Elsevier Editorial System(tm) for Journal of Chromatography B
Manuscript Draft

Manuscript Number: JCB-14-234R1

Title: Development of a UPLC-MS/MS Bioanalytical Method for the Pharmacokinetic Study of (-)-Epiafzelechin, a Flavan-3-ol with Osteoprotective Activity, in C57BL/6J Mice

Article Type: Full Length Article

Keywords: (-)-epiafzelechin, intravenous, intraperitoneal, pharmacokinetic, UPLC-MS/MS, bioanalytical method

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Development of a UPLC-MS/MS Bioanalytical Method for the Pharmacokinetic Study of (-)-*Epi*afzelechin, a Flavan-3-ol with Osteoprotective Activity, in C57BL/6J Mice

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Abstract:

(-)-*Epi*afzelechin is a flavan-3-ol commonly found in plant source. Biological studies suggested that (-)-*epi*afzelechin may have anti-inflammatory, anti-oxidant and bone protective effect. However, its *in vivo* efficacy remains to be demonstrated. A specific detection method for (-)-*epi*afzelechin was successfully developed by using UPLC-MS/MS to quantify the amount of (-)-*epi*afzelechin presence in mice plasma after a liquid-liquid extraction by ethyl acetate. The separation was achieved by using a reversed phase C18 column with a 16 minutes gradient elution protocol consisting water (0.1% v/v formic acid) and 0-70% ACN (0.1% v/v formic acid). The lower limit of quantitation for (-)-*epi*afzelechin was found to be 12.5 ng/ml. This method exhibited a good linearity (r^2 = 0.992). The intra-day and inter-day precision were within 12 % while the accuracy was between 97.6% and 113.4%. A quantity of 10 mg/kg synthetic (-)-*epi*afzelechin were administrated to C57BL/6J mice by intravenous (i.v.) and intraperitoneal (i.p.) injections and the blood was collected at different time points. The plasma was then analyzed by the UPLC-MS/MS method and the plasma drug concentration-time curves for i.v. and i.p (-)-*epi*afzelechin injection were constructed. The maximum concentrations (C_{max}) of (-)-*epi*afzelehin in blood by i.v. and i.p. injection were found to be 12.7 μg/ml and 5.8 μg/ml, respectively, while the time for reaching C_{max} in i.p. injection was found to be 15 minutes. The distribution half-lives of (-)-*epi*afzelechin after i.v. and i.p. injection were found to be 7.2 minutes and 12.4 minutes, respectively. Some of the PK parameters were found to be similar in both i.v. and i.p. injections of (-)-*epi*afzlechin owing to its high solubility in water.

Keywords: (-)-*Epi*afzelechin, intravenous, intraperitoneal, pharmacokinetics, UPLC-MS/MS

1. Introduction:

(-)-*Epi*afzelechin (**1**, Figure 1) is a flavan-3-ol found in plants such as *Celastrus orbiculatus, Cassia sieberiana, Camellia sinensis, Typha capensis* and *Drynariae fortunei* [1-4]. Many of these plants have been used in herbal medicine. Biological studies of (-)-*epi*afzelechin suggested that it possesses bone protective [2, 4], anti-inflammatory [1] and anti-oxidative effects [5, 6]. Chemical structural identification and characterization also suggested that (-)-*epi*afzelchin derivatives and its oligomers could exert different biological activities [2, 7, 8], and (-)-*epi*afzelechin is the building block of these active compounds. An example is the traditional Chinese medicine Gu-Sui-Bu [*Drynaria fortunei* (kunze) J. Sm] which has been commonly used to manage bone diseases [9]. Extract of Drynaria Rhizoma has been found to have stimulating effect on the proliferation and differentiation of osteoblastic MC3T3-E1 cells [10] and improve the bone mineral density of ovariectomized (OVX) mice [11]. Through a bioassay-guided separation, several flavan-3-ols including (-)-*epi*afzelechin itself and its derivatives were isolated from the rhizomes of *D. fortunei* [12]. These (-)-*epi*afzelechin derivatives showed potent proliferative effects on ROS 17/2.8 osteoblastic cells in a concentration range of 10^{-15} to 10^{-9} M, comparable to that of 17β-estradiol. (-)-*Epi*afzelechin was also isolated from Huangshan Maofeng tea and found to stimulate the cell proliferation and differentiation rate of rat osteoblasts, as well as significantly increase the area of mineralization of bone nodules [3]. However, these studies mainly focused on the *in vitro* effects of (-)-*epi*afzelechin. To the best of our knowledge, there has not been any *in vivo* demonstration of the osteoprotective effects of (-)-*epi*afzelechin or its derivatives. This may be partly due to the fact that isolation of these active components from natural source is quite tedious and the amounts are limited [12]. We have been interested in the chemistry and biochemistry of (-)-*epi*afzelechin and its derivatives. In 2004, we reported on the enantioselective synthesis of afzelechin and *epi*afzelechin [13]. Recently, we also reported on the chemical synthesis and biological study of 4β-carboxymethyl-*epi*afzelechin acid, an osteoprotective compound from the rhizomes of *Drynaria fortunei* [14]. Thus, we are in a position to have pure (-)-*epi*afzelechin or its derivatives by total synthesis. Our long term objective is to examine the *in vivo* osteoprotective effects of these compounds.

In this study, a bioanalytical method for quantifying (-)-*epi*afzelechin in mice plasma by ultra performance liquid chromatography coupled to triple quadrupole mass spectrometer (UPLC-MS/MS) has been developed and validated. This facilitated the pharmacokinetic (PK) study from intravenous (i.v.) and intraperitoneal (i.p.) administrations of (-)-*epi*afzelechin to mice. It is expected that the validated bioanalytical method and the PK profile could be useful for future *in vivo* efficacy study of (-)-*epi*afzelechin.

2. Materials and method:

2.1 Chemicals and reagents

Acetonitrile (ACN), ethanol (EtOH), ethyl acetate (EA), hexane (HEX), isopropyl alcohol (IPA) and methanol (MeOH) were purchased from Tedia (Tedia Co, OH, USA). Deionized water was purified by Mini-Q A10 Ultrapure Water Purification System (Millipore, MA, USA). (+)-Catechin (**2**, Figure 1), ethylenediaminetetraacetic acid (EDTA) and formic acid were purchased from Sigma Aldrich (Sigma, MO, USA). (-)-*Epi*afzelechin was synthesized according to literature procedures and characterized by ESI-MS and NMR spectroscopy [12]. The enantiomeric purity of (-)-*epi*afzelechin was verified by high performance liquid chromatography (Model 1100, Agilent, USA) equipped with analytical chiral AD column (Ø4.6mm). The flow rate was kept at 1 mL/min and the mobile phase was maintained at 70:30 of HEX and IPA for isocratic elution. The synthetic (-)-*epi*afzelechin was analyzed through a diode array detector at the wavelength of 220 nm. The enantiomeric excess was found to be 73%.

2.2 UPLC-MS/MS conditions

Waters acquity H class ultra-high performance liquid chromatography coupled to Micromass ultimate quattro triple quadrupole mass spectrometer (Waters, MA, USA) was used to detect the analyte. Waters acquity UHPLC BEH C18 (1.7 μm, 2.1 x 50 mm) column was used in the stationary phase while water (0.1% formic acid v/v) and ACN (0.1% formic acid v/v) were used in the mobile phase. The flow rate was kept at 0.4 mL/min. During separation, a gradient elution protocol was used as follow: 0-1 min 100% water; elution gradient: 1-11 min 0 → 70% ACN; and regeneration: 11-16 min 100% water. The sample was dissolved in MeOH and kept in the auto sampler at 4 ^{o}C. 2 μL of sample was injected for analysis. MS was operated in positive electrospray ionization (+ESI) mode with the supply of 150 L/h cone gas, 550 L/h desolvation gas, 150 ^{o}C source temperature, 350 ^{o}C desolvation temperature, 30 V cone voltage and 3 kV of capillary voltage. The collision voltage was configured at -18 eV with C.I.D. argon gas pressurized at 5.5 x 10^{-5} Torr. The ions were monitored by multiple reaction monitor (MRM) mode for (+)-catechin (m/z 291 → 139) and (-)-*epi*afzelechin (m/z 275 → 139).

2.3 Standard solution preparation

(+)-Catechin and (-)-*epi*afzelechin stock solutions at 10 mg/ml were freshly prepared by dissolving 10 mg of (+)-catechin and (-)-*epi*afzelechin, respectively, in 1 ml of MeOH and stored at -20 ^{o}C. Their working solutions were prepared by serial dilution using MeOH.

2.4 Animal care and pharmacokinetic study

One hundred and twenty young female C57BL/6J (20-25 g) mice were obtained from the University of Hong Kong and used for this study. Mice were housed at the centralized animal facilities in the Hong Kong Polytechnic University with a 12 hours light dark cycle. The housing

temperature and relative humidity were controlled at 22 ^{o}C and 55%, respectively. The animals were freely accessed to water and diet. All experimental procedures were approved by the Animal Ethics Committee of the Hong Kong Polytechnic University. (-)-*Epi*afzelechin was freshly weighed and prepared in PBS at 2.5 mg/mL solution and filtered by 0.22 μm syringe filter. The mice were administered with 10 mg/kg of (-)-*epi*afzelechin injection solution via i.v. or i.p. injection and the blood samples were collected at 5, 10, 15, 30, 60, 120, 240, 360 and 720 minutes. Five animals (n=5) were used in each time point of the pharmacokinetic study. Five minutes before blood sampling, the mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine via i.p. injection. The animals were then fallen into deep sleep and lost consciousness. The blood was collected by cardiac puncture using 1 cc syringe with 25 gauge needle. The animal was then sacrificed by cardiac stick exsanguinations after most of the blood was drawn. The blood samples were mixed with EDTA and centrifuged for 15 minutes at 3,500 x g to yield plasma. The plasma was kept at -20 ^{o}C for further UPLC-MS/MS analysis after extraction. The pharmacokinetic parameters were calculated by Summit PK solution v2.0.3.

2.5 Plasma sample extraction

A 5μL solution of 5 μg/ml (+)-catechin as internal standard (IS) was mixed with 50 μL of plasma sample by vortex. Afterwards, an amount of 100 μL of EA was added to 50 μL of plasma and vortexed for 30 sec. The mixture was centrifuged at 3,500 x g for 5 min. The supernatant was collected by a new eppendorf tube and the pellet was extracted again by 100 μL of EA for two times to yield 300 μL supernatant. The supernatant was dried by stream of dry air and re-dissolved in 50 μL of methanol for UPLC-MS/MS analysis.

2.6 Method validation

The method for determining (-)-*epi*afzelechin was validated according to the guidance from US Food and Drug Administration [15] and International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) [16]. The quality controls (QC) were prepared by mixing blank plasma with serially diluted (-)-*epi*afzelechin solution at desired concentrations and 500 ng/ml of IS.

2.6.1 Calibration curve:

(-)-*Epi*afzelechin standard solutions at low concentration range: 0, 12.5, 25, 50, 100, 500 and 1000 ng/ml; and high concentration range 0, 500, 1000, 1250, 2500 and 5000 ng/ml were prepared by mixing suitable amount of serially diluted (-)-*epi*afzelechin solution in blank plasma solution containing 500 ng/ml IS. These standard solutions were analyzed by UPLC-MS/MS after extraction. The results were analyzed by (1) least squares method without passing through the origin, (2) least squares method passing through the origin, and (3) $1/y^2$ weighted least squares linear regression. The least squares linear regression was constructed by Prism4 and the weighted least squares linear regression was constructed by SPSS v7.0.

2.6.2 Accuracy and precision:

QC containing 12.5, 50, 250 and 1000 ng/ml of (-)-*epi*afzelechin with 500 ng/ml IS were analyzed by UPLC-MS/MS after extraction for five times at the same day. The % coefficient variation (CV) of the five determinations was calculated for the intra-batch precision. The same experiment was repeated in another day with another batch of QC samples for inter-day precision determination. The intra-day and intra-day accuracy were determined by comparing the actual value and the value obtained from the calibration curve.

2.6.3 Recovery:

QC containing 50, 250 and 1000 ng/ml of (-)-*epi*afzelechin with 500 ng/ml of IS were extracted according to the procedure for plasma sample extraction. The amounts of extracted and non-extracted (-)-*epi*afzelechin in the same extracted plasma matrix were analyzed by UPLC-MS/MS and the peak area were used to calculate the recovery rate.

2.6.4 Stability:

QC containing 50, 250 and 1000 ng/ml of (-)-*epi*afzelechin were placed in different environment for long term low temperature, short term room temperature and three freeze/thaw cycles stability tests. These samples, including a set of freshly prepared samples, were extracted by plasma sample extraction protocol and analyzed by UPLC-MS/MS. For post-preparative stability test, extracted plasma blanks containing 50, 250 and 1000 ng/ml of (-)-*epi*afzelechin with 500 ng/ml of IS were analyzed by UPLC-MS/MS after keeping in the auto-sampler at 4 ^{o}C for 8 hours. The peak areas of freshly prepared QC and treated QC were used to calculate the stability.

2.6.5 Matrix effect:

QC containing 50, 250 and 1000 ng/ml of (-)-*epi*afzelechin with 500 ng/ml of IS were prepared in MeOH with or without extracted plasma matrix for UPLC-MS/MS detection. The peak areas of QC with or without matrix were used to calculate the matrix effect.

3. Results and discussion:

3.1 Method development

We chose the relatively new technique of ultra performance liquid chromatography (UPLC) because it offers the advantages of high resolution, decrease in time of analysis and reduced consumption of solvent [17]. We had selected (+)-catechin (**2**, Figure 1A) as IS for the quantification of (-)-*epi*afzelechin (**1**, Figure 1A) for the reason that they have similar chemical structures and likely similar hydrophilic properties. Furthermore, different detection methods including one in human plasma [18] have been validated [18-21] to quantify catechins. These suggest that (+)-catechin is a suitable candidate for use as an IS. For method of detection, we had chosen tandem mass spectrometry because of its high sensitivity and selectivity. The protonated

molecular ions of (+)-catechin and (-)-*epi*afzelechin, [M+H$^+$] *m/z* 291 and 275 respectively, were observed in positive mode of ESI-MS. These precursor ions were fragmented by tandem MS and yielded the same daughter ion *m/z* 139 (Figure 1D & 1E). This allowed the use of multiple reaction monitoring (MRM) for (+)-catechin and (-)-*epi*afzelechin at 291 → 139 and 275 → 139 respectively, to increase the detection specificity. Formic acid was added to enhance the protonation in mass spectrometric detection. The use of formic acid could also increase the resolution of (-)-*epi*afzelechin from (+)-catechin in liquid chromatography [21]. The retention times of (+)-catechin and (-)-*epi*afzelechin were found to be 3.32 and 4.11 minutes in this gradient elution protocol (Figure 2B). The peaks of (+)-catechin and (-)-*epi*afzelechin did not overlap, and therefore the MRM mode detecting the same daughter ion would not affect the quantification.

3.2 Selectivity and linearity

The selectivity of the detection method was determined by using six different sources of plasma. A representative chromatogram of the extracted plasma blank is shown in Figure 2A. There was no interfering peak found in the corresponding retention time indicating as well that there was not any indigenous (-)-*epi*afzelechin and *(+)*-catechin present in the plasma. The results suggested that the basal diets for the animals did not contain our target analytes. The responses of solutions of (-)-*epi*afzelechin ranging from 12.5 to 5000 ng/ml were determined. We had inserted blank sample in between high concentration QC samples and no carry-over was observed. Three linear regression models, least squares linear regression without passing through origin, least squares linear regression passing through origin and weighted least squares linear regression, were used to construct the calibration curves [22]. The results suggested that linear regression passing through origin and weighted least squares linear regression could fulfill the bioanalytical method validation requirements of U. S. Food and Drug Administration [15]. A simple regression model is always preferred and therefore the linear regression model passing through the origin was used. This method exhibited a good linearity (r^2 = 0.992) and the equations of the calibration curve for low concentrations and high concentrations were $y = 0.007\ x$ and $y = 0.0006\ x$, respectively.

3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) concentrations were determined from the signal to noise ratio (S:N) of dilute samples. The LOD of (-)-*epi*afzelechin was found to be 4 ng/ml with a S:N of 3. The Limit of Quantification (LOQ) of (-)-*epi*afzelechin was found to be 24 ng/ml with a S:N of 20. The Lower Limit of Quantification (LLOQ) of (-)-*epi*afzelechin was found to be 12.5 ng/ml which was the lowest concentration used in the calibration curve. It had a S:N of 10.

3.4 Precision and accuracy

The precision and accuracy of this method is shown in Table 1. The intra-day and inter-day accuracy of the determination of (-)-*epi*afzelechin were more than 99.3% and 97.6%, respectively. Reproducibility, presented as % coefficient variation (CV), of the method showed that the intraday batch precision ranged from 4.52 to 11.99 % while the inter-day batch precision ranged from 6.78 to 11.16 %. These suggested that the method could generate accurate and reproducible results.

3.5 Recovery and matrix effect

The plasma recovery rate of (-)-*epi*afzelechin ranged from 99.6% to 104.5% over the three concentrations as shown in Table 2. The matrix effect is within acceptable range. The relative content of (-)-*epi*afzelechin and IS ranged from 93.61% to 102.70% and 105%, respectively, in the presence of matrix.

3.6 Stability of samples

The relative (-)-*epi*afzelechin content of plasma samples stored at -20°C for 30 days and room temperature for 12 hours ranged from 95.06% to 98.28% and 98.76% to 100.83%, respectively. The relative (-)-*epi*afzelechin content of plasma sample after undergoing three freeze and thaw cycles ranged from 98.51% to 103.60%. The post-preparative stability of extracted (-)-*epi*afzelechin ranged from 94.2% to 102.7%. These results suggest that (-)-*epi*afzelechin is stable in both unextracted and extracted samples.

3.7 Pharmacokinetic study

The validated bioanalytical method of UPLC-MS/MS was then applied to the pharmacokinetic study of (-)-*epi*afzelechin in young female C57BL/6J (20-25 g) mice. Two administration routes, i.v. and i.p. injections, were used in this study. Their major difference was the absence of barrier in i.v. injection. In addition, the properties of (-)-*epi*afzelechin could also affect the PK profile in these two administration routes. The oral administration of (-)-*epi*afzelechin to mice was not attempted at this time because of the large amount of (-)-*epi*afzelechin required for the experiments, especially for future *in vivo* study.

The plasma drug concentration-time curves of single bolus i.v. and i.p. injection are shown in Figure 3 and their corresponding PK parameters are shown in Table 3. The maximum concentration (C_{max}) of i.v. and i.p. administration of (-)-*epi*afzelchin were found to be 10.6 μg/mL and 6.0 μg/mL, respectively. For i.v. injection, all the injected (-)-*epi*afzelechin could get into the blood directly and without delay. For i.p. injection, the time for reaching maximum concentration (T_{max}) was found to be 15 minutes. Evidently, in i.p. administration, the injected (-)-*epi*afzelechin could be easily transported to the blood vessels in the abdomen region and distributed to the whole body through blood. The distribution half-life ($T_{1/2,\ \alpha}$) for i.v. and i.p. injection were 7.0 and 12.6 minutes, respectively, while the elimination half-life ($T_{1/2,\ \beta}$) of i.v. and i.p. were 39.0 minutes and 101.0 minutes, respectively. The short $T_{1/2,\ \alpha}$ suggested that (-)-

*epi*afzelechin could distribute to different body compartments rapidly. Both the distribution and elimination half-life of i.v. injection were shorter than those of i.p. injection. This may be due to the (-)-*epi*afzelechin reservoir in the abdomen region continuously supplying (-)-*epi*afzelechin to blood and counteracting the distribution and elimination.

The drug exposure in both i.v. and i.p. injection were similar. The AUC (0-t) and AUC (0-∞) in i.v. injection were found to be 134.6 μg-min/mL and 136.8 μg-min/mL, respectively, while in i.p. injection they were 148.2 μg-min/mL and 149.9 μg-min/mL, respectively. This suggested that the bioavailability of (-)-*epi*afzelechin was essentially 100% in i.p. injection. The clearances (CL) in i.v. and i.p. injections were found to be 73.6 mL/min/kg and 65.6 mL/min/kg, respectively. All these results suggests that the i.p. administration of (-)-*epi*afzelechin should have a nearly similar effect to the i.v. administration and can be used as a viable route for *in vivo* study in mice.

4. Conclusion

In summary, a UPLC-MS/MS detection method has been developed to detect (-)-*epi*afzelechin in mice plasma samples. This method was applied to the PK study of i.v. and i.p. administration of (-)-*epi*afzelechin to mice. The results suggested that the i.p. administration of (-)-*epi*afzelechin has a pharmacokinetic profile similar to the i.v. administration and maybe useful for further *in vivo* study.

Acknowledgement

This work was supported by the General Research Fund (GRF) (PolyU 5632/09M and PolyU 5003/05P) of the HKSAR Government, the Research Studentship of the Hong Kong Polytechnic University for Dr. Kenneth KC Wong as well as the National Key Basic Research Development Plan **(**973 plan, 2012CB723506).

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Figure Legend

Figure 1 Chemical structure (A) of (-)-*epi*afzelechin (1) and (+)-catechin (2); total ion scan (TIC) of (+)-catechin (B) and (-)-*epi*afzelechin (C); and daughter ion scan of (+)-catechin (D) and (-)-*epi*afzelechin (E)

(+)-Catechin and (-)-*epi*afzelechin dissolved in ACN with 0.5% v/v formic acid was injected to the ionizer at a flow rate 10μL/min by Harvard syringe pump. (A & B) MS was operated in +ESI mode with the supply of 50 L/h cone gas, 400 L/h desolvation gas, 80 ^{o}C source temperature, 150 ^{o}C desolvation temperature, 30 V cone voltage and 3 kV of capillary voltage. (C & D) For daughter ion scan, the parent ions at m/z 291 and m/z 275 were selected and underwent fragmentation to yield daughter ions. The collision energy was configured at 18 eV collision voltages with C.I.D. argon gas pressured at 5.5 x 10^{-5} Torr.

Figure 2 TIC of plasma blank (A); and simultaneous detection (B) of (-)-*epi*afzelechin and (+)-catechin by UPLC-MS/MS

(A) 50 μL of blank plasma or spiked plasma was extracted by 100 μL of EA for three times. Solvent were dried and re-dissolved in 50 μL of MeOH for LC-MS/MS analysis. (B) 2.5 ng of (-)-*epi*afzelechin and 5 ng of (+)-catechin were dissolved in 50 μL of mice blank plasma extracted reconstitute. 2 μL of sample was detected by UPLC-MS/MS using gradient elution – initiation: 0-1 min 100% water; elution gradient: 1-11 min 0→70% ACN; and regeneration: 11-16 min 100% water. MS detection of (-)-*epi*afzelechin and (+)-catechin were operated in +ESI mode with the supply of 150 L/h cone gas, 550 L/h desolvation gas, 150 ^{o}C source temperature, 350 ^{o}C desolvation temperature, 30 V cone voltage and 3 kV of capillary voltage. The collision energy was configured at -18 eV collision voltage with C.I.D. argon gas pressure at 5.5 x 10^{-5} Torr. The ions were monitored by MRM mode for (-)-*epi*afzelechin (m/z 275 → 139) and (+)-catechin (m/z 291 → 139)

Figure 3 Semi-log plasma (-)-*epi*afzelechin concentration-time curve after a single bolus i.v. (A); and i.p. (B) injection of 10 mg/kg (-)-*epi*afzelechin

Young female C57BL/6J mice were administrated with 10 mg/kg (-)-*epi*afzelechin via i.v. and i.p. injection. After 5, 15, 30, 60, 120, 240, 360 and 720 minutes, mice were sacrificed by cardiac stick exsanguinations during blood collection. The blood were treated with EDTA and centrifuged to yield plasma. (-)-*Epi*afzelechin was extracted from the plasma by EA and underwent UPLC-MS/MS analysis. The (-)-*epi*afzelechin concentrations in plasma were plot against different time point. n=5

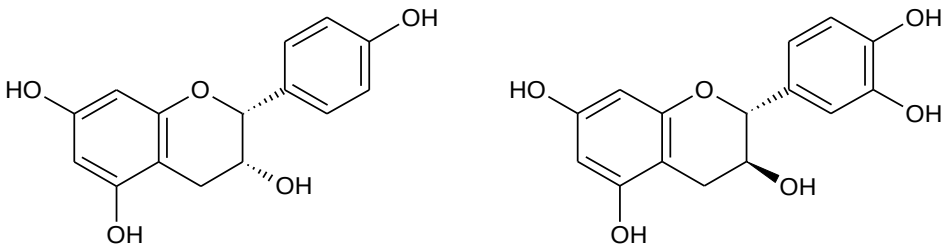
Highlights:

- UPLC-MS/MS can be used to quantify (-)-*epi*afzelechin in mice plasma
- This bioanalytical method was validated
- A pharmacokinetic study of (-)-*epi*afzelechin was performed in mice
- Intravenous and intraperitoneal are valid administration routes for *in vivo* study

Figures and Tables

Figure 1

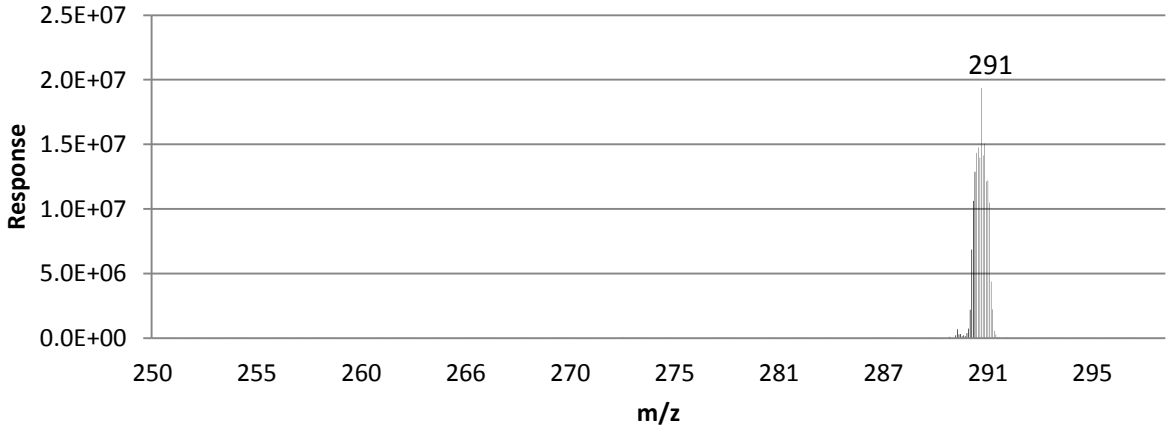
A



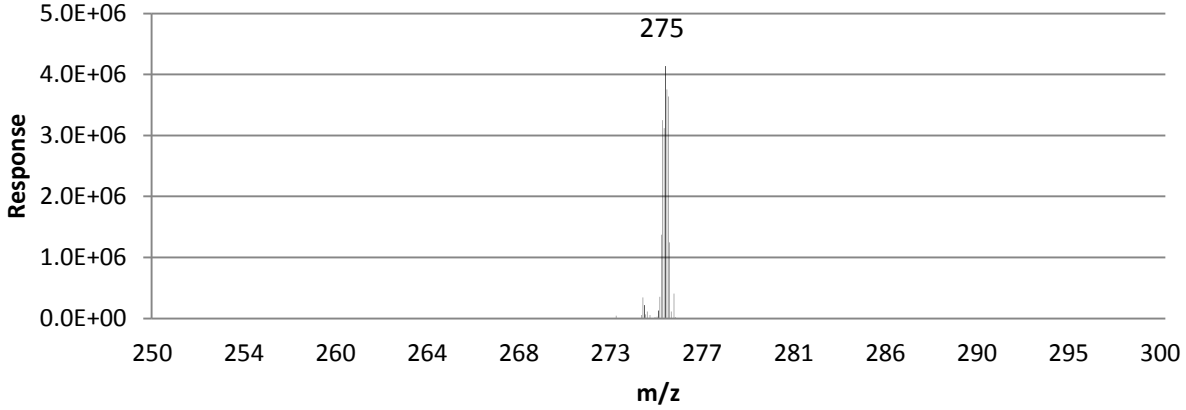
(-)-*Epi*afzelechin (1)

(+)-Catechin (2)

B



C



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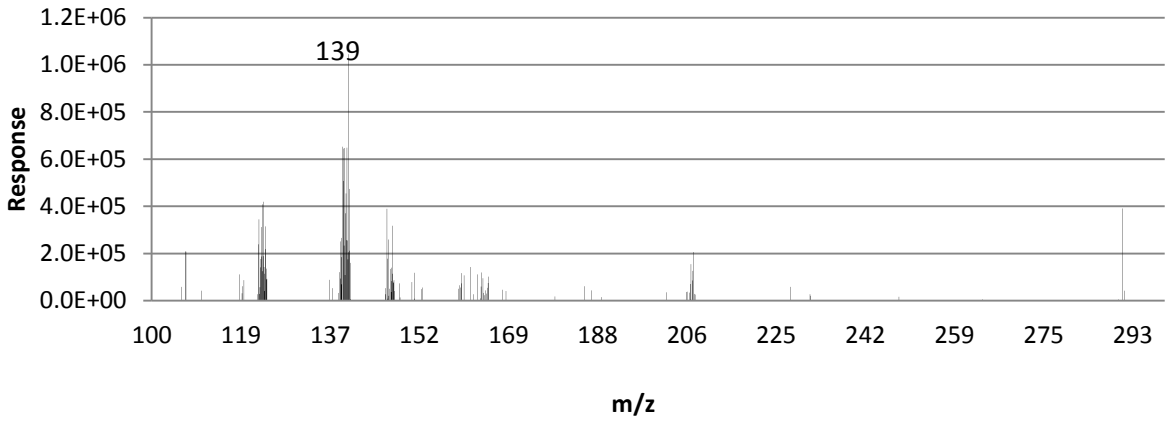

1.2E+06
1.0E+06
8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
Response
139
100
119
137
152
169
188
206
225
242
259
275
293
m/z

E

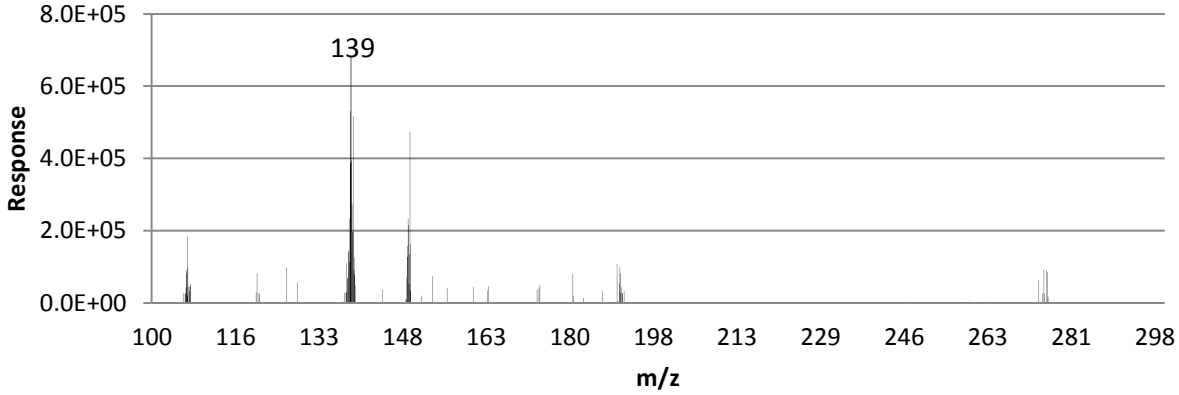
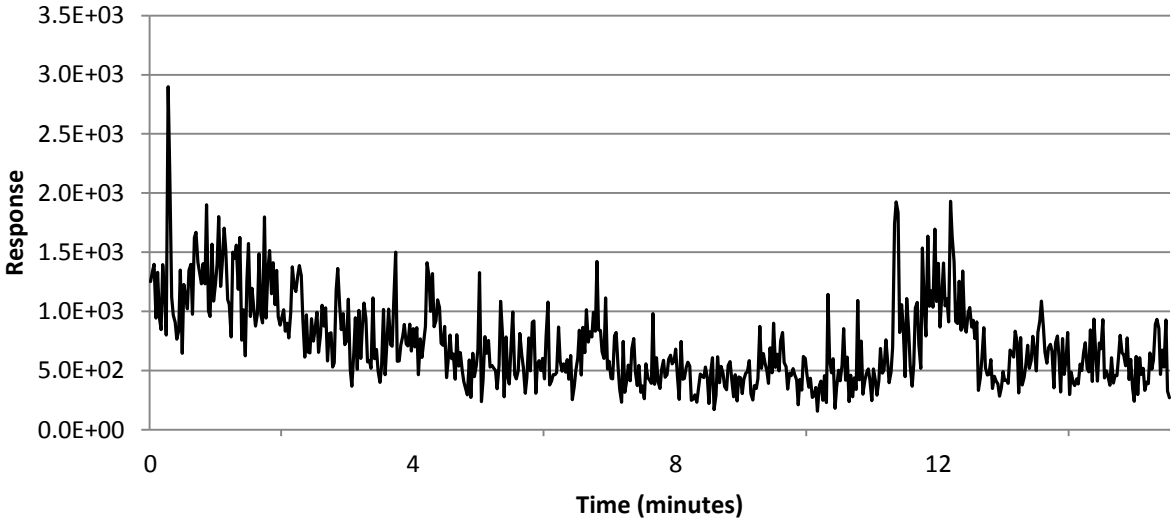

8.0E+05
6.0E+05
4.0E+05
2.0E+05
0.0E+00
Response
139
100
116
133
148
163
180
198
213
229
246
263
281
298
m/z

Figure 2

A


3.5E+03
3.0E+03
2.5E+03
2.0E+03
1.5E+03
1.0E+03
5.0E+02
0.0E+00
Response
0
4
8
12
Time (minutes)

B

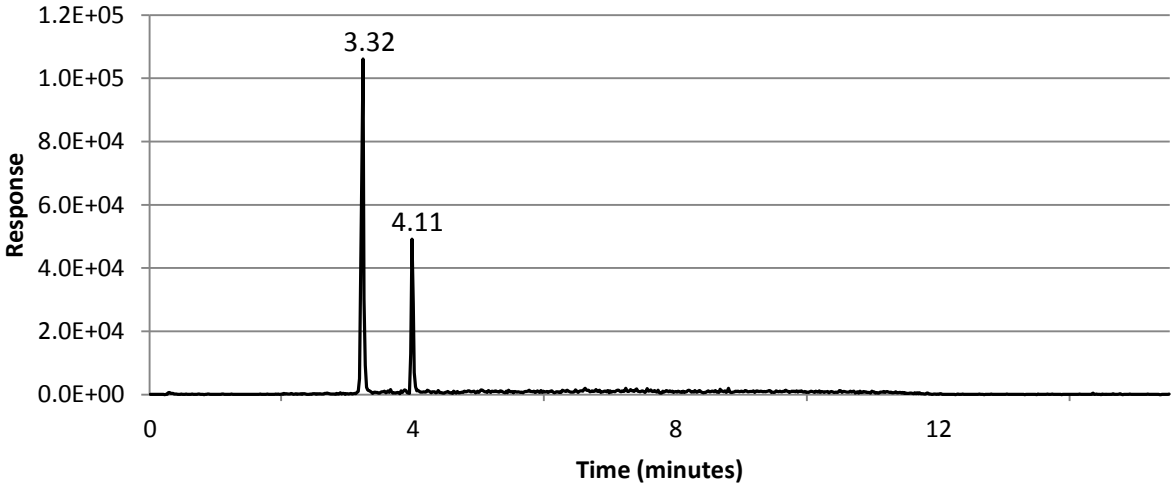
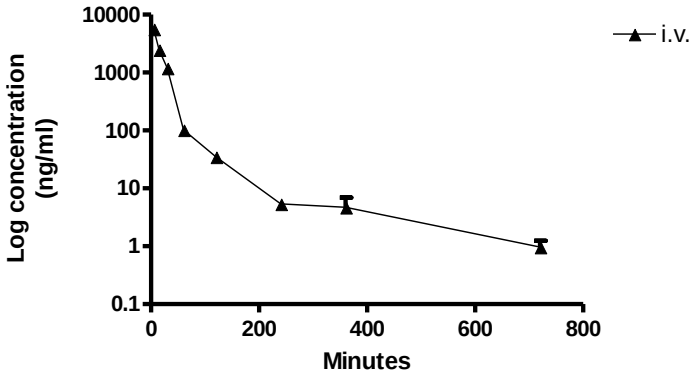
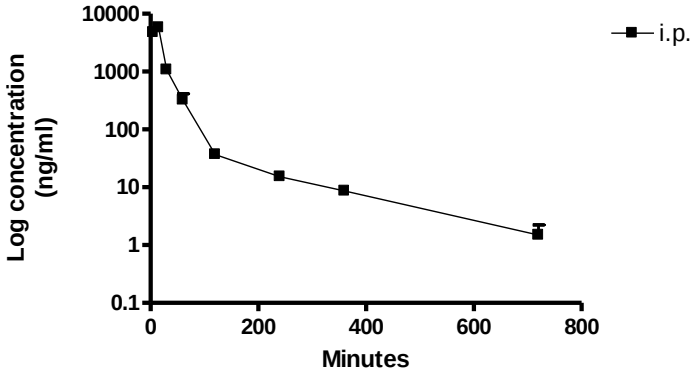

1.2E+05
1.0E+05
8.0E+04
6.0E+04
4.0E+04
2.0E+04
0.0E+00
Response
3.32
4.11
0
4
8
12
Time (minutes)

Figure 3

A


10000
1000
100
10
1
0.1
Log concentration (ng/ml)
0
200
400
600
800
Minutes
i.v.

B


10000
1000
100
10
1
0.1
Log concentration (ng/ml)
0
200
400
600
800
Minutes
i.p.

	Intra-day			Inter-day		
Actual (-)-*epi*afzelechin (ng/ml)	Found Mean ± SD	RSD (% CV)	Accuracy (%)	Found Mean ± SD	RSD (% CV)	Accuracy (%)
12.5 (LLOQ)	14.05 ± 1.69	11.99	112.4	12.20±1.80	11.16	97.6
50	54.14 ± 4.23	7.81	108.3	52.16±1.85	8.08	104.3
250	287.00 ± 17.25	4.52	113.4	276.91±20.20	7.54	113.1
1000	960.58 ± 16.72	7.50	99.3	977.50±63.43	6.78	99.2

Table 1. Intra-day and inter-day accuracy and precision of (-)-*epi*afzelechin (n=5)

	Conc. (ng/ml)	Extraction Recovery (%)	Matrix Effect	Post-preparative stability (%) 4 ^{o}C stored at auto sampler for 8 hours	Stored at -20 ^{o}C for 30 days stability	Stored at room temperature for 12 hours stability	Freeze thaw cycle stability
		Average (%) ± RSD (%)					
(-)-*epi*afzelichin	50	99.7± 7.99	100.04 ± 7.60	98.2 ± 7.60	95.06 ± 5.07	98.06±8.81	98.51±3.84
	250	99.6± 6.91	102.70 ± 6.45	102.7 ± 2.29	98.28 ± 5.10	98.76±12.39	107.61±11.40
	1000	104.5 ± 8.24	93.61 ± 4.92	94.2 ± 4.80	96.01 ± 5.72	100.83±5.43	103.60±10.60

Table 2. Recovery rate, matrix effects and stability of (-)-*epi*afzelechin (n=5)

	i.v. injection	i.p. injection
T_{max}	-	15 minutes
C_{max}	10.6 ± 4.9 μg/mL	6.0 ± 0.2 μg/mL
AUC (0-t)	134.6 ± 14.6 μg-min/mL	148.2 ± 4.6 μg-min/mL
AUC (0-∞)	136.8 ± 13.1 μg-min/mL	149..9 ± 4.9 μg-min/mL
$T_{1/2, \alpha}$	7.0 ± 0.9 min	12.6 ± 6.6 min
$T_{1/2, \beta}$	39.0 ± 15.9 min	101.0 ± 3.8 min
CL (area/kg)	73.6 ± 7.5 mL/min/kg	65.6 ± 2.2 mL/min/kg

Table 3 Pharmacokinetic parameters of i.v. and i.p. injections of (-)-*epi*afzelechin (10mg/kg) in mice (n=5). These parameters were obtained either from the direct observation from the plasma (-)-*epi*afzelechin concentration time curve or by Pharmacokinetic software – Summit® PK solution.