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Quantitative analysis of [Dmt^1]DALDA in ovine plasma by capillary liquid chromatography-nanospray ion-trap mass spectrometry

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Abstract

The synthetic opioid peptide analog Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA; Dmt = 2′,6′-dimethyltyrosine) is a highly potent and selective mu opioid-receptor agonist. A very sensitive and robust capillary liquid chromatography/nanospray ion-trap (IT) mass spectrometry method has been developed to quantify [Dmt^1]DALDA in ovine plasma, using deuterated [Dmt^1]DALDA as the internal standard. The standard MS/MS spectra of d_0- and d_5-[Dmt^1]DALDA were obtained, and the collision energy was experimentally optimized to 25%. The product ion $[M + 2H\text{-}NH_3]^{2+}$ (*m*/*z* 312.2) was used to identify and to quantify the synthetic opioid peptide analog in ovine plasma samples. The MS/MS detection sensitivity for [Dmt^1]DALDA was 625 amol. A calibration curve was constructed, and quantitative analysis was performed on a series of ovine plasma samples.

Keywords: [Dmt^1]DALDA; Opioid peptide

1. Introduction

The synthetic peptide [Dmt^1]DALDA (Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2′,6′-dimethyltyrosine) is a highly potent and selective mu opioid-receptor agonist [1–4]. The peptide's molecular weight of 639.4 Da and net charge of +3 at physiological pH make it a very polar molecule that is unlikely to cross a biological membrane. It has potential as an analgesic drug that is relatively free of side effects because of those physicochemical properties. In order to obtain the pharmacokinetic data of the peptide, it is necessary to design an experimental method that optimizes the sensitivity and specificity to quantify the peptide in a complex biological fluid. A synthetic, stable isotope-incorporated peptide (deuterated [Dmt^1]DALDA: Dmt-D-Arg-Phe(d_5)-Lys-NH_2) was used as the internal standard to quantify [Dmt^1]DALDA in ovine plasma samples.

Combined liquid chromatography and tandem mass spectrometry (LC/MS/MS) has been widely used for the quantitative analysis of trace levels of pharmaceutical drugs [5–7], metabolites [8,9], and peptides [10–13]. The on-line LC/MS/MS method minimizes the number of processing steps, could save analysis time, and is used for the high-throughput quantitative analysis of a large number of samples. Also, on-line chromatography-mass spectrometry minimizes sample transfers, reduces sample loss, and improves the detection sensitivity.

Traditionally, a triple-quadrupole (QqQ) mass spectrometer is widely used for quantitative analysis, and is considered to be robust and reliable [9]. In recent years, ion-trap (IT) [9,14] and quadrupole time-of-flight (Q-TOF) mass spectrometers [12,15,16] were investigated for quantitative analysis. An ion-trap mass spectrometer has the advantage [9,17] of providing full-scan MS/MS spectra instead of monitoring only one (or a few) product ions. A triple-quadrupole system can also provide full-scan MS/MS spectra. However, the full-scan MS/MS sensitivity is much better in a conventional ion-trap system such as the Finnigan LCQ. The

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disadvantages of an ion-trap system are that an ion-trap, at any one time, can contain only a limited number of ions, and has a low-mass cut-off due to the physical limitation [18] in efficiently trapping the product ions below a certain percentage of the precursor ion mass (28% for an LCQ [9]). Quantitative analysis with Q-TOF mass spectrometry provides better mass accuracy and resolution. In this manuscript, we describe the quantitative analysis of [Dmt^1]DALDA, using a Thermo Finnigan LCQ ion-trap mass spectrometer.

2. Experimental

2.1. Chemicals and materials

[Dmt^1]DALDA was prepared as reported elsewhere [1]. For the synthesis of deuterated DALDA [H-Dmt-D-Arg-Phe(d_5)-Lys-NH_2], L-d_5-phenylalanine [Phe(d_5)] (C/D/N Isotopes, Montreal, Que., Canada) was converted to the Boc-protected form, and was used in the solid-phase synthesis of the deuterated peptide (d_5-[Dmt^1]DALDA), based on a protocol described elsewhere [1].

Formic acid was purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Acetontrile (HPLC-grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were purchased from Pierce (Rockford, IL, USA). Oasis HLB 1 cc (30 mg) extraction cartridges (solid-phase extraction (SPE) cartridge) were purchased from Waters Corporation (Milford, MA, USA). Spin-X centrifuge tube filters (0.45 μm) were purchased from Corning Costar Corporation (Cambridge, MA, USA).

2.2. Standard solution

Standard stock solutions of d_0- and d_5-[Dmt^1]DALDA were prepared in 10% acetonitrile (containing 0.3% TFA) at a concentration of 1 mg/ml. The solutions were stored at 4 °C. The working solutions were prepared daily at a concentration of 1 pmol/μl.

2.3. Ovine plasma samples

Ovine plasma samples were obtained from Dr. Hazel Szeto. The sheep were injected with [Dmt^1]DALDA (60 μg/kg). Plasma samples were obtained at 12 time-points (0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min). The plasma samples were frozen (−80 °C), and were sent to the University of Tennessee Health Science Center for analysis.

2.4. Ovine plasma and sample preparation

The frozen plasma samples were thawed (4 °C), and an aliquot (200 μl) was taken. Salts and compounds with a high molecular weight were removed by protein precipitation and SPE [19,20]. An appropriate amount of d_5-[Dmt^1]DALDA (6.4 ng) was added to each plasma sample (100 μl), and the mixture was mixed thoroughly (20 min). Acetonitrile (200 μl) was added to precipitate proteins, and the mixture was vortexed (5 min) and centrifuged (20,000 × *g*; 5 min). Acetonitrile was evaporated from the supernatant, and TFA (final concentration was 0.1%) was added to the solution. An Oasis HLB 1 cc (30 mg) extraction cartridge was used to enrich the peptides and to remove salts. The cartridge was pre-wetted with methanol (1 ml), and was equilibrated with 0.1% TFA for approximately 30 s (1 ml). The sample (containing 0.1% TFA) was loaded, and the cartridge was washed with TFA (0.1%, 1 ml). Methanol (1 ml) was used to elute the peptide (approximately 1 ml/min). The eluant was evaporated in a Vacufuge Concentrator (Eppendorf, model 5301, Westbury, NY, USA). Formic acid (0.1%, 20 μl) was used to reconstitute the solution. The solution was centrifuged (12,000 × *g*, 5 min), using a Spin-X centrifuge tube filter to remove particles. A sample (5 μl) was injected, and a volume (4 μl, which is the loop volume) was loaded onto the column and was used for LC/MS/MS analysis.

2.5. Liquid chromatography

An on-line Thermo Finnigan Surveyor HPLC system was used. The capillary HPLC columns were home-packed. Empty capillary HPLC columns, Picofrit (75 μm inner diameter) columns, were purchased from New Objective Inc. (Woburn, MA, USA). The media was Magic C_{18} magic packing material (5 μm diameter, 200 Å pore size) obtained from Microm Bioresources Inc. (Auburn, CA, USA).

C_{18} beads were packed (approximately 8.0 cm in length) under high pressure in a home-built apparatus. Isocratic chromatographic separation was performed; the mobile phase was 36% acetonitrile in 0.1% formic acid. The flow rate was approximately 0.5 μl/min.

2.6. Mass spectrometry

A Thermo Finnigan LCQ mass spectrometer (San Jose, CA, USA) was operated in the positive-ion mode. The instrument was equipped with a nanospray ionization source. The source voltage was 2.0 kV, the capillary voltage was 23.0 V, the heated capillary temperature was 80 °C, and the tube-lens offset was −25 V. The full-scan MS/MS mode was performed, and the $[M + 2H]^{2+}$ ions of the two peptides (d_0, d_5) were selected as the precursor ions. The maximum ion-injection time was 300 ms, and each scan consisted of 3 microscans. The precursor ion isolation width was 6 Th (1 Th = 1 *m*/*z* unit) to allow the transmission of the $[M + 2H]^{2+}$ ions of d_0- and d_5-[Dmt^1]DALDA (*m*/*z* 320.7 for d_0-[Dmt^1]DALDA, *m*/*z* 323.2 for d_5-[Dmt^1]DALDA). The collision energy was experimentally optimized at 25% to maximize the intensity of the product ions at *m*/*z* 312.2 and 314.7. The product ion at *m*/*z* 312.2 derived from the

precursor ion at *m/z* 320.7, and *m/z* 314.7 from *m/z* 323.2. The ratio of d_0/d_5-[Dmt^1]DALDA was determined from the ratio of the areas under the curve of the selected product ions (*m/z* 312.2 and 314.7) in the extracted ion chromatograms. The instrument software Xcalibur was used to process the data.

2.7. Peptide recovery with different ion-pairing reagents

TFA, HFBA, and formic acid were tested for the optimal ion-pairing reagent. Methanol (1 ml) was used to prewet the SPE cartridge, and the ion-pairing reagent (0.1%, 1 ml) was used to equilibrate the cartridge. One pmol of d_0-[Dmt^1]DALDA (1 μl of a 1 pmol/μl solution) diluted into 0.1% ion-pairing reagent (1 ml) was loaded, and the SPE cartridge was washed with 0.1% ion-pairing reagent (1 ml). The d_0-[Dmt^1]DALDA was eluted at approximately 1 ml/min with methanol (1 ml). One pmol d_5-[Dmt^1]DALDA was added to the eluant, and the mixture was dried (Vacufuge Concentrator). The residue was reconstituted in 0.1% formic acid (15 μl). LC/MS/MS was used to perform the quantitative analysis ($n = 2$).

2.8. Calibration curve

A series of calibration standards was prepared. Five blank plasma samples (200 μl each) were spiked with five different ratios of d_0- and d_5-[Dmt^1]DALDA. The concentration of d_5-[Dmt^1]DALDA was fixed at 64 ng/ml, and the d_0-[Dmt^1]DALDA concentrations were 16, 32, 64, 96, and 128 ng/ml. Four measurements were performed for each concentration, and each average was used.

3. Results and discussion

3.1. Full-scan MS/MS spectra of the $[M + 2H]^{2+}$ ions of synthetic d_0- and d_5-[Dmt^1]DALDA

To develop a peptide quantification method, the MS behavior of the synthetic peptide must be studied carefully. The d_0-[Dmt^1]DALDA concentration is very low in the fetal sheep samples in these studies, and in the later time-point plasma samples of the maternal sheep. It is difficult to detect and quantify the peptide with off-line HPLC and MALDI-TOF mass spectrometry, and the post-source decay (PSD) of MALDI-TOF has a limited capability to generate the product ions that are needed to obtain the molecular specificity for the qualitative and quantitative analysis of d_0- and d_5-[Dmt^1]DALDA. However, the product ions are readily obtained by LC/MS/MS in an Finnigan LCQ. In order to optimize detection sensitivity, the most abundant ion in the MS spectrum was selected as the precursor ion for MS/MS. The MS/MS spectrum was used to select those product ions that could differentiate between the d_0-[Dmt^1]DALDA and d_5-[Dmt^1]DALDA, and that could be used for quantification.

The MS spectra of 1 pmol of d_0- and of d_5-[Dmt^1]DALDA were obtained. The $[M + H]^+$ (*m/z* 640.4), $[M + 2H]^{2+}$ (*m/z* 320.7), and $[M + 3H]^{3+}$ ions (*m/z* 214.1) of d_0-[Dmt^1]DALDA are seen in Fig. 1(a). The most abundant ion is the $[M+2H]^{2+}$ ion. Fig. 1(b) shows the $[M+H]^+$ (*m/z* 645.4), $[M + 2H]^{2+}$ (*m/z* 323.2), and $[M + 3H]^{3+}$ ions (*m/z* 215.8) of d_5-[Dmt^1]DALDA. The $[M + 2H]^{2+}$ ion is also the most abundant ion. Therefore, the pair of $[M + 2H]^{2+}$ ions (*m/z* 320.7 and 323.2) was selected as the precursor ions to generate the product ions for quantification.

The product ions of the $[M + 2H]^{2+}$ precursor ion of d_0-[Dmt^1]DALDA are shown in the MS/MS spectrum in Fig. 2(a). Ten product ions (z_1, a_1, b_1-NH_3, $[M+2H-NH_3]^{2+}$, b_2-NH_3, b_2, z_3, a_3, b_3-NH_3, and b_3) were detected. The fragmentation pattern of d_0-[Dmt^1]DALDA is shown in Fig. 3. A similar MS/MS spectrum for d_5-[Dmt^1]DALDA is obtained (Fig. 2(b)).

Because the product ions z_1, a_1, b_1-NH_3, b_2-NH_3, and b_2 from d_0-[Dmt^1]DALDA have the same *m/z* value as the corresponding ions from d_5-[Dmt^1]DALDA, those ions cannot be used for identification and quantification. However, five other product ions (z_3, a3, $[M + 2H-NH_3]^{2+}$, b_3-NH_3, and b_3) from d_0-[Dmt^1]DALDA can be used to differentiate between d_0-[Dmt^1]DALDA and d_5-[Dmt^1]DALDA. Those product ions can be used to confirm the amino acid sequence, and to quantify the peptide.

3.2. Collision energy

A solution of 1 pmol/μl of d_0-[Dmt^1]DALDA was infused by syringe into the LCQ at a flow rate of 0.5 μl/min. The collision energy was varied from 15 to 95% in increments of 5%. For each collision energy, 1 min scan spectra were used. The product ions (a_3, b_3, $[M + 2H-NH_3]^{2+}$ and b_3-NH_3) from the $[M + 2H]^{2+}$ precursor ion were studied. The intensity (counts) versus collision energy is shown in Fig. 4. When the collision energy was 25%, the $[M+2H-NH_3]^{2+}$ ion had the highest intensity. However, for the other three ions, the best collision energy was 35%. Because the intensity of the $[M + 2H-NH_3]^{2+}$ ion at collision energy 25% is much higher than the intensity of the other three ions at collision energy 35%, the $[M + 2H-NH_3]^{2+}$ ion was used for quantification. For the product ions ($[M + 2H-NH_3]^{2+}$, a_3, b_3, b_3-NH_3) of d_5-[Dmt^1]DALDA, there was a similar result (not shown) of intensity versus collision energy. The $[M+2H-NH_3]^{2+}$ ion from d_5-[Dmt^1]DALDA also has the highest intensity at collision energy of 25%. Therefore, the pair of $[M + 2H-NH_3]^{2+}$ product ions (*m/z* 312.2.7 and 314.7) was used to calculate the d_0/d_5-[Dmt^1]DALDA ratio in all quantification experiments, and the collision energy was set to 25%.

3.3. Limit of detection for standard d_0-[Dmt^1]DALDA

The LCQ limit of detection for synthetic d_0-[Dmt^1]DALDA in solution (0.1% formic acid) is 625 amol

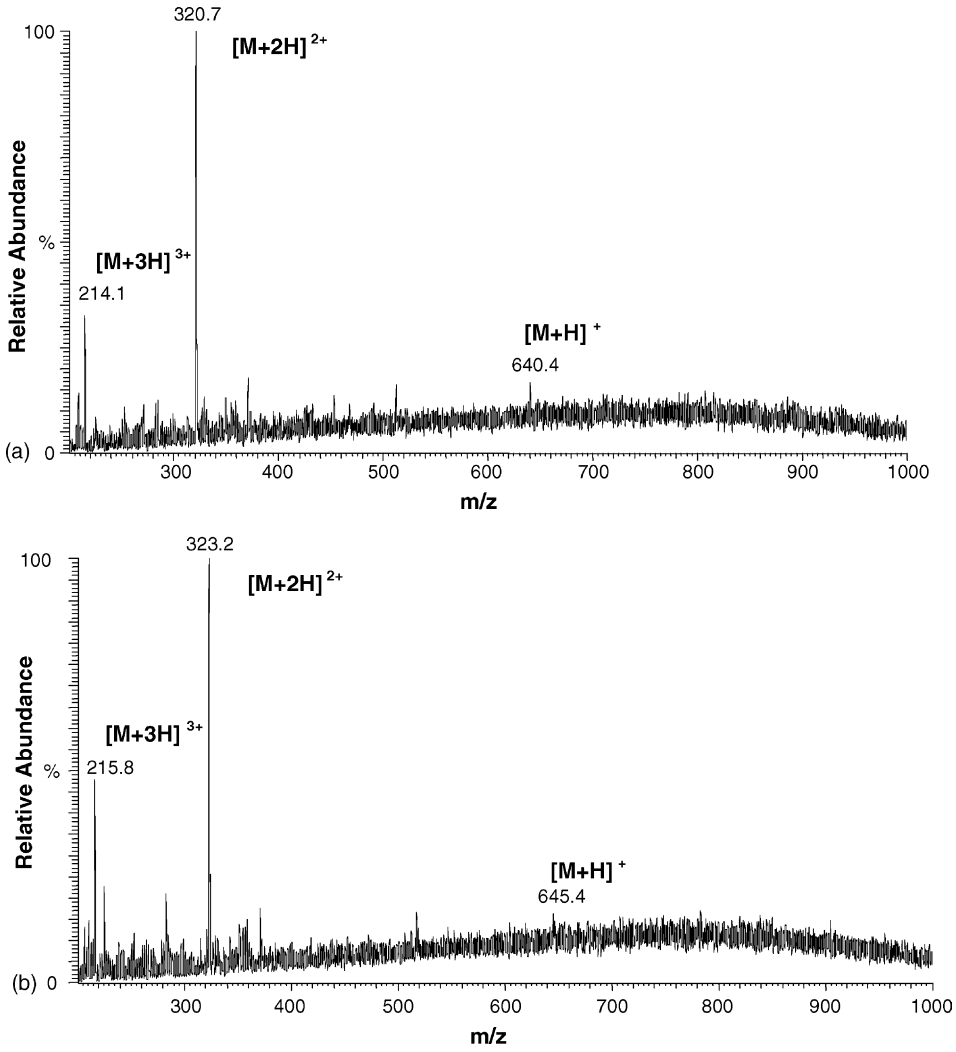

Fig. 1. MS spectra of (a) d_0-[Dmt^1]DALDA and (b) d_5-[Dmt^1]DALDA.

(0.1 ng/ml, $S/N = 3{:}1$). Compared to the detection sensitivity of d_0-[Dmt^1]DALDA from MALDI-TOF (50 fmol) and Q-TOF (12.5 fmol) [12], the LC/MS/MS method on the LCQ system is more sensitive. The lower limit of quantification (LLOQ) of d_0-[Dmt^1]DALDA in ovine plasma is 0.64 ng/ml ($S/N = 5{:}1$). With this LCQ method, one might be able to quantify the low concentration of d_0-[Dmt^1]DALDA in fetal sheep plasma samples, and in the very late time-point of maternal sheep plasma samples.

3.4. Peptide recovery and calibration curve

The results from the peptide recovery study, using different ion-pairing reagents are shown in Table 1. TFA (0.1%) provided 80% recovery, and HFBA (0.1%) and formic acid (0.1%) provided 30% and 20% recovery, respectively. Therefore, TFA (0.1%) was selected as the ion-pairing reagent in the ovine plasma quantification experiments.

The calibration curve was constructed. The equation of the best-fit line was $y = 0.017 \times -0.022$ ($r^2 = 0.999$), where y is the d_0/d_5-[Dmt^1]DALDA ratio, and x is the d_0-[Dmt^1]DALDA concentration. Good linearity was achieved.

3.5. Intra-day and inter-day assay

Intra- and inter-day precision and accuracy were determined by assaying the spiked ovine plasma samples; the final concentrations of d_0-[Dmt^1]DALDA in those samples were 16, 32, 64, and 128 ng/ml. Accuracy was expressed as

Table 1
The recovery of d_0-[Dmt^1]DALDA with different ion-pairing reagents ($n = 2$)

Ion-pairing reagent	Recovery (%)	Standard deviation (%)
0.1% TFA	80	8.5
0.1% HFBA	30	7.1
0.1% Formic acid	20	2.8
H_2O	4	1.4

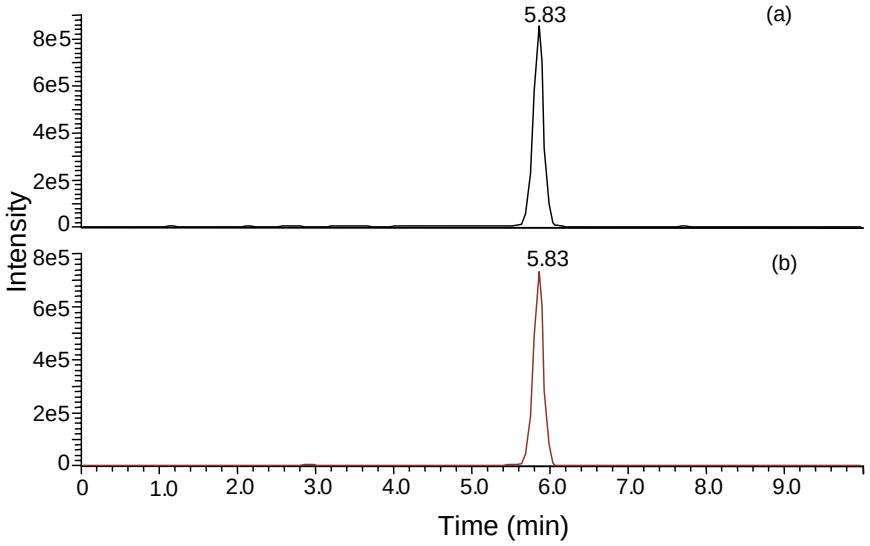

Fig. 2. MS/MS product-ion spectra of (a) d_0-[Dmt^1]DALDA; the precursor ion is the $[M + 2H]^{2+}$ ion; (b) d_5-[Dmt^1]DALDA; the precursor ion is the $[M + 2H]^{2+}$ ion.

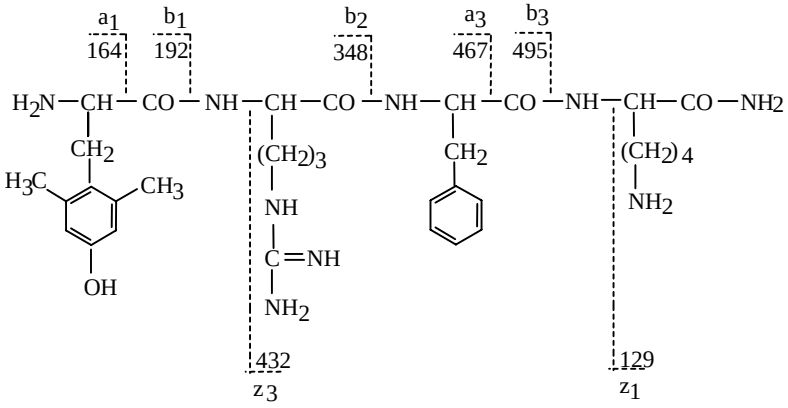

Fig. 3. The fragmentation pattern of d_0-[Dmt^1]DALDA.

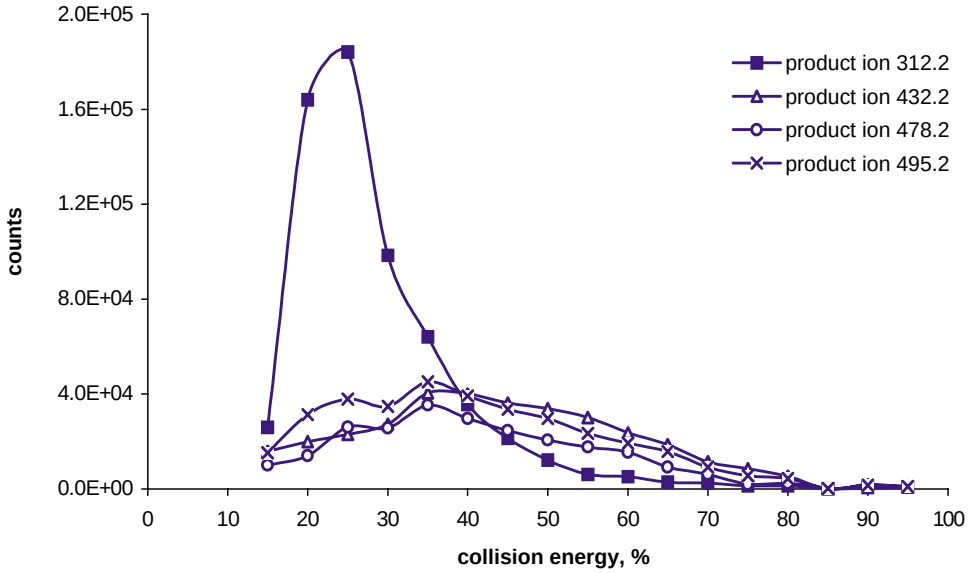

Fig. 4. The ion intensity vs. collision energy profile for the product ion produced from the $[M + 2H]^{2+}$ precursor ion of d_0-[Dmt^1]DALDA.

Table 2
Intra-day precision and accuracy for the LC/MS/MS analysis of d_0-[Dmt^1]DALDA in sheep plasma ($n = 6$)

Theoretical concentration (ng/ml)	Measured concentration (ng/ml)	Precision[a] (CV%)	Accuracy[b] (%)
16.0	15.2	7.7	−5.1
32.0	34.4	5.4	7.4
64.0	69.1	5.5	7.9
128.0	138.7	8.6	8.4

[a] Precision is expressed as the coefficient of variation (CV%; standard deviation/measured concentration).
[b] Accuracy is the measured concentration/theoretical concentration × 100.

Table 3
Inter-day precision and accuracy (across 21 days) for the LC/MS/MS analysis of standard d_0-[Dmt^1]DALDA in sheep plasma ($n = 6$)

Theoretical concentration (ng/ml)	Measured concentration (ng/ml)	Precision[a] (CV%)	Accuracy[b] (%)
16.0	15.0	9.3	−6.5
32.0	34.9	6.2	9.2
64.0	69.4	7.5	8.5
128.0	138.2	9.8	8.0

[a] Precision is expressed as the coefficient of variation (CV%; standard deviation/measured concentration).
[b] Accuracy is the measured concentration/theoretical concentration × 100.

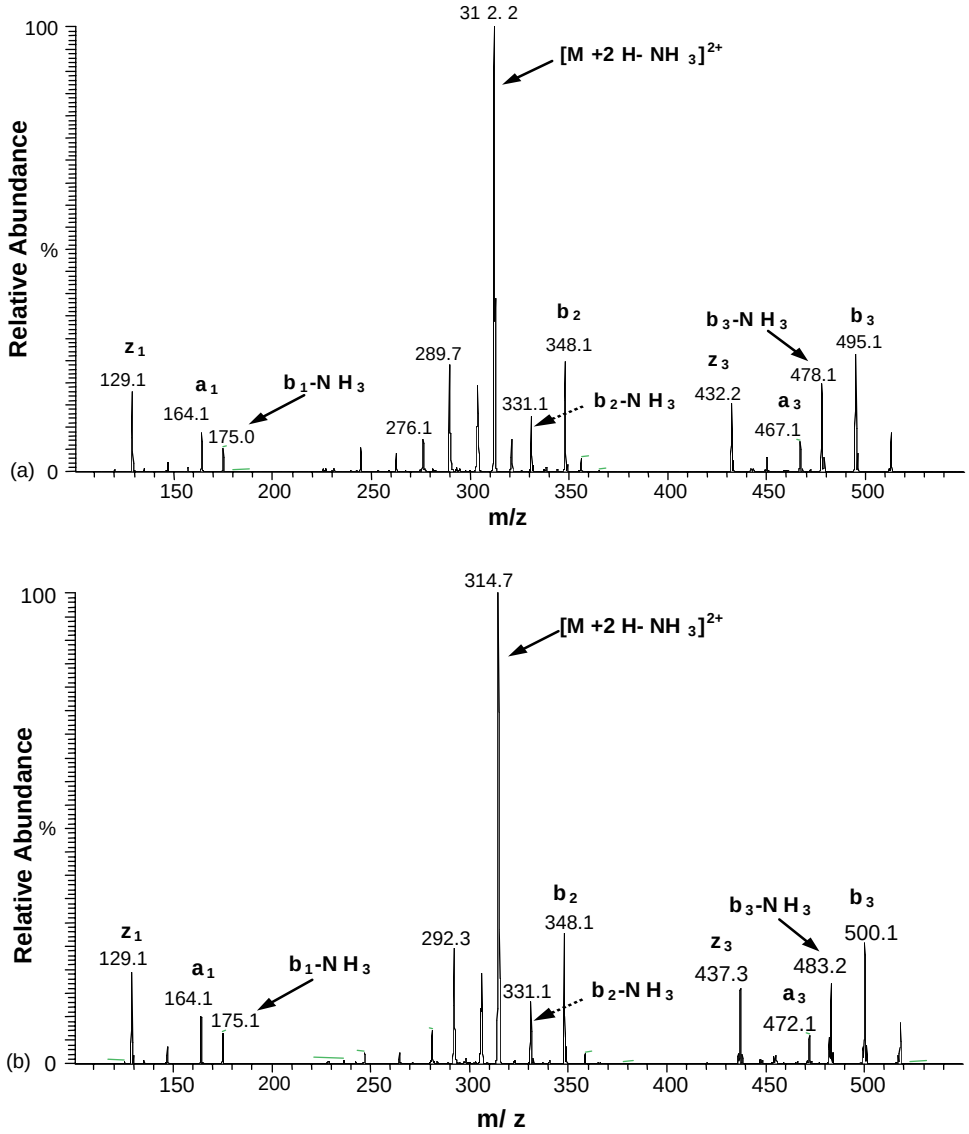

Fig. 5. The extracted product-ion chromatograms of a plasma sample from a sheep that was injected with d_0-[Dmt^1]DALDA; the sample is from the 90 min time-point. (a) m/z 312.2; the $[M + 2H{-}NH_3]^{2+}$ product ion from the $[M + 2H]^{2+}$ precursor ion of d_0-[Dmt^1]DALDA; (b) m/z 314.7; the $[M + 2H{-}NH_3]^{2+}$ product ion from the $[M + 2H]^{2+}$ precursor ion of d_5-[Dmt^1]DALDA ion.

the percentage deviation from the theoretical concentration, and the precision was expressed by CV%. The intra-day assay performance data are shown in Table 2; the accuracy was within ±8.4%, and the precision was within 8.6%. Table 3 shows the inter-day assay performance data across 21 days; the accuracy was within ±9.2%, and the precision was within 9.8%.

3.6. Analysis of [Dmt^1]DALDA in ovine plasma samples

The sheep plasma sample at time-point 0 (immediately before the sheep was injected with the d_0-[Dmt^1]DALDA) was used as the control. The extracted product-ion chromatograms (*m/z* 312.2 and 314.7) of the control plasma were obtained (not shown). All chromatography peaks were within the noise level, and the abundance was very low (<3e3 count); thus, the ions at *m/z* 312.2 and 314.7 from the control plasma sample do not interfere with the quantitative assay. Furthermore, no product ions of d_0- and d_5-[Dmt^1]DALDA were detected in the corresponding MS/MS spectrum (not shown). Fig. 5 shows the extracted product-ion chromatograms of the 90 min plasma sample from a sheep that was injected with d_0-[Dmt^1]DALDA. Abundant peaks occur at the known retention time (5.83 min) of [Dmt^1]DALDA. The ions at *m/z* 312.2 (Fig. 5(a)) and 314.7 (Fig. 5(b)) are the $[M + 2H{-}NH_3]^{2+}$ product ions from the $[M + 2H]^{2+}$ precursor ion of d_0- and d_5-[Dmt^1]DALDA, respectively. The corresponding MS/MS spectrum (Fig. 6) demonstrated the molecular specificity of the quantitative analysis of d_0- and

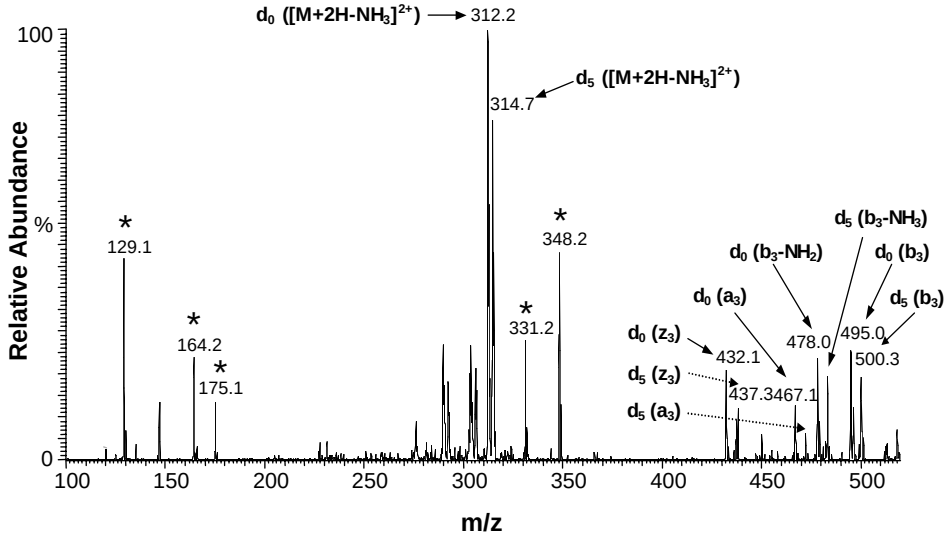

Fig. 6. MS/MS spectrum of the two $[M + 2H]^{2+}$ precursor ions (*m/z* 320.7, 323.2) from a sheep plasma sample; the sheep was injected with d_0-[Dmt^1]DALDA. The sample is from the 90 min time-point; d_5-[Dmt^1]DALDA was added as the internal standard during sample processing. Asterisks indicates the product ions that do not differ between the d_0-[Dmt^1]DALDA and d_5-[Dmt^1]DALDA.

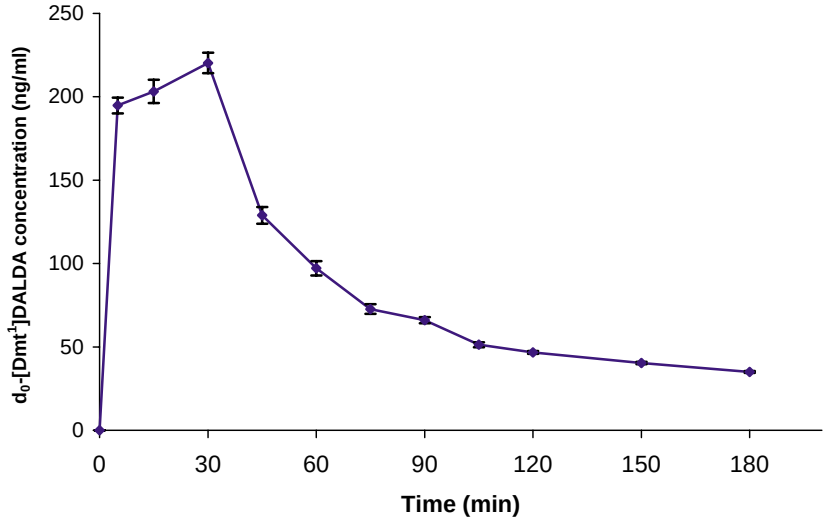

Fig. 7. Ovine plasma concentration-time profile of d_0-[Dmt^1]DALDA. The vertical bars indicate ±S.E.M. Each time-point is the average of four measurements.

d_5-[Dmt^1]DALDA; namely, the peptide that is quantified is indeed d_0-[Dmt^1]DALDA. Because the precursor window was set to transmit the $[M + 2H]^{2+}$ precursor ions of d_0- and of d_5-[Dmt^1]DALDA, the product ions of both precursor ions occur in the spectrum (Fig. 6). The $[M + 2H]^{2+}$, a_3, b_3, b_3-NH_3, and z_3 ions confirm the amino acid sequence of d_0-[Dmt^1]DALDA, and prove the existence of d_0-[Dmt^1]DALDA at that time-point.

The pharmacokinetic data were obtained by measuring a series of timed ovine plasma samples. Fig. 7 contains the ovine plasma concentration versus time-profile of d_0-[Dmt^1]DALDA.

4. Conclusion

An LC/MS/MS quantitative analysis method, using a nanospray ion-trap mass spectrometer coupled to capillary liquid chromatography, was used to quantify d_0-[Dmt^1]DALDA in ovine plasma samples. The method provides a high level of sensitivity, accuracy, precision, and molecular specificity. The high level of detection sensitivity is needed in the studies of fetal sheep plasma samples.

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