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**The sensitive determination of two major mercapturic acid  
metabolites of 1,3-butadiene in human urine based on the isotope  
dilution ultrahigh performance liquid chromatography-tandem mass  
spectrometry**

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**Abstract:**

1,3-Butadiene is widely used as a chemical intermediate to produce chemicals and is a worldwide environmental pollutant. The major urinary metabolites of 1,3-butadiene are 3,4-dihydroxybutyl mercapturic acid (DHBMA) and monohydroxy-3-butenyl mercapturic acid (MHBMA), which are usually used as biomarkers for exposure to this carcinogenic substance. Here, we developed and validated a selective and sensitive method for the quantitative determination of DHBMA and MHBMA in human urine. Firstly, two mercapturic acids were extracted from urine samples using solid phase extraction. Then, they were separated with an Acquity BEH C18 column using gradient elution. Finally, the analytes were identified and quantified based on isotope dilution ultrahigh performance liquid chromatography-tandem mass spectrometry. Under the optimal conditions, the recovery rates were ranged from 87.1% to 107.9% with coefficient of variation  $\leq 14.8\%$ . The limits of detection were 0.14 and 0.16 ng mL<sup>-1</sup> for DHBMA and MHBMA, respectively. Owing to the good accuracy, precision and high sensitivity, the proposed method is well suitable for application in simultaneous determination of mercapturic acids in human urine.

**Keywords:** 1,3-Butadiene; Mercapturic acids; Ultrahigh performance liquid chromatography-tandem mass spectrometry; Isotope dilution method; Quantitative determination

## 1. Introduction

1,3-butadiene (BD), a major industrial chemical, has been widely used in the production of synthetic rubbers, latexes and polymers.<sup>1,2</sup> BD is known to have carcinogenic effect for humans, therefore, it has been classified as Class 1 carcinogen by the International Agency for Research on Cancer (IARC).<sup>3,4</sup> Since BD is ubiquitous in the environment, humans are commonly suffered from BD exposure due to its presence at workplaces, in polluted air, in automobile exhaust and in tobacco smoke.<sup>5-8</sup> Nowadays, a lot of researches are focused on developing sensitive methods to evaluate BD exposure for humans. For this purpose, two major metabolites of BD in urine, 3,4-dihydroxybutyl mercapturic acid (DHBMA) and monohydroxy-3-butenyl mercapturic acid (MHBMA), are usually used as biomarkers for exposure to this carcinogenic substance.<sup>3,8-11</sup>

Numerous methods for the determination of mercapturic acids in human urine have been previously published.<sup>2,3,7-9</sup> Among these methods, high performance liquid chromatography-mass spectrometry (HPLC-MS/MS) has been widely used in many recent studies.<sup>1,8,12,13</sup> Compared with other analytical methods, HPLC-MS/MS could provide information on molecular weight, peak purity and partial structure. Thus, it was usually applied, in particular, to increase the reliability of target identification.<sup>14</sup> Ultra-high performance liquid chromatography-tandem

mass spectrometry (UPLC-MS/MS), a further development of HPLC-MS/MS technology, has attracted a great deal of attentions in the fields of medical and analytical chemistry.<sup>15-18</sup> For example, the UPLC-MS/MS technology has been successfully used for the sensitive detection of fructooligosaccharides in infant formula in our previous study.<sup>19</sup> Advantages of UPLC-MS/MS are relatively short retention time, good reproducibility and high sensitivity.

Nowadays, the isotope dilution method is known as one of the most reliable strategy of modern quantitative analysis.<sup>20-25</sup> The structural analogues of the target analytes are directly used as internal standards, which have the same chemical and physical properties as the targets, but are not present in natural samples.<sup>26,27</sup> The internal standards could be separated from target standards based on their different molecular mass. Moreover, an own internal standard for each target analyte is an ideal approach to account for variable recoveries and matrices, which improve the quantitative determination of trace compounds in real samples.<sup>26</sup>

In a word, the aim of this study was to develop a fast and sensitive isotope dilution UPLC-MS/MS method for the simultaneous identification and quantification of DHBMA and MHBMA in human urine. The performance of the proposed method was well validated and subsequently used to determine the concentrations of DHBMA and MHBMA in urinary samples.

## 2. Experimental

### 2.1. Chemicals and Reagents

DHBMA (N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine, purity  $\geq$  98%) and MHBMA (mixture of N-acetyl-S-1-(hydroxymethyl-2-propenyl)-L-cysteine and N-acetyl-S-2-(hydroxymethyl-3-propenyl)-L-cysteine, purity  $\geq$  95%) were obtained from Toronto Research Chemicals (Ontario, Canada). The internal standards D<sub>7</sub>-DHBMA (N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine-d<sub>7</sub>, purity  $\geq$  98%) and D<sub>6</sub>-MHBMA (mixture of N-acetyl-S-1-(hydroxymethyl-2-propenyl)-L-cysteine and N-acetyl-S-2-(hydroxymethyl-3-propenyl)-L-cysteine, purity  $\geq$  95%) were also purchased from Toronto Research Chemicals. Acetonitrile was purchased from Merck (Darmstadt, Germany). Ammonium acetate was supplied by Sigma-Aldrich (St. Louis, MO, USA). Deionized water (resistivity 18.2 MX cm<sup>-1</sup>) was produced by a Milli-Q water purification system (Millipore Co., USA), and was used for sample preparation, standard solutions, and mobile phase in UPLC-MS/MS. All other reagents are of analytical grade.

### 2.2. Apparatus

A Xevo TQ-S MS triple quadrupole mass spectrometer (Waters,

Ireland) was equipped with an ESI source and an Acquity UPLC system (Waters, Singapore). Masslynx software V4.1 (Waters, USA) was used to control the instrument and for data acquisition. An Acquity BEH C18 column was used for separation (2.1 mm × 100 mm, 1.7 µm) (Waters, Ireland).

### 2.3. Sample preparation

The urine samples of 102 volunteers were collected and tested for their DHBMA and MHBMA concentrations. The urine samples were stored at −20 °C until analysis (maximum 2 weeks).

Prior to the analysis, frozen urine samples were allowed to equilibrate to room temperature. The samples were adjusted to pH 2.0 with hydrochloric acid, and then centrifuged at 10,000 rpm for 5 min at 10 °C. An aliquot of 1 mL was transferred to polyethylene tube. Then 100 µL of the working solution of the internal standards were added to each sample. After vortex mixing, a rapid solid phase extraction (SPE) procedure was used here. The SPE was performed in a 24-port Visiprep SPE Vacuum Manifold (Supelco, USA). The SPE cartridges were Oasis HLB (WAT106202, 6cc/200mg) and purchased from Waters (USA). The Oasis HLB phase cartridges were activated with 3mL methanol and 3mL 0.1% formic acid. After that, 2mL 0.1% formic acid and 2 mL methanol were eluted through each cartridge under vacuum, respectively. The solvents were evaporated under a nitrogen stream. The residue was

dissolved in 1 mL 0.1% formic-methanol (v/v=9:1), passed through a 0.22  $\mu\text{m}$  membrane filter and transferred into a vial for the UPLC-MS/MS analysis. For each sample, three replicates were performed under the same conditions. In addition, urinary creatinine concentrations were determined by the HPLC method according to the national standard of China (Standard No. WS/T 98-1996). All procedures were approved by the ethical committee of the Shandong Center for Disease Control and Prevention, Shandong, China.

#### 2.4. UPLC-MS/MS detection

An Acquity BEH C18 column (2.1 mm  $\times$  100 mm, 1.7 $\mu\text{m}$ ) maintained at 40  $^{\circ}\text{C}$  was used for separation by an UPLC system directly coupled to a Waters Xevo TQ-S tandem mass spectrometer. A 5.0  $\mu\text{L}$  sample solution was injected at a flow rate of 0.30 mL  $\text{min}^{-1}$ . The gradient elution procedure is shown in Table 1.

Table 1 is here.

In order to obtain sensitive detection of target analytes, the MS/MS parameters were optimized at first. A solution of each target analyte and internal standard in methanol with a concentration of 200 ng  $\text{mL}^{-1}$  was injected into the MS/MS device through direct infusion via a syringe pump. Then, the MS/MS parameters were optimized automatically through the IntelliStart<sup>TM</sup> procedure by the Masslynx software V4.1 software. Negative ion detection was used in the multiple reaction



monitoring (MRM) transitions with the following parameters. Briefly, source temperature was maintained at 150 °C and desolvation gas temperature at 400 °C. The capillary voltage was 2.07 kV, and a cone voltage of 30 V was used. The cone gas flow and the desolvation gas flow were 150 L h<sup>-1</sup> and 950 L h<sup>-1</sup>, respectively. The collision gas flow was set at 0.15 mL min<sup>-1</sup>. The value of collision gas pressure was set at  $3.10 \times 10^{-3}$  mBar. In addition, ultrapure nitrogen was used as desolvation gas, cone gas, and nebulizing gas. Ultrapure argon was used as collision gas.

## 2.5. Calibration and Method Validation

A common question for calibration procedure is that of the absence of a “blank” biological specimen (human urine sample devoid of BD metabolites). As previously discussed in reference, DHBMA could be found in the urine of non-smokers and non-occupationally exposed individuals to BD, therefore, there are no true blank urine samples.<sup>13</sup> This problem solved, as published works reported, by pooling urine samples from non-occupationally exposed (or non-smoking) individuals, and using the pooled urine to build the calibration standards.<sup>2,3,10,13,28</sup> In this study, 12 blank urine samples from non-smoking, non-occupationally exposed persons were mixed and pooled. For a further step, six independent sets of calibration curves, in methanol and in urine pool sample were prepared with concentrations of 10, 50, 100, 500 1000 and 2000 ng mL<sup>-1</sup> of DHBMA, and 1, 5, 10, 50, 100 and 200 ng mL<sup>-1</sup> of

MHBMA, respectively. Besides, all the samples contained a fixed amount of D<sub>7</sub>-DHBMA (100 ng mL<sup>-1</sup>) and D<sub>6</sub>-MHBMA (10 ng mL<sup>-1</sup>). Additionally, urine pool sample was used as a blank, which was analyzed periodically for each batch of ten samples. Calibration samples were processed as described in Section 2.3. Linear calibration curves were acquired by plotting the quotients of the peak areas of the target analytes to the peak areas of the corresponding labeled internal standards as a function of the spiked concentrations. These graphs were utilized for calculating the unknown concentrations of the target analytes in urine samples. The procedure was the same as those of the published studies, where the use of labeled internal standards has been proven to be very efficient for compensation of influences due to different urinary matrices.<sup>2,9,10</sup>

Accuracy and precision of the method were calculated by the recovery experiments, which was determined by analyzing pooled urine samples spiked with 10, 50, 100, 500, 1000 ng mL<sup>-1</sup> of DHBMA and 1, 5, 10, 50, 100 ng mL<sup>-1</sup> of MHBMA, containing a fixed amount of D<sub>7</sub>-DHBMA (100 ng mL<sup>-1</sup>) and D<sub>6</sub>-MHBMA (10 ng mL<sup>-1</sup>). The creatinine concentrations for individual urine samples were ranged from 0.30 to 2.78 g L<sup>-1</sup>. The samples were analyzed in five replicates and the average was used. The inter-day precision was performed by repeating this process on five consecutive days. The limits of detection (LOD) and

quantification (LOQ) were calculated according to the amount of target analyte which gave a signal-to-noise ratio (S/N) greater than 3 and 10, respectively.<sup>8,26</sup> The coefficient of variation (CV) was calculated as the  $(SD \times 100)/\text{mean}$ .

### 3. Results and discussion

#### 3.1. Optimization of instrumentation conditions

##### 3.1.1. Mass spectrometry

In the present work, an isotope dilution UPLC-MS/MS methodology was developed and employed to quantify two mercapturic acids (DHBMA and MHBMA) in human urine samples. The parent ion of each target compound was a  $[M-H]^-$  ion in the first quadrupole, therefore, the MS/MS system was operated in negative electrospray ionization mode. The results of the MS/MS parameters for the target analytes and internal standards were summarized in Table 2, which were optimized automatically through the IntelliStart<sup>TM</sup> procedure of the instrument by direct infusion via a syringe. Figure 1 showed the fragment MS/MS spectra recorded in the instrument at the value of collision energy employed for detection. The selected daughter ion fragments were those with the maximum intensities for each target compound ensuring maximum sensitivity. It was obviously that DHBMA displayed three daughter ions ( $m/z$  121,  $m/z$  128,  $m/z$  103) with comparatively high

intensities. Therefore, three mass transitions were used for identification and detection. Especially, the mass transition ( $m/z$  121) with the highest intensity was used for quantification, whereas other two mass transitions ( $m/z$  128,  $m/z$  103) were used for qualification. The MHBMA standard substance was a isomeric mixture of 1-MHBMA and 2-MHBMA, which was used in almost all published studies.<sup>2,9-11,29,30</sup> They have the same molecular mass and cleavage pattern upon electrospray ionization, which produce primary fragment ions with the same molecular mass. As shown in Figure 1, the most prominent daughter ion for MHBMA standard substance was  $m/z$  103 followed by  $m/z$  128 and  $m/z$  73. According to the results of the previous works, the daughter ion of  $m/z$  73 is unique to 1-MHBMA but not 2-MHBMA.<sup>3,29</sup> In other words, 1-MHBMA showed three significant daughter ions ( $m/z$  103,  $m/z$  128,  $m/z$  73). By contrast, 2-MHBMA showed two significant daughter ions which had no fragment at  $m/z$  73. Therefore, for MHBMA, two daughter ions ( $m/z$  103,  $m/z$  128) showed high intensities and then two mass transitions were used for detection. The one ( $m/z$  103) with higher intensity and the other one ( $m/z$  128) with lower intensity was used for quantification and qualification, respectively. It was obvious that the values of precursor ion attenuation for daughter ions were almost 100% for both DHBMA and MHBMA. In addition, the graphs of the intensity of the selected quantifier and qualifier fragment ions as a function of collision energy for target analytes were

produced automatically by the IntelliStart<sup>TM</sup> procedure (shown in supplement materials).

Table 2 is here.

Figure 1 is here.

### 3.1.2. Optimization of UPLC conditions

During sample preparation for UPLC-MS/MS analysis, a short centrifugation step was performed at first, which aimed to precipitate proteins and particles in human urine samples. Then, a SPE clean-up procedure was used for purifying the target analytes. For a further step, the analytical columns of the UPLC system were optimized after the optimization of MS parameters. Mercapturic acids are difficult to retain on traditional reversed phases (RP) because of their high polarity.<sup>2,9</sup> As previously reported in references, chromatographic separation of these compounds was mostly based on more polar RP-columns like “polar embedded” stationary phases or stationary phases with shorter chain lengths (C8 column).<sup>2,31-33</sup> Owing to high amounts of aqueous mobile phase and low organic solvents, separation on these columns might result a low sensitivity. Moreover, DHBMA usually showed a poor retention on common C8 column because of its high polarity with two adjacent hydroxyl groups.<sup>9</sup> The Acquity BEH C18 column, filled with ethylene bridge hybrid particles, could overcome the disadvantages mentioned above and obtain favorable separation performance.<sup>34,35</sup> Therefore, the

Acquity BEH C18 column was chosen as analytical column in this study.

For a further step, two Acquity BEH C18 columns with different lengths were tested for their separation efficiencies, which were shown in Figure 2. The lengths of two columns were 100 and 50 mm, respectively. Considering the peak shape and separation efficiency, the MRM chromatograms of both analytes using the longer column are more satisfactory than those using the shorter column under the same UPLC gradient program and mobile phase composition.

Figure 2 is here.

In order to obtain good resolution and high sensitivity, the mobile phase was also optimized, which played an important role in the ionization efficiency before the target analytes enter the MS/MS system.<sup>36</sup> Here, the influence of mobile phase (0.10% formic acid in water/methanol, 0.10% formic acid in water (5 mM ammonium acetate)/methanol and 0.1% formic acid in water (20 mM ammonium acetate)/methanol) were investigated. The addition of ammonium acetate to aqueous mobile phase was found to give better separation efficiency and peak shape than that without the addition of ammonium acetate. For a further step, different concentrations of ammonium acetate to aqueous mobile phases were investigated, as shown in Figure 2. Under the optimization MS parameters, it was found that the mobile phase containing 0.1% formic acid in water (5 mM ammonium

acetate)/methanol showed the strongest MS signals of all targets, and was selected as the mobile phase.

### 3.2. Method validation

Under the optimization conditions, the representative MRM chromatograms of DBHMA and MHBMA were shown in Figure 3. It is obviously that the target peaks was very sharp and showed almost no interferences, indicating a good selectivity. It was remarkable that MHBMA standard substance contained a mixture of 1-MHBMA and 2-MHBMA, which gave two peaks with different intensities for both isomers in the chromatogram of MRM transitions. This also appeared for the internal standard of D<sub>6</sub>-MHBMA. According to the results of the published studies, in human urine samples, only one isomer of MHBMA was predominant or observed.<sup>2,9,29</sup> This phenomenon was probably caused by differences between the purchased standard substance and the enzymatic in vivo reaction.<sup>9</sup> Therefore, the results were actually the sum of 1-MHBMA and 2-MHBMA in the studies mentioned above used MS/MS as the detection method.<sup>9,10,30</sup> In the present study, as no pure MHBMA standard of one isomer could be obtained, the peaks could not be attributed to a single isomer. For this reason, the sum of two peaks was used for the quantification of MHBMA in human urine samples. In a word, further study in this field seems to be necessary.<sup>2</sup>

Figure 3 is here.

In addition, the total run time of this method was 11 minutes, which enabled a fast and reliable simultaneous determination of DHBMA and MHBMA. Thus, the presented method was suitable for routine analyses of large amount of human urine samples.

As discussed in published studies, the use of labeled internal standards has been proven to be very efficient for compensation of influences due to different urinary matrices, which has the ability to guarantee a reliable determination of the mercapturic acids in different human urines.<sup>2,9,10</sup> Linear calibration curves were obtained by plotting the quotients of the peak areas of the target analytes to the peak areas of the corresponding labeled internal standards as a function of the concentrations spiked. The linearity of the UPLC-MS/MS method for DHBMA and MHBMA was demonstrated over a broad range of concentrations. As shown in Table 3, the coefficients of correlation ( $R^2$ ) for two calibration curves were both higher than 0.998. These graphs were utilized for calculating the unknown concentrations of DHBMA and MHBMA in urine samples. The LODs were estimated to be 0.14 ng mL<sup>-1</sup> for DHBMA and 0.16 ng mL<sup>-1</sup> for MHBMA, respectively. The LODs were lower than those based on LC-MS/MS or hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-MS/MS).<sup>2,9,10</sup>

Table 3 is here.



The precision and accuracy of the method were determined using five different concentrations. The inter-day precision and accuracy was performed by repeating this process on five consecutive days. The results demonstrated that the accuracy for DHBMA and MHBMA ranged from 87.1% to 107.9%. The good accuracy results indicated that the different urine matrix had almost no influence on the analytical result, which was mainly due to the use of isotopic internal standards for DHBMA and MHBMA. The intra- and inter-day precision was expressed as CV, which was less than 14.8%. The precision and accuracy data for DHBMA and MHBMA were shown in Table 4.

Table 4 is here.

### 3.3. Application to human urine samples

The developed method was applied to determine the concentrations of DHBMA and MHBMA in human urine samples. The specific mass transitions summarized in Table 1 and the correct retention times in association with the internal standards were utilized for the identification and quantification of the target analytes. The stability of the analytes was checked firstly. For this purpose, two quality control samples were suffered from three freeze-thaw cycles on three different days. The samples were analyzed and the results were shown in Table 5. It is obviously that no significant deviation of the contents was observed and the values of CV were less than 10%. Therefore, the possible

decomposition of the analytes in human urine during several freeze-thaw cycles could be ignored.

Table 5 is here.

The urine of 102 volunteers were collected and analyzed with this UPLC-MS/MS method for their DHBMA and MHBMA concentrations. DHBMA was detected in all human urine samples, which varied from 12.8 to 1288.3  $\mu\text{g L}^{-1}$  with a mean value of 500.5  $\mu\text{g L}^{-1}$ . Regarding MHBMA, 95 of the 102 urine samples was detected. The highest value and mean value of MHBMA in samples were 83.8 $\mu\text{g L}^{-1}$  and 20.7 $\mu\text{g L}^{-1}$ , respectively. A detailed report of the results will be published soon.

**4. Conclusion**

In this study, a selective and sensitive method for the simultaneous determination of DHBMA and MHBMA has been developed and satisfactorily validated. By a combination of UPLC-MS/MS technique and isotope dilution method, the presented method was fast, precise and reliable. Especially, the utilization of isotopically labeled internal standards of the target analytes was able to compensate for matrix-related effects as ion enhancement or ion suppression as well as analyte losses due to sample preparation. Therefore, the method is suitable for the routine analysis, and could be a reference measurement procedure for the simultaneous determination of the main urinary metabolites of BD in human urine.

## Acknowledgement

This study was financed by the Medical and Health Technology Project of Shandong province of China (2013WS0165 and 2014WS0382).

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Tables:

**Table 1:** The conditions of UPLC gradient elution program. Solvent A: 0.10% formic acid in water (5 mM ammonium acetate); Solvent B: methanol.

Step	Time (min)	Flow rate (mL min <sup>-1</sup> )	Solvent A (%)	Solvent B (%)	Curve
1		0.30	98.0	2.0	
2	3.00	0.30	98.0	2.0	6
3	6.00	0.30	30.0	70.0	6
4	7.50	0.30	10.0	90.0	6
5	8.50	0.30	10.0	90.0	6
6	11.00	0.30	98.0	2.0	1

**Table 2:** MRM parameters of DHBMA, MHBMA and their stable isotopes.

Compounds	Parent ion (m/z)	Daughter ion (m/z)	Dwell time (s)	Cone voltage (V)	Collision energy (V)
DHBMA	250	103	0.043	44	14
DHBMA	250	121	0.043	44	14
DHBMA	250	128	0.043	44	12
D <sub>7</sub> -DHBMA	257	128	0.043	26	14
MHBMA	232	103	0.043	24	14
MHBMA	232	128	0.043	24	8
D <sub>6</sub> -MHBMA	238	109	0.043	36	12

**Table 3:** The coefficient of correlation ( $R^2$ ), LOD, LOQ and linearity for UPLC-MS/MS analysis of DHBMA and MHBMA.

Compounds	$R^2$	Concentration (ng mL <sup>-1</sup> )		
		LOD	LOQ	Linearity
DHBMA	0.9998	0.14	0.42	10–2000
MHBMA	0.9987	0.16	0.49	1–200

**Table 4:** Precision and accuracy data for UPLC-MS/MS analysis of DHBMA and MHBMA

Compounds	Added (ng mL <sup>-1</sup> )	Intra-day (n = 5)			Inter-day (n = 25)		
		[Mean ± SD] (ng mL <sup>-1</sup> )	Accuracy (%)	Precision (CV%)	[Mean ± SD] (ng mL <sup>-1</sup> )	Accuracy (%)	Precision (CV%)
DHBMA	10	10.3 ± 1.02	103.0	9.90	10.3 ± 1.47	103.0	14.3
	50	48.4 ± 1.97	96.8	4.07	50.8 ± 7.53	101.6	14.8
	100	87.1 ± 4.18	87.1	4.80	90.4 ± 2.9	90.4	3.21
	500	539.7 ± 4.79	107.9	0.89	497.7 ± 25.9	99.5	5.20
	1000	975.0 ± 8.21	97.5	0.84	1012 ± 29.2	101.2	2.89
MHBMA	1	1 ± 0.099	100.0	9.90	1.04 ± 0.15	104.0	14.4
	5	5.2 ± 0.42	104.0	8.08	4.74 ± 0.52	94.8	11.0
	10	9.9 ± 1.18	99.0	11.9	9.04 ± 1.32	90.4	14.6
	50	49.4 ± 3.4	98.8	6.89	49.1 ± 2.24	98.2	4.56
	100	100.3 ± 2.72	100.3	2.71	102.9 ± 0.78	102.9	0.76

**Table 5:** Stability of the analytes on three different days after freeze-thaw cycles.

Compounds	Samples	day 1 (ng mL <sup>-1</sup> )	day 2 (ng mL <sup>-1</sup> )	day 3 (ng mL <sup>-1</sup> )	CV%
DHBMA	S1	617.2	607.1	630.6	1.91
	S2	661.1	629.0	645.3	2.49
MHBMA	S1	11.7	12.5	11.4	4.79

	S2	13.0	12.8	11.0	8.98
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**Caption to Figures:**

**Fig. 1:** The pictures of the fragment MS-MS spectra recorded in the instrument at the value of collision energy employed for detection. A: DHBMA, B: D<sub>7</sub>-DHBMA, C: MHBMA, and D: D<sub>6</sub>-MHBMA.

**Fig. 2:** The MRM chromatograms of DHBMA and MHBMA under different lengths Acquity BEH C18 columns (A: 2.1 mm × 50 mm, 1.7µm; B: 2.1 mm × 100 mm, 1.7µm) and mobile phases (B: 0.10% formic acid in water (5 mM ammonium acetate)/methanol; C: 0.10% formic acid in water (20 mM ammonium acetate)/methanol). The concentrations of DHBMA and MHBMA in working solution were 500 and 50 ng mL<sup>-1</sup>, and D<sub>7</sub>-DHBMA and D<sub>6</sub>-MHBMA were 100 and 10 ng mL<sup>-1</sup>, respectively.

**Fig. 3:** Typical chromatogram of MRM transitions of DHBMA and MHBMA. The concentrations of DHBMA and MHBMA were 500 and 50 ng mL<sup>-1</sup>, and D<sub>7</sub>-DHBMA and D<sub>6</sub>-MHBMA were 100 and 10 ng mL<sup>-1</sup>, respectively.



**Supplement Materials:**

**Supplement Material 1:** The graphs of the intensity of the fragment ions as a function of collision energy-DHBMA

**Supplement Material 2:** The graphs of the intensity of the fragment ions as a function of collision energy-D<sub>7</sub>-DHBMA

**Supplement Material 3:** The graphs of the intensity of the fragment ions as a function of collision energy- MHBMA

**Supplement Material 4:** The graphs of the intensity of the fragment ions as a function of collision energy-D<sub>6</sub>-MHBMA