed with an Acquity ultra performance liquid chromatography system (UPLC) coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). LC separation was conducted with an Acquity BEH C18 column (2.1 mm × 50 mm; 1.7 μm; Waters). The mobile phases were A (ultrapure water) and B (methanol). The injection volume was 10 μL. A total flow rate of 0.3 mL/min was used with a gradient elution starting with 30% mobile phase B, followed by a 4 min linear gradient to 100% mobile phase B, which was continued for 2.0 min. The system was re-equilibrated for 3 min between runs. Samples were ionized in negative electrospray ionization mode (ESI). The capillary voltage was 2.5 kV. The source temperature and desolvation temperature were set at 150 °C and 400 °C. The nitrogen flow rate was 700 L/h, and ultra-pure argon was used as the collision gas at a flow rate of 0.13 mL/min.

2.3.2. Preparation of stock and standard solution

Approximately 10 mg of BPAF powder was accurately weighed and dissolved in methanol to yield a final concentration of 1.0 mg/mL and then stored at −20 °C in amber glass vessels. Working standards were prepared by diluting the stock solution in methanol/water (50:50, v/v) to a final concentration of between 0.1 μg/L and 500 μg/L.

2.3.3. Preparation of biological matrices sample

Aliquots of 100 mg of homogenized tissue, 50 mg of freeze-dried feces, or 200 μL of serum/urine samples were transferred into 5 mL polypropylene centrifuge tubes containing 500 μL of 0.2 mol/L acetate buffer (pH 5.2). After which, 20 μL of glucuronidase/arylsulfatase from *Helix Pomatia* (Roche Diagnostics GmbH, Mannhein, Germany) was added and mixed thoroughly with a vortex mixer. The mixture was incubated overnight at 37 °C. After the sample cooled to room temperature, 1.5 mL of acetonitrile was added, and the mixture was sonicated at room temperature for 15 min. This extract was centrifuged at 9000 × *g* for 10 min at 4 °C. The supernatant was transferred into another 5 mL tube, and the residue was re-extracted with 1.5 mL of acetonitrile. The combined supernatants were diluted with a 4-fold volume of water and purified with an Oasis MAX cartridge (60 mg, 3 mL) that was preconditioned with 3 mL of methanol and 3 mL of water. After the sample was loaded, the cartridge was sequentially washed with 1 mL of water containing 5% ammonia, 1.5 mL of methanol, and 1 mL of methanol/water (40:60, v/v) containing 2% formic acid. The target drug was eluted from the cartridge with 1 mL of methanol containing 2% formic acid. The eluate was dried under a gentle stream of nitrogen, and the residual was reconstituted in 1 mL of methanol/water (50:50, v/v) for analysis. For feces samples, the reconstituted solution was diluted with a 100-fold volume of methanol/water (50:50, v/v) before instrumental detection.

2.4. Method validation

Three types of standard calibration curves were prepared for the method assessment: (1) a neat standard curve (plotted using methanol/water (50:50, v/v) dissolved standard solutions from 0.1 μg/L to 100 μg/L), (2) matrix-matched standard curves (plotted using standards spiked in extracts of blank samples that were spiked before LC–MS/MS analysis), and (3) matrix-fortified standard curves (plotted using extracts of blank samples spiked before pretreatment).

2.4.1. Linearity and range

The linearity of the response was studied using the matrix-fortified standard curves (using extracts of blank samples spiked at six concentrations before pretreatment, ranging from the quantification limit to 1000 μg/kg). The peak areas of the selected quantification MRM transitions were used to construct matrix-fortified calibration curves, which were used for quantitative determinations.

2.4.2. Recoveries and precision

Recoveries and precision were determined by analyzing biological samples spiked at three levels in six replicates. Recoveries were calculated by comparing the peak areas of blank samples spiked before pretreatment to the counterparts spiked before LC–MS/MS analysis. The precision, expressed as percent relative standard deviation (RSD%), was determined using six replicates of different spiked biological matrices.

2.4.3. Detection limit and quantification limit

The method detection limit (MDL) and the method quantification limit (MQL) were defined as the minimum detectable amount of analyte from real samples in MRM mode with signal-to-noise ratios of 3:1 and 10:1.

2.4.4. Matrix effects

Matrix effects (ion suppression or ion enhancement) were ubiquitous during the LC–MS analysis due to the ionization competition between co-eluting compounds in the chromatographic system. In this study, the matrix effects were evaluated using the strategy applied by Matuszewski et al. [5] with slight modification. The modifications included subtracting the ratio between the slope of the matrix-matched standard curves and the slope of neat solution curves and then multiplying by 100 to obtain a percentage. The signal was enhanced if the value was negative, whereas the signal was suppressed if the value was positive.

2.5. Quality control

Special precautions were taken to prevent intralaboratory contamination that could occur from sample collection and assay. All glassware, scissors, knives and tweezers were rinsed in a washing machine and baked at 400 °C for 4 h in a muffle furnace (L9/11/B 170, Nabertherm Industrial Furnaces Limited, Lilienthal/Bremen, Germany) before use. Background contamination was evaluated during sample preparation and detection using procedural blanks (blank solvents) and blank matrix extracts (blank samples), which were treated along with every set of processed tissues (ten samples).

3. Results and discussion

3.1. Optimization of LC–MS/MS

The MS/MS acquisition parameters were optimized in ESI negative mode by the direct infusion of a standard solution (500 μg/L) *via* the syringe pump at a flow rate of 20 μL/min in combination with methanol/water (50:50, v/v) at a flow rate of 0.1 mL/min. This process provided a stable response during the optimization. Diagnostic fragment ions were selected, and the relevant parameters were optimized for maximum sensitivity. The precursor ion of BPAF was $[M-H]^-$ at *m*/*z* 335.2. The product ions *m*/*z* 265.0 and 197.0 correspond to the loss of either one or two CF_3 from the BPAF. The *m*/*z* 335.2 > 265.0 transition was chosen as the quantitative transition. The optimal cone voltage was 30 V, and the collision energy was 25 eV and 30 eV for *m*/*z* 265.0 and 197.0, respectively. The spectrum obtained for the BPAF fraction under the given MS parameters is shown in Fig. 2.

Following the optimization of the MS/MS parameters, the composition of the mobile phase (*i.e.*, methanol–water and acetonitrile–water) and the concentration of ammonium hydroxide (usually employed in reversed-phase chromatography under negative ESI mode) were compared. The results suggested that the responses using methanol–water as mobile phase were higher than those using acetonitrile–water and methanol–water containing

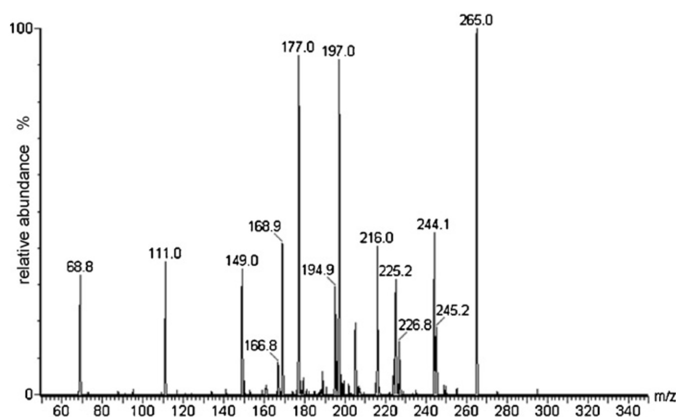

Fig. 2. Typical collision-induced dissociation (CID) spectra for BPAF (cone voltage: 30 V, collision energy: 25 eV).

0.1% ammonium hydroxide. Thus, methanol–water was selected as the mobile phase.

3.2. Optimization of sample preparation

3.2.1. Enzymatic hydrolysis

Many of the compounds in the tissues and urine were present in conjugated forms. Previous studies have shown that BPA glucuronide was the major metabolite of BPA in rats, accompanied by small amounts of other conjugated forms including BPA sulfate and BPA diglucuronide [6–8]. Analytical methods for the determination of these compounds in bio-samples usually involve enzymatic hydrolysis to transform the conjugated form to the free form. The usefulness of this technique for animal samples is controversial because the proportion of cleavable conjugated forms in tissue has been described as being very low [9,10]. In this study, the levels of BPAF in the tissues and excreta were determined using two different pretreatment procedures, one with enzymatic hydrolysis and the other without enzymatic hydrolysis (Table 1). A statistical analysis was performed by SPSS 16.0 to compare the difference in BPAF concentrations between samples with and without enzymatic hydrolysis, using the Wilcoxon Signed Ranks Test. Significantly elevated concentrations of BPAF were found in tissues pretreated with enzymatic hydrolysis ($Z = -4.937$, $p < 0.001$). The results in Table 1 indicated that a high proportion of BPAF was represented by conjugated forms in liver, kidney and serum samples (approximately 86.0%, 90.9%, and 99.1%, respectively). Therefore, enzymatic hydrolysis was used in this study to ensure accuracy.

3.2.2. Purification

The non-selective nature of the extraction procedures and the complexity of the sample matrices result in complex extracts that require further purification. For bio-samples, proteins and lipids should be removed before analysis to avoid contamination and damage to the analysis system.

Based on the structure of BPAF and the increased selectivity of anion exchange phases for compounds with acidic groups, Oasis MAX cartridges were selected for the cleanup in the experiments. Oasis MAX is a polymeric reversed-phase, anion exchange mixed-mode sorbent that allows for the retention and release of weakly acidic compounds. Acetonitrile–water (25:75, v/v) was used as the loading solvent to ensure that BPAF was retained without any loss. One milliliter of 5% ammonia–water was used to remove proteins and to enhance the ion-exchange interaction between BPAF and the sorbent. The interferences retained by hydrophobic interaction were removed with 1.5 mL of methanol. Furthermore, 1 mL of 2% formic acid in methanol–water (40:60, v/v) was used to remove

Table 1
Concentration of BPAF in orally dosed rats ($n = 4$).

Tissues and excreta	Concentration (μg/kg[a])							
	Without enzymatic hydrolysis				After enzymatic hydrolysis			
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 1	Rat 2	Rat 3	Rat 4
Liver	78.1	141.0	437.5	190.5	1210.2	1376.3	1637.5	1496.6
Muscle	8.1	10.0	12.8	17.1	44.3	20.1	17.6	19.5
Adipose	30.3	30.3	39.9	40.1	62.7	47.2	42.1	57.8
Testis	15.3	16.0	21.5	24.0	66.0	50.0	68.0	47.0
Kidney	31.4	28.3	56.3	45.5	880.3	387.0	372.5	431.3
Serum	3.4	3.2	3.9	4.3	1075.3	358.8	312.4	431.3
Urine	31.5	17.0	17.6	25.5	177.6	47.5	25.7	59.8
Feces	424682.4	449908.6	337844.6	146468.7	545266.9	469864.7	370004.5	223646.9

[a] The unit of concentration for serum and urine was μg/L.

Table 2
Analytical performance of the LC–ESI-MS/MS method for different matrices.

Matrices	Linearity range[a] (μg/kg[b])	r^2 [a]	MQL[a] (μg/kg)	Slopes of three different curves[c]			Matrix effect[e] (%)
				Neat standard curves	Matrix-matched standard curves[d]	Matrix-fortified standard curves	
Liver	1.0–1000.0	0.9989	1.0	610.6	550.1	491.6	9.9 ± 0.9
Muscle	1.0–500.0	0.9999	1.0	550.6	522.0	457.9	5.2 ± 1.1
Adipose	1.0–500.0	0.9999	1.0	565.3	515.6	463.0	8.8 ± 0.6
Testis	0.5–250.0	0.9994	0.5	626.9	601.2	454.1	4.1 ± 1.6
Kidney	1.0–500.0	0.9999	1.0	596.5	524.9	474.2	12.0 ± 4.3
Serum	0.5–1000.0	0.9998	0.5	1267.7	1195.4	948.8	5.7 ± 3.0
Urine	1.0–250.0	0.9984	1.0	898.4	818.5	808.8	8.9 ± 3.5
Feces	3.0–1000.0	0.9991	3.0	337.6	271.5	228.8	19.6 ± 5.8

[a] Linearity range, r^2 and MQL were obtained from the matrix-fortified standard curves, which were used for quantitative determinations.
[b] The unit was μg/L for serum and urine.
[c] Slope = peak area of BPAF/mass concentration of BPAF (μg/kg for tissues, μg/L for serum and urine).
[d] Data represent mean ($n = 3$).
[e] Calculated as [1 − (the slope of matrix-matched standard curves/the slope of neat standard curves)] × 100%. Data represent mean ± SD ($n = 3$).

acidic compounds that were more polar than BPAF, which maximized the versatility and selectivity of the method. The results indicated that no target analyte was eluted during the wash procedure. After elution with 1 mL of 2% formic acid in methanol, the eluents were dried under a gentle stream of nitrogen, and the residues were reconstituted.

3.2.3. Reconstituted solvents

Reconstituted solvents with different ratios of methanol–water were assessed. The results suggested that satisfactory peaks and good sensitivities were achieved when methanol/water (50:50, v/v) was used. Obvious peak fronting was observed when pure methanol was used as the reconstituting solvent, which might indicate that the target compounds could be partially eluted with stronger solvents upon injection.

3.3. Method validation

The linearity range of each matrix is summarized in Table 2. The correlation coefficients (r^2) of the matrix-fortified calibration curves were all greater than 0.99. The MQLs of the method were 0.5 μg/kg for testis and serum matrices; 1 μg/kg for liver, muscle, adipose, kidney and urine samples; and 3 μg/kg for feces.

Recovery studies were performed at three fortification levels, which were selected according to the sensitivity of each matrix (Table 3). The mean recoveries ranged from 71.0% to 102.3% with relative standard deviations of no more than 13.2% ($n = 6$).

Different samples were analyzed to evaluate the impact of the matrix on the final LC–MS/MS method. In this study, all of the matrix effects were present as signal suppression at a level of <15% in all of the tissue and urine samples after SPE purification, except

Table 3
Recovery of BPAF in different matrices ($n = 6$).

Matrices	Spiking level (μg/kg)[a]	Recovery (%)	Relative standard deviation (%)
Liver	1.0	92.1	13.1
	10.0	83.7	4.1
	100.0	78.7	2.3
Muscle	1.0	94.3	8.4
	10.0	96.2	3.8
	100.0	90.0	7.5
Adipose	1.0	91.3	8.2
	10.0	86.8	6.5
	100.0	87.6	7.8
Testis	0.5	98.6	11.7
	5.0	83.9	3.6
	50.0	72.9	8.0
Kidney	1.0	89.0	7.0
	10.0	95.8	1.8
	100.0	90.2	11.1
Serum	0.5	95.8	5.6
	5.0	90.0	4.5
	50.0	84.2	1.1
Urine	1.0	84.1	9.0
	5.0	93.8	10.1
	50.0	102.3	5.0
Feces	5.0	71.2	13.2
	20.0	71.0	6.2
	200.0	84.6	6.6

[a] The unit of concentration for serum and urine was μg/L.

for feces (approximately 60–70%). The reason for the stronger matrix suppression in feces was not fully understood, though it may originate from the more complex composition and higher organic matter content of the feces sample. However, because BPAF was present in the feces at very high concentrations, usually >10 mg/kg, the real sample extracts were diluted with a 100-fold volume of methanol/water (50:50, v/v) before instrumental detection, which effectively decreased the matrix effect (19.6%) and avoided the residual contamination.

3.4. Determination of BPAF in rats orally dosed

The developed method was applied to the analysis of BPAF in the tissues and excreta of orally dosed rats. No BPAF was detected in procedural blanks and blank matrix extracts. The concentrations of BPAF in different matrices of exposed rats were shown in Table 1. High levels of BPAF were detected in the liver, kidney and serum samples. The significant enhancement of the BPAF concentration after enzymatic hydrolysis in the serum, liver and kidney samples implies that the liver is the major organ responsible for metabolism and that the kidney plays an important part in the excretion of the metabolites of BPAF. The highest level of BPAF was observed in the feces, which indicates that most of the BPAF was excreted as the nonconjugated form. Fecal excretion was the major route of elimination, and urinary excretion was the secondary route, but the high proportion of prototype in feces is not clear whether the compound was poorly absorbed from the intestine or if its metabolites were deconjugated by the intestinal micro flora *in vivo*, which was found to occur with conjugated BPA [11].

In addition, it can be noted that the concentrations of BPAF in some tissues varied quite a lot among the four replicates, such as liver without enzymatic hydrolysis and serum after enzymatic hydrolysis. This phenomenon was unlikely to arise from the fluctuations and contaminations in our sample preparation and detection, since the percent relative standard deviations of the recovery studies were no more than 13.2% (Table 3) and no BPAF was detected in our procedural blanks and blank matrix extracts. These variations may probably be due to the fact that the sample replicates of each tissue collected from four different SD rats, and the metabolism of BPAF can be quite distinct between individuals.

4. Conclusions

An SPE cleanup followed by LC–ESI-MS/MS detection procedure was developed for the identification and quantification of BPAF in orally dosed rats. The method was successfully applied to the analysis of the target compound in different biological matrices. To the best of our knowledge, this is the first report on the determination of BPAF in biological samples.

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