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PO-CON1522E

LC-MS/MS analysis of the new antioxidant identified from a soft-body extract of *Crassostrea gigas*

ASMS 2015 WP 103

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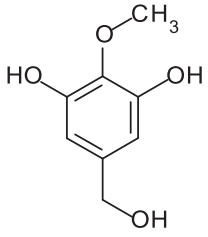
Introduction

A cerebral oxidation stress makes them induce insomnia and etc. So, it is desirable to take antioxidants with high penetration through blood-brain barrier (BBB) for the prevention of insomnia.
We had found a new antioxidant, 3,5-dihydroxy-4-methoxybenzyl alcohol (E6), from a soft-body extract of *Crassostrea gigas*, which has antioxidation ability about 2.4 times more than that of alpha-tocopherol and L-ascorbic acid and then we synthesized and identified E6.
This time, we developed the method to analyze E6 sensitively by LC-MS/MS and we verified the penetrating ability through blood-brain barrier of E6.

Figure 1 Crassostrea gigas



C8H10O4
Mw 170

Figure 2 Structure of 3,5-dihydroxy-4-methoxybenzyl alcohol

Methods and Materials

Crassostrea gigas were purchased from the market. Standard of 3,5-dihydroxy-4-methoxybenzyl alcohol (E6) was made by synthesis. MS condition of E6 was optimized on compound-dependent parameter (Q1 Pre Bias, collision energy, Q3 Pre Bias) and MRM transition and then they were analysed on LC/MS/MS condition as follows. HPLC system was connected to triple quadrupole mass spectrometer LCMS-8040. Chromatographic separations were carried out using reversed phase column, Shim Pack VP ODS (2.0mmI.D. x 150mm) maintained at 40 Celsius degrees. Sample was eluted at 0.25mL/min with a binary gradient system consisting of solvent A, 0.5% acetic acid and solvent B, acetonitrile, and applied to mass spectrometer with electro ion spray source, then analysed with negative MRM mode.

UFMS
ULTRA FAST MASS SPECTROMETRY
Speed Beyond Comparison

Ultra Fast Polarity Switching
- 15 msec
Ultra Fast Scanning
- 15,000 u/sec
Ultra Fast MRM
- Max. 555 transition /sec

Figure 3 LCMS-8040 triple quadrupole mass spectrometer

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Table 1 Analytical Conditions

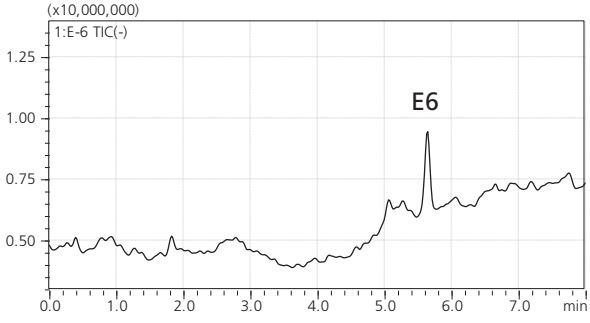
HPLC (Prominence HPLC system)	
Column	: Shim-pack VP-ODS (250 mmL x 2.0 mmI.D., 5 µm)
Mobile Phase A	: 0.05% Acetic acid
Mobile Phase B	: Acetonitrile
Gradient Program	: 5 %B (0 min) - 100 %B (8-12 min) - 5 %B (12.1-20 min)
Flow Rate	: 0.21 mL / min
Column Temperature	: 40 Celsius
Injection Volume	: 5 uL
Mass (LCMS-8040 triple quadrupole mass spectrometry)	
Ionization	: ESI, Negative (-3.5 kV)
Nebulizing Gas Flow	: 1.5 L / min
Drying Gas Pressure	: 10 L / min
DL Temperature	: 250 °C
BH Temperature	: 400 °C

Result

LC-MS/MS Method development for E6

First, MRM method was optimized using standard sample of E6. A molecule ion [M-H]- was detected at m/z 169 with FIA, electro spray ionization, negative mode. Product ion scan resulted m/z 154, 137, 125 as fragment ion. Then, Q1>Q3=169>154 was obtained as the optimal MRM transition for quantitation.

(a) Q1 TIC chromatogram



(b) Q1 spectrum

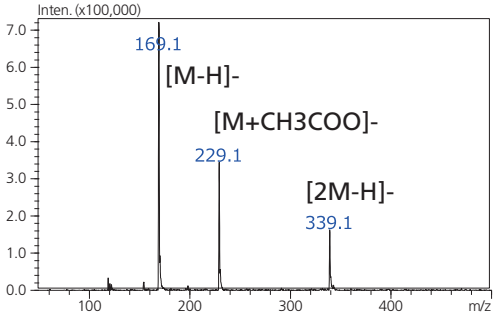


Figure 4 Q1 scan data of E6 standard

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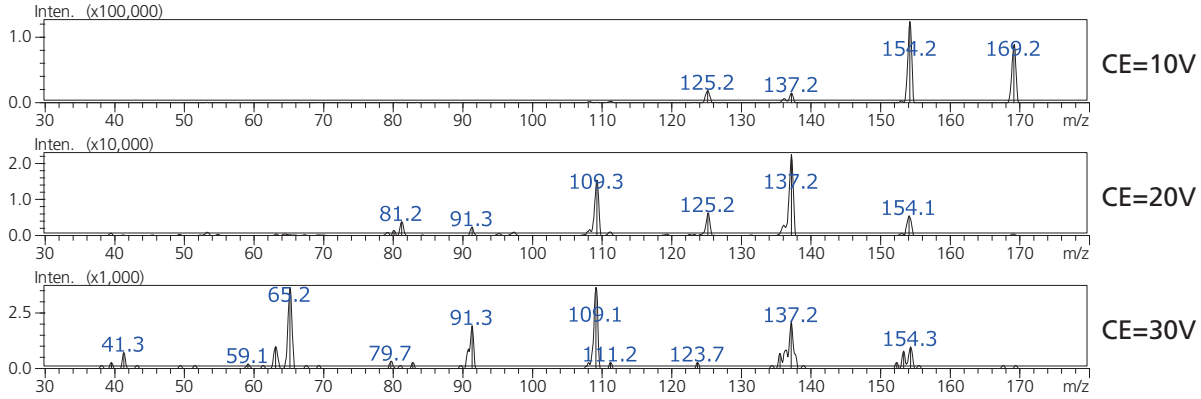

Figure 5 Product ion scan spectra of E6 standard

In the auto optimization for MRM method, multi product ion scannings are acquired for the selection of suitable fragment ions and collision energy. It is able to be executed with single flow injection analysis owing to the Ultra Fast Scanning.

Table 2 Analytical Conditions; optimized MRM transition of E6

Event	Compound	Q1 (m/z)	Q3 (m/z)	Dwell time (msec)	CE (V)	
1	E6	169	154	100	14	Quantitative ion
2	E6	169	137	100	24	Reference ion
3	E6	169	125	100	22	Reference ion

Next, dilution series of E6 was made and they were analyzed by LC-MS/MS. As a result, it was confirmed that the linearity of calibration curve and repeatability for E6 was excellent and E6 could be detected high sensitively from 1ng/mL.

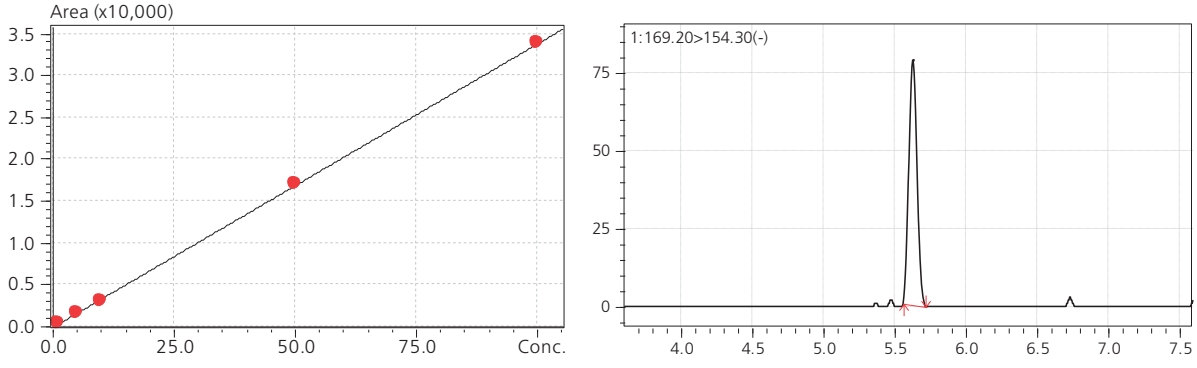

Figure 6 Calibration curve (1~1000 ng/mL) of E6 and MRM chromatogram of E6 1ng/mL (standard)

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Analysis of E6 kinetics *in vitro* / *in vivo*

To estimate the penetration through blood-brain barrier, the first experiment was implemented using a BBB kit (PharmaCo-Cell Company Ltd., Japan). After adding 100uM E6 to the blood side, E6 concentration of the blood side and the brain side was analyzed by HPLC. As a result, it was suggested that E6 had the penetration through blood-brain barrier.

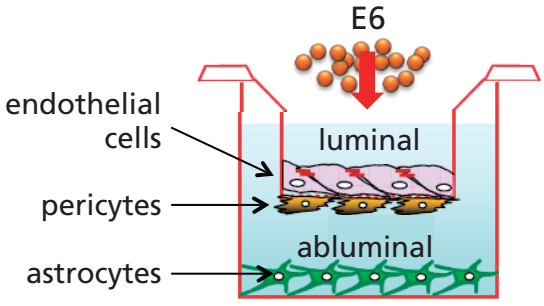

Figure 7 BBB kit™

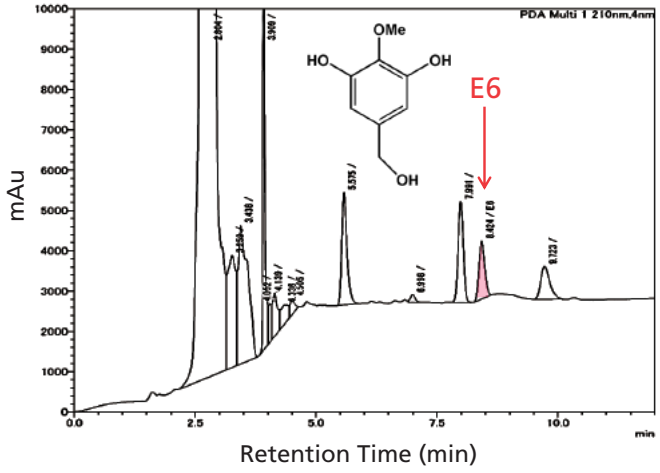

Figure 8 HPLC chromatogram of sample on BBB kit brain side to which E6 was added

The second experiment; E6 was administered to mouse orally and E6 concentrations in the plasma and in the brain before and after administration were analyzed by LC-MS/MS. As a result, E6 was detected both in the plasma sample and in the cerebral sample.

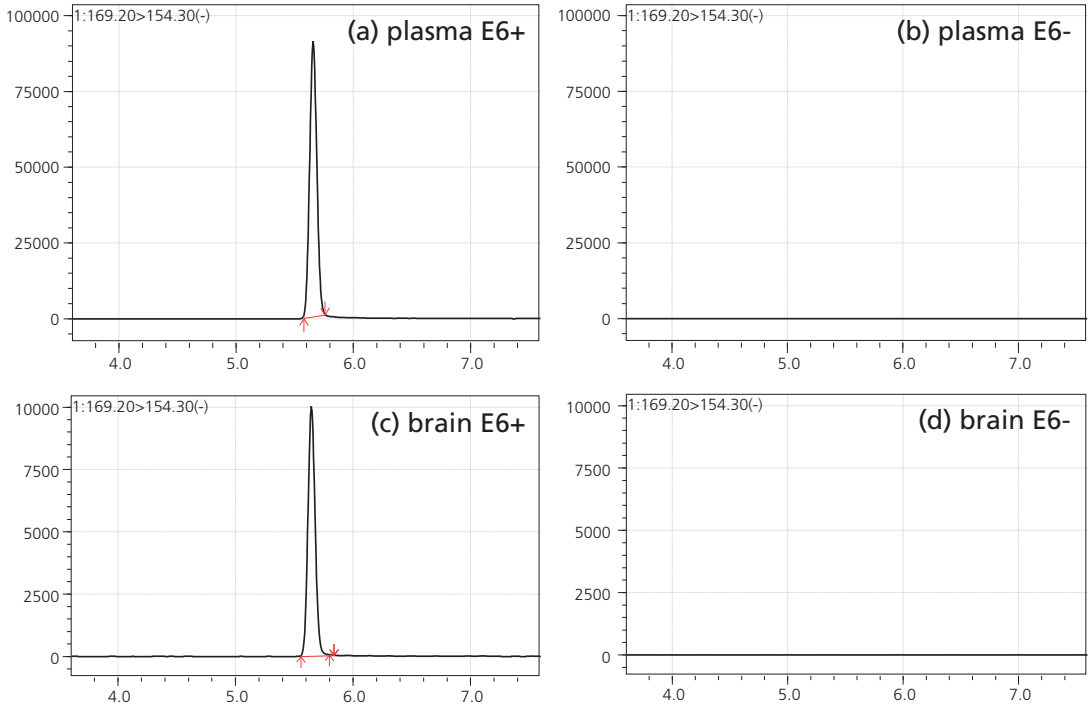

Figure 9 MRM chromatograms of (a) plasma sample after E6 oral administration, (b) plasma sample control, (c) brain sample after E6 oral administration and (d) brain sample control; all samples were diluted by the addition of water (x100) in order to weaken the effect of ion suppression before LCMS analysis

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E6 was not detected in mouse plasma and brain samples before E6 administration (control samples). This meant that E6 did not exist endogenously in the living body of a mouse.
E6 concentration in the plasma and brain time course samples after its oral administration was analyzed (Figure 10). It was found that E6 was detected in both samples of 10 minutes after E6 administration and E6 level in brain resulted highest at 30 minutes after administration while plasma 10 minutes.

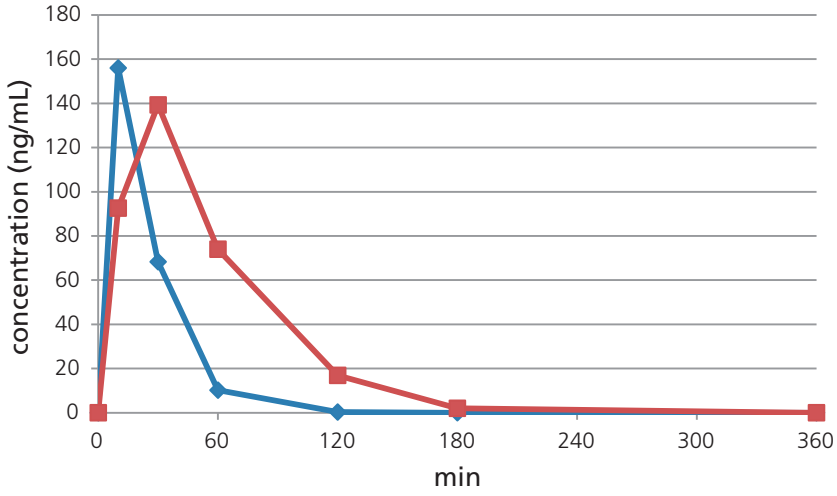

Figure 10 Results of time course test for E6 level (blue) in the plasma samples (red) in the brain samples

Conclusions

- High sensitive analysis method of E6, from a soft-body extract of Crassostrea gigas, by LC-MS/MS was developed.
- *In vivo* experiment, E6 was proved to have the ability of penetration through blood-brain barrier.

First Edition: May, 2015

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