

## Liquid chromatography-tandem mass spectrometric analysis of oleracone D and its application to pharmacokinetic study in mice

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**Abstract:** We have demonstrated a sensitive analytical method of measuring oleracone D in mouse plasma using a liquid chromatography-tandem mass spectrometry (LC-MS/MS). Oleracone D and oleracone F (internal standard) in mouse plasma samples were processed using a liquid-liquid extraction method with methyl *tert*-butyl ether, resulting in high and reproducible extraction recovery (80.19-82.49 %). No interfering peaks around the peak elution time of oleracone D and oleracone F were observed. The standard calibration curves for oleracone D ranged from 0.5 to 100 ng/mL and were linear with  $r^2$  of 0.992. The inter- and intra-day accuracy and precision and the stability fell within the acceptance criteria. The pharmacokinetics of oleracone D following intravenous and oral administration of oleracone D at doses of 5 mg/kg and 30 mg/kg, respectively, were investigated. When oleracone D was intravenously injected, it had first-order elimination kinetics with high clearance and volume of distribution values. The absolute oral bioavailability of this compound was calculated as 0.95 %, with multi-exponential kinetics. The low aqueous solubility and a high oral dose of oleracone D may explain the different elimination kinetics of oleracone D between intravenous and oral administration. Collectively, this newly developed sensitive LC-MS/MS method of oleracone D could be successfully utilized for investigating the pharmacokinetic properties of this compound and could be used in future studies for the lead optimization and biopharmaceutic investigation of oleracone D.

**Key words:** oleracone D, LC-MS/MS, pharmacokinetics, plasma stability, protein binding

### 1. Introduction

*Portulaca oleracea* L. is a well-known annual plant found distributed in temperate, tropical and subtropical

regions.<sup>1</sup> It has been used for some time in many countries for the alleviation of various diseases due to its pharmacological effects that include anti-inflammatory,<sup>2-4</sup> antioxidative,<sup>5,6</sup> anti-tumor,<sup>7</sup> antiaging,

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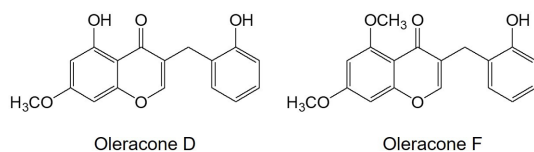


Fig. 1. Chemical structure of oleracone D and oleracone F (internal standard, IS).

and hypolipidemic effects.<sup>1,5</sup> These effects may be due to the flavonoid constituents of *Portulaca oleracea* L.<sup>8-11</sup> Yang *et al.*<sup>9,10</sup> were able to isolate a novel series of flavonoids, named oleracone C, D, E, and F (Fig. 1), from *Portulaca oleracea* L. and investigated their activities. Comparing the antioxidant activity of these oleracones by evaluating their radical scavenging effect, oleracone D was the most effective among the tested oleracones.<sup>5,9,10</sup> Oleracone D also had the highest anticholinesterase activity; however, oleracone C and E also had anticholinesterase activity with lower inhibitory efficacy.<sup>10</sup> Oleracone E and F had a similar lifespan extension potential from the lifespan assay using age-synchronized nematodes.<sup>5</sup> The results demonstrated that these oleracone derivatives have potent antioxidative activity and may exhibit a therapeutic potential on the aging process. For further assays for investigating its activity and toxicity, the development of an analytical method for monitoring oleracone D concentration in a biological matrix is a necessary progression.

Pharmacokinetics of oleracone C in rats and the analytical detection method for plasma oleracone C concentrations have previously been reported.<sup>2</sup> The plasma concentration of oleracone C had an elimination half-life ( $T_{1/2}$ ) of 0.3 h and showed low oral bioavailability of 8.3%. For the determination of plasma oleracone C, the liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed in the standard range of 10-2000 ng/mL, with a volume of 100  $\mu$ L of rat plasma samples processed using four volumes of methanol precipitation method.<sup>2</sup> Although oleracone D had better *in vitro* antioxidative and neuroprotective effects than those of oleracone C, the analytical method and pharmacokinetic study of oleracone D have not been previously reported.

Therefore, this study aimed to develop and validate a sensitive and reproducible LC-MS/MS method for measuring concentrations of oleracone D and apply this analytical method to a pharmacokinetic study of oleracone D in mice.

To achieve a substantial extraction recovery and negligible matrix effect from a biological matrix such as plasma, the most widely used sample preparation methods are protein precipitation (PPT) and liquid-liquid extraction (LLE).<sup>12</sup> Since LLE has an advantage in lowering interferences from the sample matrix and increasing the sensitivity of the analyte,<sup>12,13</sup> LLE method was, therefore, utilized in this study for the sample preparation method for the analysis of oleracone D in 30  $\mu$ L of mouse plasma samples. Additionally, we aimed to validate the analytical method for its linearity, selectivity, accuracy, precision, stability, recovery, and matrix effects according to the U.S. Food and Drug Administration Guideline for Bioanalytical Method.<sup>14</sup>

## 2. Experimental

### 2.1. Chemicals and reagents

Oleracone D and oleracone F (Fig. 1) were synthesized with a purity of >99.0% using a previously described method by Yoon *et al.*<sup>5</sup> The purity was confirmed using nuclear magnetic resonance spectroscopy and mass spectroscopy and oleracone F was used as the internal standard (IS). Acetonitrile, water, and methanol were purchased from Tedia (Fairfield, CT, USA). Formic acid and methyl *tert*-butyl ether (MTBE) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MA, USA). All solvents and chemicals were of HPLC and of reagent grade.

### 2.2. Preparation of the stock and working solutions

Stock solutions were prepared individually by dissolving oleracone D and oleracone F in methanol at concentrations of 1 mg/mL. The oleracone D working solutions were prepared by serial dilution of the stock solution with acetonitrile to final concentrations of 0.5, 1, 2, 5, 20, 50, 100 ng/mL for the calibration standards and 1.5, 10, and 75 ng/mL for the quality control (QC)

samples. The oleracone F solution was prepared at a concentration of 5 ng/mL, with a dilution of the stock solution with water. The stock and working solution of oleracone D were stored at -80 °C during the analysis.

### 2.3. Preparation of calibration standards and quality control samples

A volume of 30 µL of the working solution for calibration standards and QC samples were evaporated and reconstituted with a 30 µL aliquot of blank mouse plasma. The final concentrations of calibration standards were 0.5, 1, 2, 5, 20, 50, 100 ng/mL and 1.5 (low QC), 10 (middle QC), 75 (high QC) ng/mL, respectively, for the QC samples.

### 2.4. Sample preparation

The calibration standards and QC samples (30 µL) were combined with 20 µL of oleracone F solution (5 ng/mL in water) and 400 µL of MTBE. The mixture was vigorously vortexed for 15 min and subsequently centrifuged at  $16,000 \times g$  for 5 min. The supernatant was transferred to a clean tube and evaporated to dryness until dry using a gentle stream of nitrogen. The residue was reconstituted in 200 µL of mobile phase, and subsequently, a 10 µL aliquot of the solution was injected into the LC-MS/MS system.

### 2.5. Instrument conditions

The LC system was an Agilent Infinity 1260 Infinite II HPLC system (Agilent Technologies, Santa Clara, CA, USA), and chromatographic separation was carried out using a Luna C18 (150 × 2.0 mm, 5 µm; Phenomenex, Torrance, CA, USA). The mobile phase was pumped with isocratic elution of water that contained 0.1 % formic acid : acetonitrile containing 0.1 % formic acid (20:80, v/v).

The flow rate was set to 0.2 mL/min with a column temperature maintained at 30 °C. The Agilent 6430 triple quadrupole MS was equipped with an electrospray ionization source and was used for mass spectrometric detection and quantitative analysis. The mass spectrometer was operated in the positive ion mode, with multiple reaction monitoring (MRM) transitions at  $m/z$  299.1→205.0 for oleracone D and at  $m/z$  313.0

→219.1 for oleracone F (IS) with optimized fragmentor of 105 or 140 V, and collision energy of 20 eV, respectively.

### 2.6. Method validations

#### 2.6.1. Selectivity and linearity

To determine the selectivity was determined by a comparison of the chromatogram of the blank plasma from six different mice and corresponding mouse blank plasma containing a lower limit of quantification (LLOQ) samples (0.5 ng/mL of oleracone D) and IS was performed.

The calibration standard curve for oleracone D (0.5-100 ng/mL) was obtained from the ratio of the peak areas of oleracone D and IS and plotted to the concentrations of oleracone D. The results were fitted using a least square linear regression analysis.

#### 2.6.2. Precision and accuracy

Two sets of QC samples (low, middle, and high QC) on five independent days were used to determine the inter-day precision and accuracy. Intra-day precision and accuracy were determined by analyzing six sets of QC samples (low, middle, and high QC) on the same days. The precision was expressed as the coefficient of variance (CV, %), whereas accuracy was expressed as the percentage of the measured QC concentration to the nominal QC concentration.

#### 2.6.3. Matrix effect and recovery

The matrix effect of oleracone D was determined by comparing the peak area obtained from the post-extraction blank plasma that was spiked with three QC concentrations of oleracone D and with the peak area from the corresponding concentration of the neat solution. The matrix effect of IS was determined in the same manner as the procedure of oleracone D using a 5 ng/mL concentration of IS.

The extraction recovery of oleracone D was determined by the comparison of the peak area obtained from the pre-extraction samples spiked with three QC concentrations of oleracone D, with that of the post-extraction blank plasma spiked with corresponding QC concentrations of oleracone D. The extraction

recovery of IS was determined in the same manner using the procedure of oleracone D using the concentration of 5 ng/mL of IS.

#### 2.6.4. Stability

The stability of oleracone D in the mouse plasma was determined from the three sets of QC samples (low and high QC) under various conditions. The bench-top stability was determined by incubating the QC samples at 25 °C for 6 h. Thereafter, the concentrations of the QC samples were determined using freshly prepared calibration standards. For the freeze-thaw stability, QC samples were analyzed after three cycles of freeze-thawing. One cycle of the freeze-thaw process involved storing the QC samples at -80 °C for over 12 h and thawing at 25 °C for 6 h. After undergoing three cycles of the freeze-thaw process, the concentrations of the QC samples were determined using freshly prepared calibration standards. The autosampler stability was evaluated by placing the extracted QC samples in the autosampler at 6 °C for 24 h, and the subsequently determined concentrations were calculated using freshly prepared calibration standards. The long term stability of oleracone D stock solution was evaluated by comparison the oleracone D stock solution stored at -80 °C for 6 months with freshly prepared oleracone D stock solution. Both oleracone D stock solutions were diluted to 500 ng/mL using mobile phase and compared the peak area of corresponding oleracone D stock solutions.

#### 2.7. Pharmacokinetic study

All experiments involving animals and the utilized protocols were approved by the Animal Care and Use Committee of the Kyungpook National University (No. 2019-0126) and were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.<sup>15</sup>

Male ICR mice (7-8 weeks old, 30-35 g) were purchased from Samtako (Osan, Korea) and acclimated to the animal facility of Kyungpook National University for 1 week with *ad libitum* access to food and water and fasted for 12 h prior to the pharmacokinetic experiments. After the intravenous administration of

oleracone D (5 mg/kg dissolved in 2 mL mixture of DMSO : saline = 20 : 80 [v/v]) via the tail vein, blood samples were collected via the Retro-Orbital plexus using a heparinized collection tube at 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h while mice were under anesthesia with isoflurane for 5 min. After the oral administration of oleracone D (30 mg/kg suspended in 5 mL of 0.5 % carboxymethyl cellulose suspension) using oral gavage, blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h, as described above. All blood samples were centrifuged at 16,000 × g for 1 min to separate plasma, and the plasma sample was stored at -80 °C until analysis. The plasma samples (30 µL) were added to 20 µL of IS solution (5 ng/mL oleracone F in water) and 400 µL of MTBE. The mixture was vigorously vortexed for 15 min and then centrifuged at 16,000 × g for 5 min. The supernatant was transferred to a clean tube and evaporated under dry using a gentle stream of nitrogen. The residue was reconstituted in 200 µL of mobile phase, and subsequently, 10 µL aliquot of the solution was injected into the LC-MS/MS system.

#### 2.8. Plasma protein binding

Protein binding of oleracone D (75 ng/mL, high QC) in mouse plasma was determined using a rapid equilibrium dialysis kit (ThermoFisher Scientific Korea, Seoul, Korea) according to the manufacturer's protocol.<sup>7</sup> Briefly, 100 µL of mouse plasma containing 75 ng/mL oleracone D was added to the inner sample chamber of a semipermeable membrane (molecular weight cut-off 8,000 Da). To the outer chamber, 300 µL of phosphate buffered saline (PBS) was added. The samples were then incubated for 4 h at 37 °C on a shaking incubator at 300 rpm, followed by 30 µL aliquot being removed from both the sample and buffer chambers. Samples were treated with equal volumes of fresh PBS or blank plasma to match the sample matrices. The matrix-matched samples (60 µL) were mixed with 40 µL of IS solution (5 ng/mL oleracone F in water) and 800 µL of MTBE. The mixture was vigorously vortexed for 15 min and subsequently centrifuged at 16,000 × g for 5 min. The supernatant was transferred to a clean tube and

evaporated under dry using a gentle stream of nitrogen. The residue was reconstituted in 200  $\mu\text{L}$  of mobile phase, and subsequently, 10  $\mu\text{L}$  aliquot of the solution was injected into the LC-MS/MS system.

### 2.9. Plasma stability

The determination of the plasma stability of oleracone D (75 ng/mL, high QC) in mouse plasma was conducted.<sup>6</sup> Briefly, 100  $\mu\text{L}$  of mouse plasma containing 75 ng/mL oleracone D was incubated for 8 h at 37  $^{\circ}\text{C}$  on a shaking incubator at 300 rpm. From this, 30  $\mu\text{L}$  aliquot was taken and added to 20  $\mu\text{L}$  of oleracone F solution (5 ng/mL in water) and 400  $\mu\text{L}$  of MTBE. The mixture was vigorously vortexed for 15 min and then centrifuged at 16,000  $\times g$  for 5 min. The supernatant was transferred to a clean tube and evaporated under dry using a gentle stream of nitrogen. The residue was reconstituted in 200  $\mu\text{L}$  of mobile phase, and subsequently, 10  $\mu\text{L}$  aliquot of the solution was injected into the LC-MS/MS system.

### 2.10. Data analysis and statistics

Calculation of the pharmacokinetic parameters was conducted using WinNonlin 5.1 software (Pharsight Co., Mountain View, CA, USA). All data are expressed as the mean  $\pm$  standard deviation (SD).

## 3. Results and Discussion

### 3.1. LC-MS/MS analysis of oleracone D

For optimization of the electrospray ionization conditions of oleracone D and IS, each compound was injected directly into the mass spectrometer ionization source. The oleracone D and IS had optimal ionization in the positive mode. The MRM transition of oleracone D was selected from the precursor ion ( $[\text{M}+\text{H}]^+$ ,  $m/z$  299.1) and the most frequent product ion ( $m/z$  205.0). The MRM transition of IS was selected from the precursor ion ( $[\text{M}+\text{H}]^+$ ,  $m/z$  313.0) and the most frequent product ion ( $m/z$  219.1) (Fig. 2).

### 3.2. Selectivity and linearity

Fig. 3 shows the typical chromatograms of double blank, zero blank, LLOQ sample (0.5 ng/mL), and plasma sample at 8 h after intravenous administration of oleracone D. The retention times for oleracone D and IS were 3.4 and 2.7 min, respectively. The signal-to-noise (S/N) ratio of oleracone D was  $> 10.0$  in the LLOQ samples, with no significant matrix interference at the retention times of oleracone D and IS in the blank samples, compared to the LLOQ samples, when observing the chromatograms (Fig. 3).

A calibration standard curve was linear within the

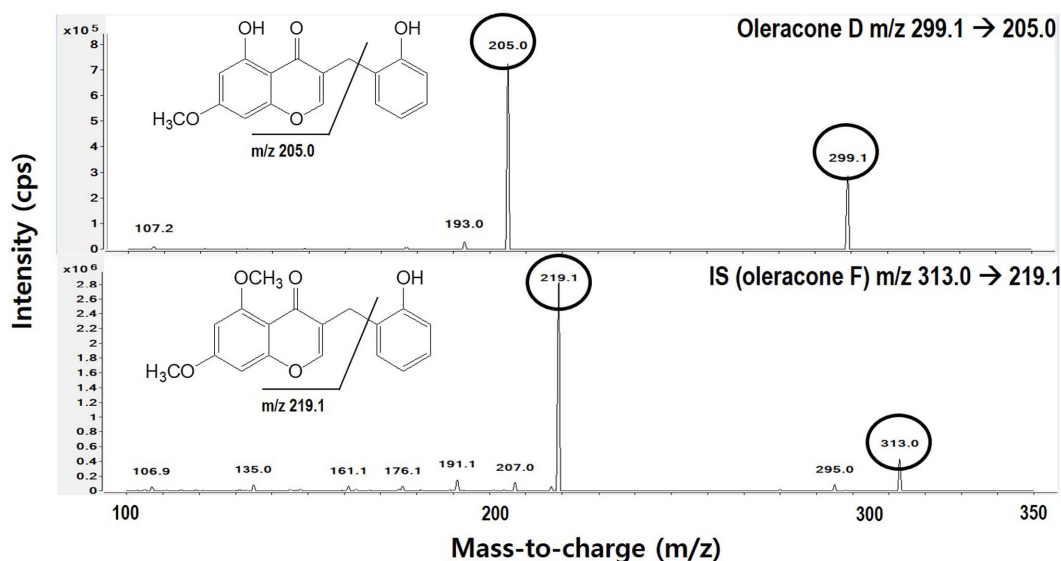


Fig. 2. Product ion scan of oleracone D and IS (oleracone F).

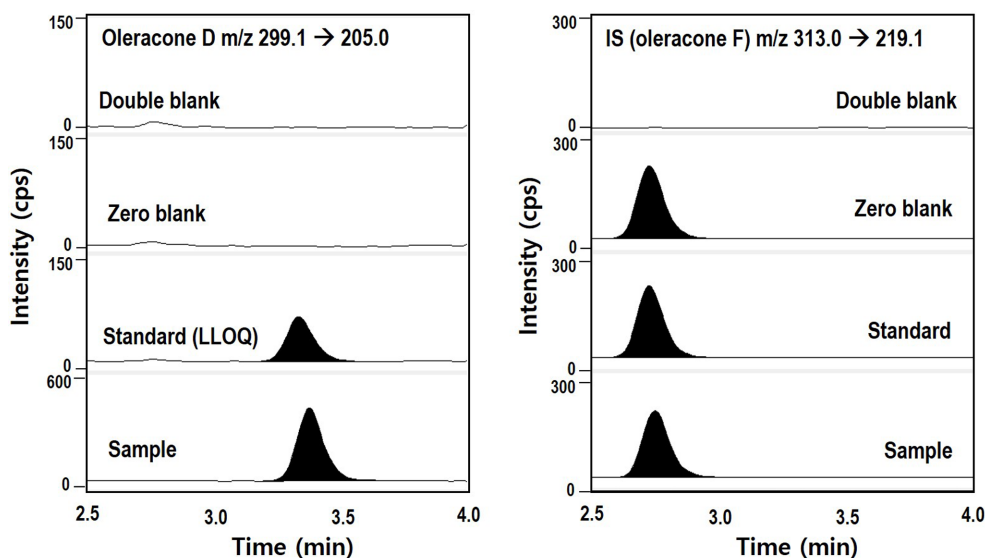


Fig. 3. Representative multiple reaction monitoring (MRM) chromatograms of oleracone D and IS (oleracone F) in mouse plasma of double blank, zero blank, lower limit of quantification (LLOQ) sample (0.5 ng/mL), and plasma sample at 8 h following intravenous injection of oleracone D.

Table 1. Calculated concentrations of oleracone D in calibration standards

Variables	Nominal concentrations (ng/mL)							Slope	$r^2$
	0.5	1	2	5	20	50	100		
Mean (ng/mL)	0.51	0.98	1.93	4.66	19.53	52.89	106.34	0.980	0.992
Accuracy (%)	102.7	97.6	96.7	93.3	97.6	105.8	106.3	-	-
CV (%)	5.3	8.7	5.7	6.7	7.2	9.5	4.1	8.08	0.83

Data represented as mean  $\pm$  SD (n = 6).

Table 2. Intra- and inter-day precision and accuracy of oleracone D

	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Intra-day (n=6)	1.5	1.34 $\pm$ 0.06	4.30	89.38
	10	9.35 $\pm$ 0.79	8.43	93.50
	75	81.32 $\pm$ 4.53	5.58	108.43
Inter-day (n=5)	1.5	1.34 $\pm$ 0.06	4.24	89.48
	10	9.08 $\pm$ 0.55	6.10	90.82
	75	72.38 $\pm$ 5.36	7.41	96.49

Data represented as mean  $\pm$  SD.

oleracone D concentration ranges of 0.5-100 ng/mL, and a coefficient of determination ( $r^2$ ) was 0.992 determined from linear regression analysis, with a weighting of  $1/\text{concentration}^2$  (Table 1).

### 3.3. Precision and accuracy

Table 2 summarizes the intra- and inter-day precision and accuracy for oleracone D from three levels of QC samples. The intra- and inter-day precision was from 4.24 to 8.43% for oleracone D and the intra-

Table 3. Extraction recoveries and matrix effects of oleracone D and IS

Analyte	Nominal concentration (ng/mL)	Extraction recovery (%)	CV (%)	Matrix effects (%)	CV (%)
Oleracone D	1.5	80.88 ± 3.17	3.91	89.21 ± 2.25	2.53
	10	82.49 ± 7.80	9.46	89.28 ± 4.16	4.66
	75	80.19 ± 4.74	5.91	106.23 ± 5.00	4.71
IS	5	94.54 ± 8.83	9.34	106.88 ± 10.14	9.49

Data represented as mean ± SD (n=3).

and inter-day accuracy was from 89.38 to 108.43%, which fell in the acceptable criteria (less than 15%).

### 3.4. Matrix effect and recovery

The results of the extraction recoveries and matrix effects are summarized in Table 3. The extraction recoveries for oleracone D were calculated at three levels of QC samples and were high and reproducible in the range of extraction recovery from 80.19 to 82.49% and CV from 3.91 to 9.46%. Therefore, the LLE method utilized in this study was capable of efficiently extracting oleracone D from the mouse plasma. The matrix effects ranged from 89.21 to 106.23%, suggesting that the coeluting substances did not interfere with the ionization of oleracone D. The low CV in the matrix effect indicated that there were no significant differences for the peak areas of oleracone D in the three concentrations of the three samples of each plasma matrix. Therefore, it was possible to exclude any matrix effect on ion suppression or enhancement. The extraction recovery and matrix effects of IS was also high and reproducible.

### 3.5. Stability

In Table 4, the results of stability experiments are presented. The accuracy of oleracone D ranged from 92.46% to 107.73% and the precision ranged from 2.33% to 5.96% for the three different conditions of stability test. As there was no significant difference observed for each measured concentration compared with nominal concentration under various stability treatment conditions, these results provided evidence that the oleracone D in the mouse plasma was stable up to 6 h at 25 °C for bench-top stability, and was stable for 24 h in the autosampler stability after

Table 4. Stability of oleracone D

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Bench-top stability (at 25 °C for 6 h)			
1.5	1.54 ± 0.04	2.38	102.78
75	76.83 ± 4.58	5.96	102.44
Freeze-thaw stability (3 cycles, from -80 °C for 12 h to 25 °C for 6 h as one cycle)			
1.5	1.49 ± 0.08	5.61	99.59
75	69.35 ± 1.62	2.33	92.46
Autosampler stability (at 6 °C for 24 h)			
1.5	1.61 ± 0.09	5.36	107.51
75	80.79 ± 2.93	3.63	107.73

Data represented as mean ± SD (n=3).

sample treatment, and also remained stable over three freeze-thaw cycles. The peak area for oleracone D stock solution stored at -80 °C for 6 months was 5025.67 ± 170.12 (n = 3) and that of freshly prepared oleracone D stock solution was 5447.22 ± 96.92 (n = 3). The mean peak area ratio was calculated as 92.26%, suggesting that the oleracone D stock solution was stable for storing at -80 °C for more than 6 months.<sup>16</sup> However, we should note that the long term plasma stability of oleracone D need to be evaluated for further preclinical studies using oleracone D.

### 3.6. Pharmacokinetics of oleracone D

Temporal profiles for the plasma concentration of oleracone D after intravenous and oral administration are shown in Fig. 4, and the relevant pharmacokinetic parameters are detailed in Table 5. The plasma concentration of oleracone D after intravenous injection demonstrated first-order elimination kinetics, with an

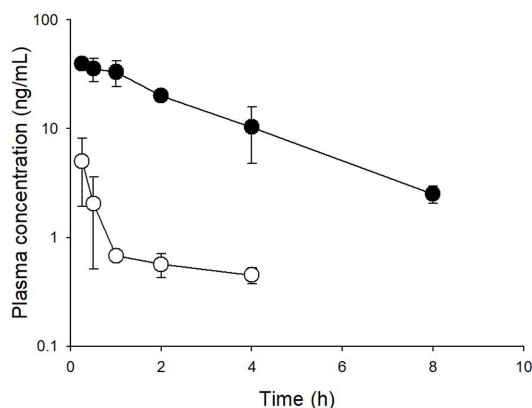


Fig. 4. Plasma concentration-time profile of oleracone D in mice following an intravenous injection of oleracone D at a dose of 5 mg/kg (●) and an oral administration of oleracone D at a dose of 30 mg/kg (○). Data are expressed as mean ± SD (n=3).

Table 5. Pharmacokinetic parameters of oleracone D in mice following intravenous injection or oral administration of oleracone D

Parameters	IV (5 mg/kg)	PO (30 mg/kg)
C <sub>0</sub> (ng/mL)	45.6 ± 5.5	-
C <sub>max</sub> (ng/mL)	-	5.0 ± 3.1
T <sub>max</sub> (h)	-	0.25 ± 0.0
AUC <sub>8h</sub> (ng·h/mL)	119.9 ± 14.8	5.1 ± 1.6
AUC <sub>∞</sub> (ng·h/mL)	127.1 ± 14.8	7.2 ± 1.5
T <sub>1/2</sub> (h)	1.9 ± 0.3	3.9 ± 1.2
MRT (h)	2.6 ± 0.5	5.4 ± 1.1
CL (L/h/kg)	39.7 ± 4.5	-
Vd (L/kg)	104.8 ± 22.7	-
BA (%)	-	0.95

C<sub>0</sub>: initial plasma concentration; AUC<sub>8h</sub> or AUC<sub>∞</sub>: Area under plasma concentration-time curve from zero to 8h or infinity; T<sub>1/2</sub>: elimination half-life; MRT: mean residence time; CL: clearance (Dose/AUC); Vd: volume of distribution (MRT×CL)

BA: Bioavailability (Dose normalized AUC<sub>PO</sub>/dose normalized AUC<sub>IV</sub>×100)

Data are expressed as mean ± SD (n=3).

elimination half-life of 1.9 ± 0.3 h. However, oleracone D had high clearance (CL) and volume of distribution (Vd) in mice, resulting in low plasma exposure, even with intravenous administration of oleracone D. In addition, the results suggested that oleracone D may undergo substantial metabolism or distribution even though the underlying mechanism requires further

Table 6. Physicochemical properties of oleracone D

Parameters	Mean ± SD (n=3)
Log P <sup>a</sup>	3.4
Solubility (μg/mL)	84.5 ± 0.4
Plasma stability for 8 h (%)	96.1 ± 1.8
Protein binding (%)	99.6 ± 0.2

<sup>a</sup>LogP value was obtained from Chemdraw data base

investigation. As oleracone D was stable for 8 h in the plasma and had very high plasma protein binding (99.6 ± 0.2%) (Table 6), the plasma instability could be excluded from the contributing factor for high CL and Vd of oleracone D in mice.

The plasma concentration of oleracone D following oral administration reached C<sub>max</sub> within the initial sampling times (i.e. T<sub>max</sub> of 0.25 h), providing evidence that the gastrointestinal absorption of oleracone D is rapid, but was eliminated by multiexponential kinetics. Considering its rapid intestinal absorption, it had better collect plasma samples at earlier time points to obtain the accurate kinetic parameters for C<sub>max</sub> and T<sub>max</sub>. However, very low levels of the plasma concentrations of oleracone D after oral administration maintained even with high doses of oleracone D within the mice (30 mg/kg), resulted in low oral bioavailability of 0.95%.

We next investigated the different elimination kinetics of intravenous and oral administration, by measuring the aqueous solubility of oleracone D (Table 6). Its solubility (84.5 ± 0.4 mg/mL) was much lower than the oral suspension (6 mg/mL). A dissolved fraction of oleracone D could be absorbed quickly due to its lipophilicity (LogP of 3.4); however, undissolved oleracone D in the intestine could be absorbed steadily when dissolved in the gastric fluid, which possibly increased the half-life of oleracone D compared with intravenous administration.

In agreement with our findings, Yang *et al.*<sup>2</sup> reported that plasma concentrations of oleracone C, a structural analog of oleracone D, eliminated quickly, with a half-life of 0.35 h, and also showed the low oral bioavailability (8.3%) in rats following intravenous and oral administration (3 mg/mL).



#### 4. Conclusions

We have developed and validated a sensitive analytical method for oleracone D using an LC-MS/MS system through inter-day and intra-day precision and accuracy, matrix effect, extraction recovery, and stability tests. We have successfully investigated the pharmacokinetic characteristics and oral bioavailability of oleracone D in mice following its intravenous and oral administration using our validated and sensitive analytical method. Although the biological activities of oleracone D on the antioxidative and antiaging effects, it showed low oral bioavailability, high CL, and Vd values, which was partly attributed to its low aqueous solubility.

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