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Quantitative determination of the diastereoisomers of hexabromocyclododecane in human plasma using liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

A sensitive, simple and feasible method has been developed and validated for the simultaneous determination of three diastereoisomers of hexabromocyclododecane (HBCD) in human plasma using liquid chromatography tandem mass spectrometry (LC–MS/MS). The simple pretreatment generally involved protein precipitation with methanol (MeOH). The separation was performed with a C18 reverse phase column. The mobile phases were 5 mM ammonium acetate (NH_4AC) in water and acetonitrile (ACN). The mass spectrometer was operated using negative electrospray ionization (ESI) source and the data acquisition was carried out with multiple reaction monitoring (MRM) mode. The analyte quantifications were performed by external standard method with matrix-matched calibration curves. The method was partially validated with the evaluations of accuracy, precision, linearity, limit of quantification (LOQ), limit of detection (LOD), recovery, matrix effect and carryover effect. With the present method, the intra-batch accuracies were 94.7–104.3%, 91.9–109.3% and 89.8–105.0% for α-, β- and γ-HBCD, respectively. And the inter-batch accuracies were ranged from 94.2% to 109.7%. Both intra-batch and inter-batch precisions (relative standard deviation, RSD, %) of the analytes were no more than 11.2%. The recoveries were from 79.0% to 108.9% and the LOQ was 10 pg/mL for each diastereoisomer. The linear range was 10–10,000 pg/mL with the linear correlation coefficient $R^2 > 0.996$. No significant matrix effect and carryover effect of the analytes were observed in this study. This method is in possession of sufficient resolution, high sensitivity as well as selectivity and convenient to be applied to the trace determination of HBCDs in human plasma.

1. Introduction

Environmental pollution caused by brominated flame retardants (BFRs) has attracted increasing public concern recently. Besides polybrominated diphenyl ethers (PBDEs), hexabromocyclododecanes (HBCDs) are also widely used additive BFRs and applied as thermal insulation materials in buildings, upholstery textiles and electrical equipment housings [1]. HBCDs have become a category of ubiquitous environmental pollutants and they have been found in a great variety of abiotic and biota matrices including river sediment [2], air [3], dust [4], sewage sludge [5], sea bird eggs [6], marine mammals [7], food [8], human breast milk [9], serum [10] and human adipose tissue [11]. There is a limited number of studies [12,13] reporting the health effects of HBCDs to human body. Rodent models have indicated that HBCDs can result in adverse effects on neurotransmitter levels [14], neurobehavioral function [15], carcinogenesis [16], thyroid dysfunction [17], and endocrine disruption [18,13]. The commercial mixtures of HBCDs mainly consist of γ-HBCD (75–89%), α-HBCD (10–13%) and β-HBCD (1–12%) [19]. Different structures of HBCD diastereoisomers could lead to the differences in polarity, dipole moment and water solubility. These different properties could result in the discrepant chemical behaviors of HBCDs in environment and in bio-medias. For better understanding the distribution, transportation, metabolism and health effects of HBCDs in human body and animals, it is essential to obtain the data of respective HBCD diastereoisomer. Though the total concentration of HBCDs can be determined with gas chromatograph coupled to electron-capture detection or gas chromatography mass spectrometry, the separation of HBCD diastereoisomers cannot be achieved with these techniques due to the thermal rearrangement and thermal decomposition at relatively high temperature during the GC separating

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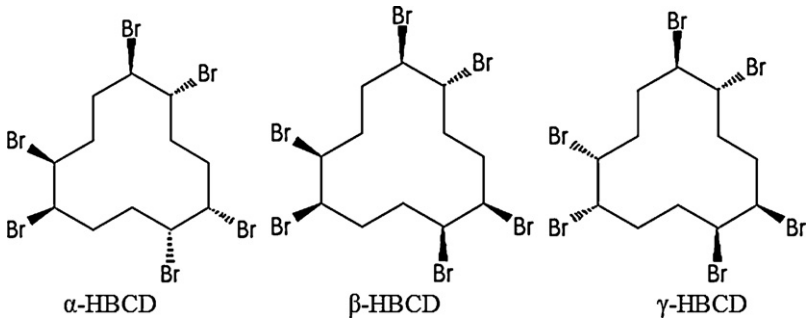

Fig. 1. Structures of α-, β- and γ-HBCD.

process [20–22]. In contrast to gas chromatography, LC–MS or LC–MS/MS can separate the HBCD diastereoisomers [23–25]. Up to now, the HBCD diastereoisomers were mostly determined in environmental samples using LC–MS or LC–MS/MS [23,26–28], and few studies [9,10] reported the methodologies for determining HBCD diastereoisomers in biofluids especially in those of humans. In addition, the previously reported methods [10,26] employed either solid phase extraction (SPE) or the similar pretreatment protocols as those for assaying PBDEs, which may lead to relatively high consumption of time, cost and sample.

In the present study, we have developed and optimized an LC–MS/MS method with minimal time and sample consumption for measuring HBCD diastereoisomers in human plasma. To the best of our knowledge, this is a relatively simple and fast method currently, achieving the LOQ at a very low concentration level.

2. Experimental

2.1. Chemicals and reagents

The stock solutions of the reference standards α-, β- and γ-HBCD (structures are listed in Fig. 1) were purchased from Cambridge Isotope Labs. (Andover, MA, USA). HPLC grade reagents ACN, MeOH, ethyl acetate, acetone and isopropanol were obtained from Merck (Darmstadt, Germany). NH_4AC was HPLC grade and purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). All the water used in this work was ultra-pure and produced by an ELGA water purifying system (ELGA LabWater Corporation, UK).

2.2. Equipments

The LC–MS/MS system consisted of an Agilent 1200 series HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) and an API 5000 (Applied Systems, Forster City, CA, USA) triple quadruple mass spectrometry equipped with an electrospray ionization interface. Separation was carried out with a 2.1 × 150 mm Atlantis® dC18 3 μm liquid chromatography column (Waters, Milford, MA, USA).

2.3. Working solutions

The concentrations of the purchased stock solutions were 50 μg/mL and the solvent used was toluene. The working solutions were prepared by serial dilution from the stock solutions with aqueous MeOH (H_2O/MeOH, 1:1, V/V). The concentrations of the calibration sample working solutions were 100–100,000 pg/mL, and those of the quality control sample (QC) working solutions were from 100 to 80,000 pg/mL. Those solutions were stored in a freezer at −20 °C and protected from light prior to usage. The pooled calibration samples and QCs were prepared by spiking the corresponding working solutions into the mixed human plasma with the dilution factor of 10.

2.4. LC–MS/MS conditions and parameters

The mobile phase A (MPA) was 5 mM NH_4AC in water and the mobile phase B (MPB) was ACN. A stepwise gradient elution program was as follows: initial–1.50 min, linear from 50% to 70% MPB; 1.50–2.50 min, linear from 70% to 80% MPB; 2.50–6.50 min, 80% MPB; 6.51–7.00 min, 100% MPB; 7.01–8.00 min, 50% MPB. The flow rate was set at 0.35 mL/min. The thermostatic oven was set at 20 °C and the autosampler was maintained at 10 °C. The injection volume was 20 μL.

The MS/MS parameters were as follows: ESI operated in negative mode; resolution of Q1 and Q3 was unit; the curtain gas, gas 1, gas 2 and collision gas were set at 20, 30 and 50 units, respectively; source temperature was 450 °C; ionization voltage was −3500 V; declustering potential, entrance potential, collision energy, and collision cell exit potential were set at −35, −10, −40 and −18 V, respectively; dwell time was 80 ms. All the gases used were high-pure nitrogen. MS acquisition was done with the MRM mode. The ion transitions for HBCDs were 640.6 → 78.8 m/z and 640.6 → 80.7 m/z. The data acquisition and processing were controlled with the software Analyst 1.4.2 (Applied Systems).

2.5. Sample pretreatment

An aliquot 100 μL of human plasma was placed in a well of a 96-well-plate followed by adding 300 μL of MeOH. Then the plate was vortex-mixed for 5 min and centrifuged for 10 min with a rotating speed of 4000 rpm at 4 °C. Thereafter, 200 μL of the supernatant was transferred to a new plate and 20 μL of the mixture was injected into the LC–MS/MS system.

2.6. Method validation

2.6.1. Calibration curves

Quantification of the three HBCD diastereoisomers was performed with external standard method. To eliminate the influence of matrix effect, the calibration samples were prepared by spiking the working solutions in mixed human plasma (nominal concentrations listed in Table 1). For each analytical batch, two groups of calibration samples were set. The first group was analyzed at the beginning of the batch and the second was analyzed at the end. The two-group calibration samples were combined to get 8-ponit linear calibration curves with the weight factor of $1/x^2$ to calculate the concentration of samples. The linear range (10–10,000 pg/mL) was ascertained to encompass the range of the concentrations expected in the real samples.

2.6.2. Accuracy and precision

Six replicates of QCs were prepared at 4 concentration levels, namely lower limit of quantification QC (LLQC, 10 pg/mL), low QC (LQC, 30 pg/mL), medium QC (MQC, 200 pg/mL) and high QC (HQC,

Table 1
Precision and accuracy of the assay for HBCDs in human plasma with the calibration samples at eight concentration levels.

Sample name	Nominal concentration (pg/mL)	α-HBCD				β-HBCD				γ-HBCD			
		Intra-batch (n = 2)		Inter-batch (n = 6)		Intra-batch (n = 2)		Inter-batch (n = 6)		Intra-batch (n = 2)		Inter-batch (n = 6)	
		Mean back-calculated concentration (pg/mL)	Mean accuracy (%)	Mean accuracy (%)	RSD (%)	Mean back-calculated concentration (pg/mL)	Mean accuracy (%)	Mean accuracy (%)	RSD (%)	Mean back-calculated concentration (pg/mL)	Mean accuracy (%)	Mean accuracy (%)	RSD (%)
Level-8	10	9.6	95.7	97.9	2.4	9.9	99.4	97.7	5.6	9.8	98.0	98.7	0.5
Level-7	20	21.5	107.0	104.5	3.2	19.8	99.0	101.1	2.1	20.8	104.0	103.3	0.9
Level-6	50	51.9	103.6	100.5	7.2	52.4	104.5	97.6	5.5	50.4	100.6	98.2	6.0
Level-5	100	100.2	100.2	101.5	4.8	101.2	101.2	99.3	1.9	98.0	98.0	102.0	8.1
Level-4	500	482.0	96.4	99.9	3.1	498.0	99.5	96.9	2.5	521.0	104.0	103.9	2.0
Level-3	2000	1975.0	98.6	103.5	3.9	1955.0	97.6	99.0	1.1	1865.0	93.4	99.5	4.9
Level-2	5000	4805.0	96.1	100.5	4.5	4995.0	99.8	104.4	3.3	5230.0	104.4	106.0	5.1
Level-1	10,000	10200.0	102.0	105.0	4.1	9845.0	98.5	101.5	3.0	9705.0	97.1	102.5	4.8

8000 pg/mL). Those QCs distributed in each analytical batch were applied to assess the intra-batch accuracy and precision of the method. The inter-batch accuracy and precision were determined by repeating the analytical batches in 3 different occasions. The accuracy was calculated in percentage ratio of the determined concentration to the nominal concentration of the QCs and calibration samples within the same batch (intra-batch) and different batches (inter-batch). RSDs were calculated to evaluate the precision. The acceptance criteria were referenced from the FDA guidance for industry [29]. For the acceptable calibration and QCs, the bias of the relevant accuracy should be within ±15% and the precision ≤15%, except LLQC samples, for which the bias should be within ±20% with the precision ≤20%.

2.6.3. LOQ and LOD

The concentration of plasma HBCDs in Chinese population has not been reported, but the HBCD concentration in human breast milk has been reported recently [30]. Therefore, we set the concentration of the LLQC as low as possible and the linear range covering the probably predicted concentrations after referencing the concentration levels of HBCDs reported in that publication [30]. The ratio of signal to noise for LLQC sample should be no less than five. The LOD was defined as the concentration in the sample can produce a signal with no less than three times height of that from blank sample.

2.6.4. Recovery, matrix effect and carryover effect

The recovery was assessed with the ratio comparing the absolute peak area from the quality control samples to that from the post-extraction spiked samples. The matrix effect was presented with the percentage of the absolute peak area from the post-extraction spiked samples to that from the neat solutions at the same nominal concentrations. The carryover effect, or named memory effect, has become an important negative problem for the analytical accuracy especially for that of relatively low concentration samples. The carryover effect was assessed by comparing the peak area at the retention time of the analyte in the blank sample (injected following the injection of the upper limit of quantification sample) to that of the LLQC sample. The precision of recoveries and matrix effect should be no more than 20%, and the value of carryover effect as well.

3. Results and discussion

3.1. Sample pretreatment optimization

To simplify the pretreatment, a simple protein precipitation protocol was employed. In comparison with the pretreatment methods previously published [10,31], which applied the time-consuming and expensive SPE pretreatments, the method used in this study was much simpler and less expensive. It has been estimated that the octanol–water partitioning coefficient ($\log K_{ow}$) of HBCDs is 5.6 [19], which could contribute to the adsorption of polypropylene tubes to HBCDs when the mixture is high aqueous. In this study, the adsorption effect of polypropylene tubes to HBCDs was tested. When the glass vials were replaced with polypropylene tubes during the spiking procedure, the recoveries of HBCDs were significantly decreased (data not shown). Therefore, during the spiking procedure, the mixture was placed in a 2-mL amber glass vial. In addition, all the stock solutions and cocktailed working solutions were stored in amber glass vials to protect from adsorption. After spiking, the mixture was mixed well and allowed to equilibrate for 2 h. MeOH was chosen as the protein precipitation solvent due to its solvency for HBCDs and suitability to the following LC separation.

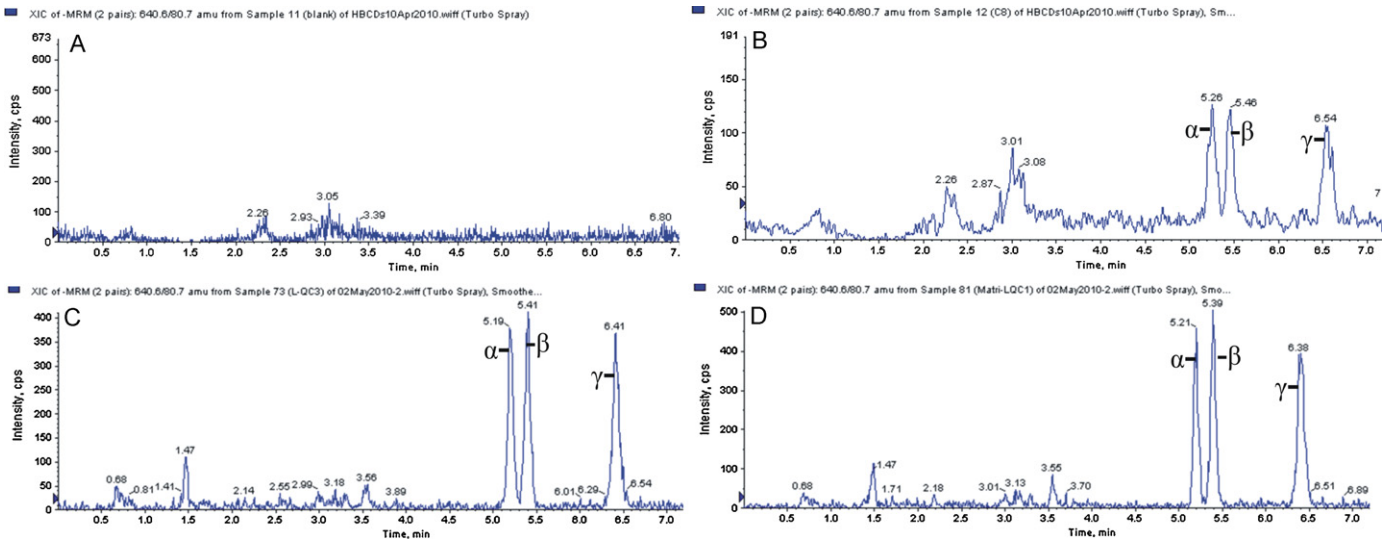

Fig. 2. Representative chromatograms of HBCDs from blank (A), LLQC (B), LQC (C) and post-extraction spiked LQC (D) samples.

During the method development, it is important to minimize the amount of samples required, especially when involving human-beings. Accordingly, we used the volume of human plasma as low as possible (100 μL) in this study and ensured the sensitivity fulfilling the assay at the LOQ concentration level. Comparing to the previously reported methods [10,31], which used the sample volumes up to 3.5 mL and 5 g, this method could offer a comparable sensitivity and selectivity with a much lower sample consumption.

No further cleanup and filtering procedures were applied in this study, which evidently simplified the method and shortened the analysis time. In addition, good recoveries were achieved and no significant matrix effect was observed in the samples processed with this pretreatment.

3.2. Optimization of LC–MS/MS conditions

Several types of reverse phase columns with different specifications and different particulate materials were tested to perform the LC separation. The HBCD diastereoisomers could not be separated by the columns with specifications of Capcellpak C18 (MG 50 × 2.0 mm, 5 μm, Shiseido, Japan) and Phenomenex polar-RP (Synergi 50 × 2.00 mm, 4 μ, Phenomenex, USA). When the two columns were used, only a total chromatogram of the three HBCD diastereoisomers was observed. The best chromatographic resolution and peak shape were achieved when the Waters C18 column was applied (Fig. 2).

For the optimization of LC separation, several mobile phases and additives were tested. The use of MeOH and ACN was evaluated for MPB and the additives such as NH_4AC as well as formic acid in MPA solution were tested also. When ACN was replaced with MeOH, the retention time was lengthened and the peak shape along with the resolution was worsened. This phenomenon could be attributed to the stronger eluting ability of ACN comparing with MeOH. Therefore, ACN was selected as the MPB solvent. The addition of 5 mM NH_4AC in MPA solution was proved to improve the peak shape and signal response, while the addition of formic acid (0.1% in MPA) could decrease the signal response. The addition of NH_4AC could enhance the conductivity of the mobile phase, which could improve the ionization efficiency during the ESI process, thus intensifying the signal response. However, the addition of formic acid could increase the acidity of the mobile phase, which could negatively affect the deprotonation of the analytes during the ESI process, thus decreasing the signal response. Accordingly, the solution 5 mM NH_4AC in water was chosen as MPA.

Once the mobile phase solutions and the column were chosen, the elution gradient was optimized to improve chromatographic resolution and shorten total analysis time. A relatively high flow rate of 0.35 mL/min was set to reduce the separation time and increase the peak heights by compressing peak widths, accordingly increasing the sensitivities. The initial percentage of MPB was set at 50% to ensure the initial ratio of organic phase in mobile phase solution was close to that in the injection mixtures. From 2.5 to 6.5 min, an isocratic elution of 80% MPB was maintained in order to ensure the analytes were effused at the same mobile phase composition. Consequently, the optimized retention times of the analytes were among the isocratic elution time range, which could help to achieve the stable ionization efficiency and matrix effect for the analytes. This can improve the experimental results especially for the experiment using external standard method or using non-isotope labeled internal standard method. After the analytes were eluted from the column, the gradient was extended to 100% MPB and maintained for 0.5 min to clean the column for preventing from further contaminations. Finally, 1 min was set to equilibrate the column. The total separation time was 8 min, which might be shorter than that of any previously reported method. The fine chromatographic resolution and peak shape were achieved with the optimized LC conditions (Fig. 2).

ESI interface is commonly applied in determination of HBCDs using LC–MS or LC–MS/MS currently. In this study, ESI was preferentially chosen as the ion source. To optimize the mass spectrometry working conditions and parameters, a cocktailed solution containing the three investigated analytes at the concentration of 4 ng/mL was assayed repeatedly by LC–MS/MS with the flow injection analysis (FIA) mode. The deprotonated ($[M-H]^-$) precursor ion 640.6 m/z was firstly determined using Q1 MS scan mode. The product ions ($[Br^-]$) (78.8 m/z and 80.7 m/z) were detected under the product ion san mode. Then, the two MRM transitions 640.6 → 78.8 m/z and 640.6 → 80.7 m/z were determined and chosen to perform the data acquisition. The MRM transitions were in accordance with the reported results [25]. The declustering potential, collision energy, collision cell entrance and exit potentials were optimized sequentially with MRM mode. The ion source parameters including the curtain gas, gas 1, gas 2, collision gas, source temperature and ionization voltage were also optimized step by step. Because the flow rate used in this study was relatively high (0.35 mL/min),

Table 2
Precision and accuracy of the assay for HBCDs in human plasma with the quality control samples at four concentration levels.

Analyte	Sample type	Nominal concentration (pg/mL)	Intra-batch (n = 6)			Inter-batch (n = 18)	
			Mean calculated concentration (pg/mL)	Mean accuracy (%)	RSD (%)	Mean accuracy (%)	RSD (%)
α-HBCD	LLQC	10	9.8	98.4	2.6	102.1	3.1
	LQC	30	31.4	104.3	4.0	108.4	5.1
	MQC	200	189.3	94.7	3.7	102.3	7.2
	HQC	8000	7713.3	96.4	11.2	104.9	7.0
β-HBCD	LLQC	10	10.9	109.3	3.9	109.7	6.4
	LQC	30	31.7	105.5	5.2	107.2	1.4
	MQC	200	184.0	91.9	6.7	96.9	4.6
	HQC	8000	7766.7	97.0	5.5	100.6	4.3
γ-HBCD	LLQC	10	10.5	105.0	9.5	102.6	4.1
	LQC	30	28.5	94.9	4.3	99.9	4.3
	MQC	200	179.6	89.8	4.4	94.2	6.0
	HQC	8000	7565.0	94.5	6.7	103.7	8.7

the source temperature was set at a relatively high value of 450 °C to enhance the evaporating efficiency and ionization efficiency, accordingly improving the sensitivity. However, in some reported methods [32,33], the applied source temperatures were 160 and 250 °C, which were relatively lower than that applied in this study. Whereas, in the study of Dodder et al. [34], the optimal source temperature was 450 °C also. These inconsistent optimal source temperatures could be attributed to the different instruments used and particularly the different flow rates applied. In the previously reported methods [32,33], the flow rates were set at a relatively low value of 0.2 mL/min, which could allow the mobile phase to be evaporated easily in the interface with a low source temperature. While in the study of Dodder et al., the flow rate was 0.5 mL/min, which could need a high source temperature to enhance the evaporating efficiency, thus promoting the instrumental sensitivity.

3.3. Quantification and identification

The transitions 640.6 → 80.7 m/z and 640.6 → 78.8 m/z were chosen for the quantification and the identification of the investigated analytes, respectively. In addition, the retention time consistency and the signal response ratio of the two transitions were used to identify the analytes.

3.3.1. Linearity, LOQ and LOD

The calibration samples and the QCs were prepared by spiking the working solutions into mixed blank human plasma and analyzed with the same method described above. In this way, the deviation of the assay would be decreased by reducing the impact caused by the matrix effect and recovery. The linearity of the assay was evaluated with the 8-point standard calibration curves. The calibration curves of the HBCDs were linear at the range of 10–10,000 pg/mL with the correlation coefficient of $R^2 > 0.996$. The observed mean back-calculated concentrations of the calibration samples with the accuracy and precision are listed in Table 1.

The signal to noise values of the HBCD peaks from the LLQC sample are more than 5 (Fig. 2), thus the LOQ of each analyte determined in this method is 10 pg/mL. The LOD of the respective HBCD was evaluated to be approximately 5 pg/mL.

3.3.2. Accuracy and precision

The accuracy and precision of this method were assessed using the QCs and proved to be satisfactory. The intra-batch accuracy values for HBCDs were found to be from 89.8% to 109.3% with RSD ≤11.2%. The inter-batch accuracy values for the HBCDs were between 94.2% and 109.7% with RSD within 1.4–8.7%. The comprehensive results of the intra-batch and inter-batch accuracy are provided in Table 2.

3.4. Recovery and matrix effect

Satisfying recoveries of the investigated HBCD diastereoisomers were achieved. The recoveries of the HBCDs at the low, middle and high concentration levels were mostly within 80.0–100.0% with the RSD less than 11.0% (Table 3). Three batches of single plasma were applied to preparing the post-extraction spiked samples at the concentration of LLQC, LQC, MQC and HQC levels. And the neat solutions were prepared with 75% MeOH (MeOH/H_2O, 3:1, V/V) at the same

Table 3
Recovery and matrix effect for HBCDs in human plasma with the quality control samples.

Analyte	Sample name	Mean recovery[a] (n = 3, %)	RSD (n = 3, %)	Mean matrix effect[b] (n = 3, %)	RSD (n = 3, %)
α-HBCD	LLQC	NA	NA	88.3	10.1
	LQC	82.1	3.5	91.0	10.1
	MQC	99.0	4.9	83.5	9.9
	HQC	96.9	2.1	102.2	6.6
β-HBCD	LLQC	NA	NA	97.5	8.3
	LQC	89.7	6.7	92.7	9.4
	MQC	88.2	9.0	95.1	10.6
	HQC	91.4	6.2	104.6	7.9
γ-HBCD	LLQC	NA	NA	73.3	6.8
	LQC	79.0	11.0	82.9	4.0
	MQC	108.9	8.0	84.9	5.4
	HQC	86.0	3.4	90.3	2.8

[a] Calculated with the recoveries from three quality control samples prepared with pooled human plasma.
[b] Calculated with the matrix effect from the quality control samples prepared with three batches of single human plasma.

concentration levels. No significant matrix effect for the HBCDs was observed in this study. The matrix effect were mainly within 80.0–100.0% with RSD ≤10.6% (Table 3). The results unambiguously indicated that the co-eluted endogenous compounds did not affect the ionization of HBCDs and not influence the determination in the assay. Therefore, the pretreatment of protein precipitation was competent to ensure the ruggedness and sensitivity of the assay.

3.5. Specificity and carryover effect

Specificity is the capability of the method to eliminate the endogenous and exogenous compounds which can interfere with the quantification of the analytes. As Fig. 2 shows, no interference peak was observed at the retention times of the investigated HBCD diastereoisomers, which illustrate that no endogenous or exogenous interference presented using the simple pretreatment procedures.

Carryover is defined as the appearance of a compound in a blank sample that does not contain the compound, especially when the blank sample is injected immediately after an injection of a sample containing high concentration of the investigated compound. To evaluate the carryover effect, a blank sample was set next to an upper limit of quantification sample. No peak appeared at the retention times of HBCDs in that blank sample. The carryover-free result of the method would be attributed to the wash procedures with the washing solutions ethyl acetate/acetone/isopropanol/ACN (4:4:1:1, V/V/V/V) and isopropanol/ACN (1:9, V/V) used successively.

4. Conclusions

A fast, simple, sensitive and reliable LC–MS/MS method has been developed and validated to determine α-, β- and γ-HBCD diastereoisomer in human plasma. The method, involves a simple protein precipitation treatment and LC–MS/MS detection, is very convenient for fast trace determination of HBCD diastereoisomers in plasma using microliter-level volume of sample. The recoveries were satisfactory and no significant matrix effect, carryover effect and interference were observed. The method has been proved to be accurate, precise and can be applied to support the investigation of HBCDs in human plasma.

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