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A liquid chromatography–tandem mass spectrometry method for the determination of nodularin-R in human plasma and its preliminary clinical application

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ABSTRACT

Background: Nodularins are an important class of hepatotoxic cyclic pentapeptides that most current methods are ill-suited to clinically monitor. Therefore, a liquid chromatography–tandem mass spectrometry (LC/MS/MS) assay for the determination of nodularin-R in human plasma was validated and applied to clinical samples in this report.

Methods: Sample cleanup and enrichment were achieved using solid phase extraction. The concentration of nodularin-R in each sample was calculated using the relative abundance area ratio of nodularin-R and a stable isotope-labeled internal standard.

Results: The validated calibration range was from 0.50 to 100 ng/ml. The intra- and inter-day precision were <6.0 and 9.8%, respectively. The accuracy at the lower limit of quantification (LLOQ) was 2.0%, while for other QC levels, it was <3.4%. The absolute concentrations determined by the LC/MS/MS assay were significantly lower than those measured using a commercial immunoassay. Finally, a variety of demographic factors were evaluated and the findings indicated that the elderly (>50 y) and people living close to seashore or lakefront areas (≤10 km) were the most susceptible to nodularin-R ($p = 0.79 \times 10^{-4}$ and 2.57×10^{-6}, respectively).

Conclusions: This report was among the first to demonstrate that clinical monitoring of nodularin-R could be achieved using LC/MS/MS.

1. Introduction

Cyanobacteria, also known as blue-green algae, are the cause of intermittent but repeated cases of water poisoning in both animals and humans [1]. In recent years, they have spread across China's east coastline and into major Chinese lakes. Cyanobacteria produce a wide range of cyanotoxins [2], among which are nodularins, a group of hepatotoxic cyclic peptides that consists of 5 amino acids, including Mdhb (N-methyldehydrobutyric acid) and the unique β-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid). To date, about ten nodularin variants have been identified [3], with nodularin-R being the first and most abundant form in natural blooms [4]. Nodularin-R contains an arginine residue at position 2 (Fig. 1). While there are non-toxic nodularin strains [5], there is evidence indicating a direct toxic effect of nodularin-R [6,7].

It is widely accepted that the toxicity of nodularin-R is due to severe inhibition of the protein phosphatases 1 (PP-1) and 2A (PP-2A), which leads to functional and structural disturbance of the liver [8]. Nodularin-R has also been reported as a direct carcinogen and apoptogen in the liver [9]. Due to a lack of exposure data, its toxicity can currently only be estimated from microcystin-LR, which has been reported to have similar toxicity to nodularin-R [6,8,10]. However, these estimated toxicity values were sometimes inconsistent with observed toxic effects [9,11]. Because the plasma (or whole blood or serum) level is often used as a straight guide to assess exposure [12], the determination of nodularin-R in human plasma may help elucidate the mechanisms underlying its induced toxicity and provide a direct evaluation of human health risks. Therefore, it is of clinical significance to monitor the content of nodularin-R.

Most of the currently available assays for the determination of nodularin-R have relied on enzyme-linked immunosorbent assays (ELISA) [13], high-performance liquid chromatography (HPLC) [14] and liquid chromatography coupled with mass spectrometry (LC/MS) [15,16]. ELISA offers good sensitivity at picogram level when compared to the other methods [17]; however, the antibodies obtained usually present a questionable specificity [18,19]. As a result, the bioassays cannot determine the concentration of individual nodularins, but only their total amount. HPLC and LC/MS generally employ long columns that achieve good resolution of analogs but with low throughput [3]. Also, matrix interference has been observed in these analyses [20,21]. Because of this, liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is likely to have a

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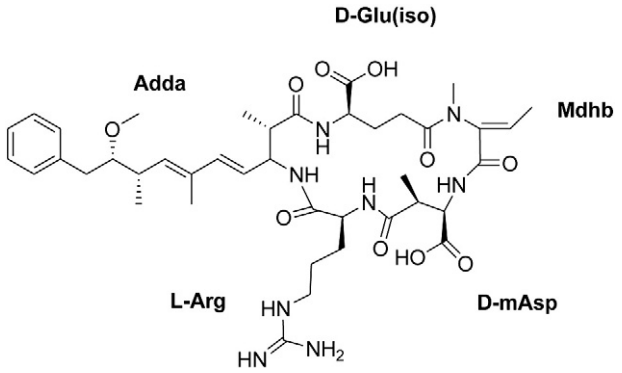

Fig. 1. Chemical structure of nodularin-R.

substantial impact on nodularin-R quantification by providing high sensitivity, selectivity and robustness for both detection and quantification. While LC/MS/MS assays have been developed for the determination of nodularin-R in several biological matrices (e.g., natural water [4,22] and animal tissues [21,23,24]), their application to clinical practice has rarely been attempted.

In this report, an LC/MS/MS assay for the determination of nodularin-R in human plasma was developed and validated. Mass spectrometric, chromatographic and extraction conditions were optimized. Moreover, this assay was applied to the quantitative analysis of nodularin-R in clinical samples, and the resulting values were compared to those obtained using a commercial immunoassay. Finally, several demographic factors that may affect the plasma concentration of nodularin-R were assessed.

2. Materials and methods

2.1. Chemicals

Nodularin-R (Cyclo[3S-amino-9S-methoxy-2S,6E,8S-trimethyl-10-phenyl-4,6-decadienoyl-D-γ-glutamyl-(2Z)-2-(methylamino)-2-butenoyl-(3S)-3-methyl-D-β-aspartyl-L-arginyl]) was from Cayman Chemical Company (Ann Arbor, MI). Stable isotope-labeled nodularin-R containing [U-13C6]-L-arginine was developed by ChinaPeptides Co., Ltd. (Shanghai, China) (Supplementary Material, Fig. 1S). Acetonitrile (ACN) was from Lingfeng Chemical Reagent Co. Ltd (Shanghai, China). Formic acid (FA) was from Xilong Chemical Industrial Factory Co. Ltd (Shantou, China). Methanol was from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All of the reagents used were of analytical grade. Water was purified and deionized by a Milli-Q system from Millipore (Bedford, USA). Analyte-free EDTA human plasma was provided by the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) and was screened for the presence of nodularin-R prior to use.

2.2. Preparation of stock solutions, calibration and quality control (QC) standards

Nodularin-R was accurately weighted, and 1 mg/ml stock solution was prepared by dissolving the weighed peptide into the working solvent, methanol:water (50:50 v:v) containing 0.25% formic acid. The solution was stored in a brown glass tube, which protected it from light, at −20 °C. The stock solution was diluted to 1000 ng/ml for the infusion solution. Isotope-labeled nodularin-R was also weighed and a 5 μg/ml stock solution was prepared in the working solvent. An internal standard solution with a concentration of 50 ng/ml was prepared by diluting the stock solution with ACN:water (70:30 v:v) containing 0.1% formic acid.

There are no studies currently available that can indicate the highest non-toxic concentration of nodularin-R, whereas nodularin-R may be in an environmental concentration range similar to microcystin-LR [21,25]. According to the corresponding values reported for microcystin-LR (i.e., 15 ng/ml [26], 5 ng/ml [27] and ppm–ppb range [17]) in vitro, the calibration range for this study was 0.50 ng/ml to 100 ng/ml. Nodularin-R spiking solutions were prepared via serial dilution with the working solvent. The concentrations used were 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00 and 10.0 μg/ml. Calibration standards were freshly made every working day by adding 10 μl of the spiking solution to 990 μl of the blank human plasma. The resulting calibration standard concentrations were 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 ng/ml. The QC standards (i.e., LLOQ (Lower Limit of Quantification), low QC, mid QC and high QC) were prepared at 0.50, 1.50, 7.50, and 80.0 ng/ml, respectively, in the blank plasma and frozen prior to use. Six unique batches of human plasma spiked with nodularin-R at the low QC concentration were compared to evaluate the matrix effect. Recovery solutions were QC samples (i.e., low QC, mid QC and high QC) at six replicates in working solvent at their final concentration levels assuming 100% recovery (i.e., a two-fold higher concentration due to SPE enrichment).

2.3. Liquid chromatography and mass spectrometry

A Waters ACQUITY UPLC system and a Quattro triple quadrupole mass spectrometer (Milford, MA, USA) were used. Liquid chromatography separations were performed on an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm; Waters) at room temperature. The mobile phase consisted of solvent A (0.1% FA:water) and solvent B (0.1% FA: methanol). A linear gradient with a flow rate of 0.25 ml/min was applied in the following manner (duration in parentheses): B 20% (0 min) → 90% (1 min) → 90% (2.5 min) → 20% (2.6 min). The injection volume was 10 μl. To avoid the carryover effect, the injection port was washed twice by the needle rinse solution (50:50 ACN: water) between injections.

The mass spectrometer was interfaced with an electrospray ion source and operated in positive multiple reaction monitoring (MRM) mode. Q1 and Q3 were both set at unit resolution. The desolvation gas flow was 801 l/h, and the cone gas flow was 50 l/h. The source and desolvation gas temperatures were held at 105 °C and 350 °C, respectively, while the electrospray capillary and cone voltages were optimized to 3.0 kV and 50 V, respectively. Data were collected and processed using MassLynx 4.1 from Micromass-Waters (Manchester, UK). The MRM transitions used for nodularin-R and the internal standard were m/z 826 → 135 and m/z 832 → 135 [28], respectively. The collision energy for nodularin-R was optimized for 50 eV, and the dwelling time was set to be 50 ms.

2.4. Extraction

Prior to extraction, all frozen samples were thawed and allowed to equilibrate to room temperature. They were then vortexed for around 15 s, and 200 μl of each sample was transferred into a 1.5 ml test tube followed by an additional 800 μl of the internal standard solution. The tubes were vortexed for about 5 min and then centrifuged twice at approximately 3250 rpm for 10 min at 5 °C. Afterward, the supernatant was transferred into an Oasis HLB cartridge (60 mg/3 ml Waters) that had been preconditioned with 3 ml methanol and then 3 ml deionized water. After the sample was loaded, the cartridge was washed with 2 ml of water and 2 ml of 10% methanol and eluted with 3 ml of 100% methanol. Finally, the eluent was evaporated to dryness and then resuspended in 100 μl of the working solvent.

2.5. Method validation

Validation of the assay involves determining its linear range, accuracy, precision, limit of quantification (LOQ), recovery, matrix effect and

stability. The detailed procedures and the acceptance criteria used to validate the assay have been described in a number of publications [29].

2.6. Application to clinical samples

To examine the applicability of the developed assay for clinical practices, human plasma samples were obtained from volunteers and stored at −20 °C. This study was approved by the institutional review board of Nanjing Medical University. One hundred volunteers were consecutively recruited between January 2010 and May 2011 at the First Affiliated Hospital of Nanjing Medical University, Nanjing, China (50 men and 50 women with a mean age and range of 42.4 and 15–65 y, respectively). They were biologically unrelated, ethnic Han Chinese from Jiangsu province, China. In addition, these volunteers, to their knowledge, were healthy and had no reason to consult their local doctors during the preceding 12 months. Informed consent was obtained from the subjects, and the collected samples were prepared and analyzed using the procedure described above. Finally, the results were compared with that of an available ELISA. So far, there is no commercial ELISA kit for the specific detection of nodularin-R or other nodularins. In this study, the assay was performed in the First Affiliated Hospital of Nanjing Medical University using a microcystins/nodularins (ADDA) ELISA kit (Abraxis, Warminster, PA) on a Model 680 Microplate Reader (BioRad, Hercules CA). According to the manufacturer's instructions, this kit detects microcystins, nodularins and many of their congeners.

3. Results and discussion

3.1. Method development

For biological samples, sample preparation is usually carried out to remove possible interference from endogenous substances and increase the selectivity and sensitivity of assays. Three common techniques used during sample preparation are protein precipitation, liquid–liquid extraction and solid phase extraction. In the case of nodularins in both water and phytoplankton matrices, solvent extraction has been widely applied [3,22], while solid phase cartridges have been previously employed for tissue sample preparation [21,23,30]. In this report, solid phase extraction was selected as the technique of choice for the sample cleanup and enrichment. Solid phase extraction has shown great promise for sample enrichment, with an approximately 10 fold improvement compared to protein precipitation (data not shown).

A BEH C18 column (1.7 μm, 2.1 mm × 50 mm) was used for the chromatographic separation. A mobile phase consisting of methanol and water with a linear gradient was found to be optimal with respect to obtaining symmetrical peak shapes and short run times for both the analyte and internal standard. The retention times for nodularin-R and the internal standard were around 1.69 min.

3.2. Characteristic fragment ions of nodularin-R in a triple quadrupole mass spectrometer

To date, sensitive analyses have been reported for peptide quantification using a triple quadrupole mass spectrometer. Recently, quantitative assays have also been successfully developed in our lab for the determination of desmopressin [28] and human epidermal growth factor [31]. All of these studies support the idea that quantitative analysis of peptides can be achieved assuming that the characteristic fragment ions formed in abundance.

In the full scan spectrum of nodularin-R, the base peak at *m/z* 826 corresponds to its protonated molecular ion (Fig. 2A). In the subsequent MS/MS analysis, low-mass fragment ions were observed upon application of the collision energy (CE) in stepwise increments and became significant at a CE of 50 eV (Fig. 2B). Among these ions, the most intense one possessed an *m/z* of 135 from the ion $[Ph\text{-}CH_2\text{-}CH(OMe)]^+$, which was derived from the heterolytic cleavage of the C8–C9 bond on Adda residue (Fig. 2C). The less intense product ion with an *m/z* of 163 from $[C_{11}H_{15}O]^+$ resulted from the fragmentation of the $[M+2H\text{-}135]^+$ ions, proceeding through a C–N bond cleavage (Fig. 2C) [32]. The prominence of the *m/z* 135 ion has also been

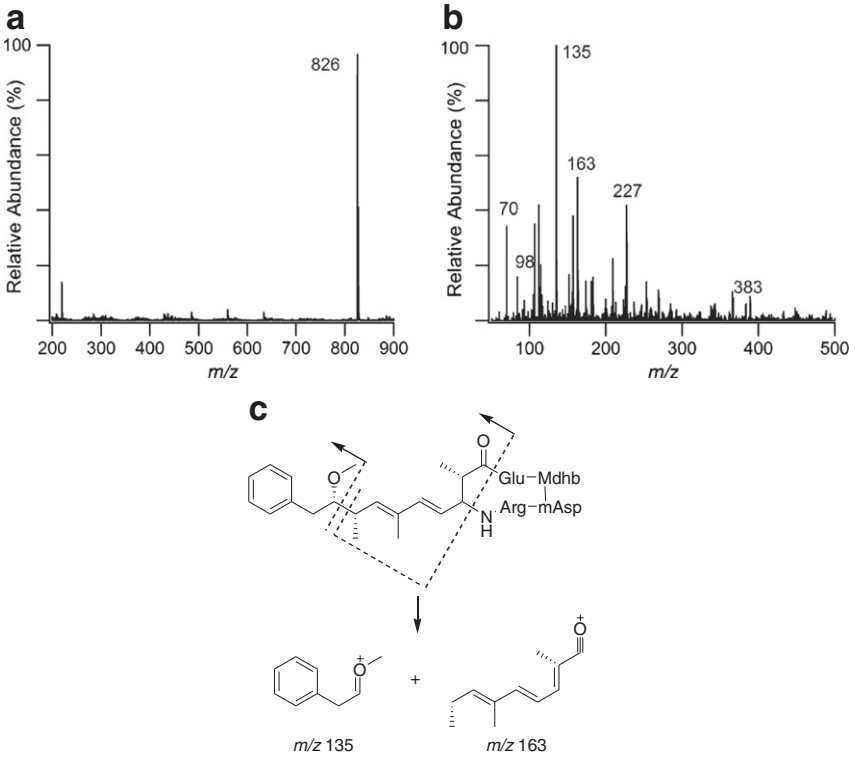

Fig. 2. Full scan spectrum (A) and product ion spectrum of nodularin-R (B). The intense fragment ions were derived from Adda residue (C).

observed in several studies, which employed the ion transition *m/z* 826→135 for nodularin-R measurements [21,23]. It is noteworthy that both the transitions of *m/z* 826→135 and *m/z* 826→163 were optimized and evaluated in this work [29,33]. As a result, nodularin-R was detected at the same retention time (data not shown). Since the transition of *m/z* 826→135 had a higher S/N ratio, we used this transition for both detection and quantification.

3.3. Linearity, limit of detection (LOD) and limit of quantification

Good reproducibility was achieved within a run in terms of the peak shape, height, area, and retention time. Calibration curves were constructed using a weighted linear regression model with a weighting factor of $1/x^2$. The linearity was good over 3 orders of magnitude (from 0.50 ng/ml to 100 ng/ml; representative calibration curve: $y = 69.4x - 0.19$; correlation coefficient: $r^2 \geq 0.9929$; see Supplementary Material, Fig. 2S). The LOD, defined as the concentration that gave a signal-to-noise ratio of 3, was estimated to be 0.22 ng/ml. The LOQ was chosen as the concentration corresponding to the lowest standard of the calibration curve that gave good accuracy and precision and was found to be 0.50 ng/ml here (Table 1), which was several folds lower than those previously reported [3,21].

As mentioned earlier, the association between the concentration of nodularin-R in plasma and human health risks is not currently clear. However, the level of nodularin-R indicative of toxicity was estimated to be >15 ng/ml for sertoli cells [26], 5 ng/ml for embryos [27] and in the range of 1 ng/ml–1 μg/ml for tissues [17]. Moreover, the estimated IC50 value of nodularin-R for the direct inhibition of protein phosphatases in vitro was 4.12 ng/ml [34,35]. Therefore, the LOQ of the assay could be sufficient to detect the toxic level of nodularin-R in human plasma.

3.4. Precision and accuracy

The precision and accuracy of the assay were assessed by measuring the response of QC samples at four concentrations with three validation runs each. The intra- and inter-day precisions were expressed as the percent coefficient of variation (%CV). The accuracy was obtained by averaging the calculated concentrations and comparing to their nominal values (%Bias). The results are listed in Table 1(A). For each QC level of nodularin-R, the intra- and inter-day precisions were <6.0% and 9.8%, respectively. Accuracy of LLOQ was 2.0% and the values for other QCs were <3.4%. Overall, the QC data indicated the current method possessed acceptable accuracy and precision for the determination of nodularin-R in human plasma.

3.5. Specificity, recovery and matrix effect

The matrix effect in electrospray ionization mass spectrometry has been frequently pointed out in studies on nodularins [21,23]. Therefore, 6 different lots of blank plasma were analyzed here to investigate whether there was any significant interference from the analyte-free human plasma. No significantly interfering peak was found at the retention time of nodularin-R in the LC/MS/MS chromatogram of the blank plasma (see Supplementary Material, Fig. 3S). A representative chromatogram of the LLOQ was also shown.

The absolute recovery of nodularin-R from human plasma was calculated by comparing the response ratios prior to extraction of blank plasma spiked with nodularin-R with the working solvent at its final concentration. The results are shown in Table 1(A). The recoveries of low, mid and high QC were 103.0%, 82.2% and 98.1%, respectively. In fact, the absolute recovery is composed of 2 parts, the extraction recovery and process efficiency. The values for extraction recovery reflect the degree of matrix suppression/enhancement, and the matrix effect calculated in this manner may be referred to as an "absolute" matrix effect [36]. Although the presence of this absolute matrix effect may be of some concern, a more important parameter in the evaluation and validation of a bioanalytical method is the absence of a "relative" matrix effect. This type of effect is usually measured by spiking six unique lots of blank plasma with low QC standards [29,36]. The results are listed in Table 1(B), and no relative matrix

Table 1
Results of method validation.

(A) Accuracy, precision and absolute recovery for nodularin-R QC samples

Nominal concentration	0.50 ng/ml	1.50 ng/ml	7.50 ng/ml	80.0 ng/ml
Mean	0.49	1.53	7.25	82.7
%Bias	−2.0	2.0	−3.3	3.4
Intra-day precision (%CV)	5.2	6.0	4.1	2.1
Inter-day precision (%CV)	4.3	3.1	8.9	9.8
Total variation (%CV)	6.2	5.7	9.1	8.5
n	17	18	18	18
Number of runs	3	3	3	3
Absolute recovery (%)	–	103.0	82.2	98.1

(B) Matrix effect

Nodularin-R	QC nominal concentration (1.50 ng/ml)					
	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
Mean	1.48	1.53	1.45	1.49	1.52	1.51
%CV	9.8	3.8	4.7	6.0	4.6	7.2
%Bias	−1.3	2.0	-3.3	-0.7	1.3	0.7
n	6	6	6	6	6	6

(C) Stability

Nodularin-R	QC nominal concentrations			
	0.50 ng/ml	1.50 ng/ml	7.50 ng/ml	80.0 ng/ml
Three cycle freeze–thaw stability				
Mean	–	1.43	–	70.1
%CV	–	7.5	–	14.3
%Bias	–	−4.7	–	−11.3
n	–	6	–	6
48 h post-preparative stability				
Mean	0.52	1.50	7.46	80.0
%CV	5.7	2.4	0.8	2.8
%Bias	4.6	0.0	−0.5	0.0
n	6	6	6	6
12 h room temperature stability				
Mean	–	1.38	–	75.5
%CV	–	2.0	–	1.5
%Bias	–	−8.0	–	−5.6
n	–	6	–	6
3 month stability at −70 °C storage				
Mean	–	1.35	–	71.8
%CV	–	4.5	–	2.2
%Bias	–	−9.7	–	−10.2
n	–	6	–	6

Table 2
Demographic data.

Data	Mean ± STD (N = 100)
Age	42.4 ± 15.1
BMI	22.9 ± 4.2
Gender	
Male	50%
Female	50%
Concentration (ng/ml)	
<0.5	51%
0.5–0.6	23%
0.6–0.7	17%
0.7–0.8	6%
>0.8	3%

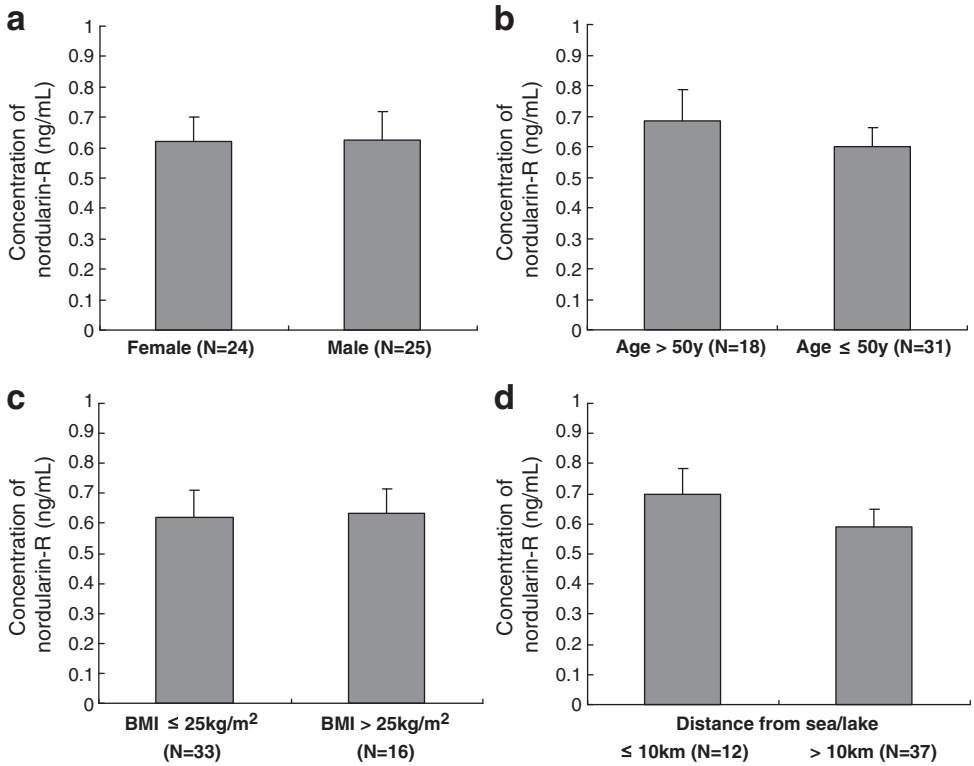

Fig. 3. The impact of demographic factors on the nodularin-R plasma concentrations. The factors investigated include age, sex, BMI and area of residence.

effect was observed. The acceptance criteria is that average accuracy expressed as %Bias for each lot tested should be $\leq \pm 15\%$ and precision expressed as %CV should be $\leq \pm 15\%$ [36,37].

3.6. Stability

Three cycle freeze–thaw, 48 h post-preparative (4 °C) and 12 h room temperature stabilities have been successfully established. The stability after 3 months at −70 °C was also evaluated. The results are shown in Table 1(C). Moreover, the stability of the stock and spiking solutions has been assessed, and both were stable for at least 6 h at room temperature and for 14 days at −20 °C.

3.7. Analysis of clinical samples

The validated analytical method was applied to measure the nodularin-R concentration in the collected clinical samples. The demographic data is presented in Table 2. For the 49 samples with concentrations greater than the LOQ, the mean concentration (mean ± SD) was 0.62 ± 0.09 ng/ml, and the maximum value was 0.86 ng/ml. The individual data from the volunteers are depicted in Supplementary Material, Fig. 4S. Even though all of the detected values were significantly lower than the highest, non-toxic, in vitro concentration of nodularin-R, the influencing factors (e.g., sex, age, body mass index (BMI) and residence) that could be correlated to the nodularin-R susceptibility of humans were evaluated. Undetectable samples were not included [12,38]. The student's *t*-test was used for statistical analysis. While the results indicate that the elderly (>50 y; 18 volunteers) are more susceptible to nodularin-R ($p = 0.79 \times 10^{-4}$), no significance was observed attributable to either sex ($p = 0.81$) or BMI ($p = 0.61$). Although we cannot provide a complete history of food consumption for each volunteer, it was found that 12 volunteers who lived either close to the seashore or in lake areas (≤10 km) had a higher nodularin-R plasma concentration than the others ($p = 2.57 \times 10^{-6}$). The mean concentrations observed for the different groups are depicted in Fig. 3.

There are a number of studies that point out that elderly people have longer chemical exposure histories and pre-existing compromised function in target organs, which can lead to greater toxin accumulation and slower detoxification/excretion rates [39,40]. In addition, people who live close to the seashore or near lakes are more likely to get their food from the sea/lake and come into contact with the source water, which are the major points in the food chain for the contamination of nodularins. This situation may get more severe because of the current blue-green algae outbreaks off of China's eastern coastline and in major Chinese lakes in recent years [41,42]. On the other hand, the present results cannot exclude the possibility that sex and BMI may have some effect on toxicity because the small sample size employed here increases the possibility of false positives (type II errors) [43]. To provide the more conclusive evidence needed for an accurate and reliable statistical analysis, a larger sample size should be explored in future.

All of the results obtained from the LC/MS/MS assay were significantly lower than those measured with the ELISA (mean concentration, 1.51 ± 0.12 ng/ml; range, 1.23–2.01 ng/ml). As mentioned earlier, this difference was probably caused by the non-specificity of the antibody in the immunoassay, which can also detect other nodularin variants, microcystins, and their conjugates and congeners. In addition, the ratios of concentrations determined by the two assays were between 1.9 and 3.2 folds. Unfortunately, there was a poor correlation between ELISA and LC/MS/MS assays ($y = 1.09x + 0.847$, $r = 0.5276$, $p > 0.05$). This may be explained by the varying amounts of interference from the samples. These phenomena were also observed in samples of mussels and flounders in Meriluoto's lab [21].

Finally, it is noteworthy that the cysteine conjugate of nodularin-R has been identified as the main detoxification product in a number of reports [21,44,45]. Therefore, this conjugate was also prepared and investigated in this work. The conjugation reaction was carried out

under non-enzymatic conditions as described previously [21,44], and the product was identified by the base peak at *m/z* 947 in its full spectrum scan. The product ion spectrum was similar to that of nodularin-R, except that the immonium ion at *m/z* 70 derived from Mdhb residue, previously shown in the spectrum of nodularin-R, was not easily found (see Supplementary Material, Fig. 5S). The MRM transition used for the conjugate was *m/z* 947→135. The validation was performed and the extracted samples were assayed. Unfortunately, no cysteine conjugate of nodularin-R could be detected in the plasma samples. Because the liver and kidney are the major sites of enzymatic detoxification [21,45], we might expect to detect quantifiable concentrations of the cysteine conjugate of nodularin-R in these human tissues upon further investigation.

4. Conclusion

In this study, a sensitive LC/MS/MS assay was developed and validated. Using this assay, nodularin-R in clinical samples was detected and quantified. Notably, the absolute concentrations determined by the LC/MS/MS assay were significantly lower than those measured with ELISA, which demonstrates a high degree of analytical specificity. In addition, there was evidence indicating a possible impact from age and place of residence on nodularin-R plasma concentrations. We show that clinical monitoring of nodularin-R could be achieved using LC/MS/MS. However, a larger number of samples combined with the measurement of physiological parameters are needed to further define the toxic plasma level of nodularin-R in humans.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cca.2012.01.039.

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