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Journal of Chromatography B, 695 (1997) 287–298

JOURNAL OF CHROMATOGRAPHY B

Mass spectrometric quantification of the mu opioid receptor agonist Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) in high-performance liquid chromatography-purified ovine plasma

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Received 29 November 1996; revised 13 March 1997; accepted 13 March 1997

Abstract

The mu opioid receptor agonist Tyr-D-Arg-Phe-Lys-Amide (*D*-Arg^2-*Lys*^{4}-*D*ermorphin$_{1-4}$amide=DALDA) was infused continuously for 2 h into sheep. The presence of DALDA in ovine plasma was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) in plasma samples that were obtained at different times during and following that infusion. A stable isotope-incorporated internal standard, deuterated DALDA (d_5-DALDA), was used for the MS quantification of DALDA via the protonated molecule ion, $(M+H)^+$, of DALDA and of d_5-DALDA. Time-course data (μg DALDA ml^{-1} plasma vs. time) were obtained. Tandem MS (MS–MS) provided the product-ion spectrum of the $(M+H)^+$ ion of DALDA in one of the samples to confirm the amino acid sequence of DALDA.

Keywords: DALDA; Tyr-D-Arg-Phe-Lys-NH_2; Opioids; Peptides

1. Introduction

Opiate alkaloid drugs are used for pain management during labor and delivery. However, because these drugs may adversely affect the mother and the fetus [1], it has been necessary to design and to test synthetic opioid peptides that minimize those adverse effects. The pharmacokinetics that are involved in a drug that is circulating in the maternal–fetal systems and the receptor activity that is displayed by the synthetic peptide must be studied during the development of drugs that reduce pain in the mother without affecting the fetus. The lipophilicity of the peptide and its transfer (passive; mediated) across the blood–brain [2] and the placental barriers are important experimental physicochemical characteristics of a peptide drug and play a role in determining the peptide's lifetime in blood. Also, the incorporation of D-amino acids eliminates the activity of certain peptidases.

The synthetic peptide Tyr-D-Arg-Phe-Lys-Amide (*D*-Arg^2-*Lys*^{4}-*D*ermorphin$_{1-4}$amide=DALDA) [3] is one of the peptides being tested in the pregnant sheep model. DALDA affects locomotor activities

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PII S0378-4347(97)00182-5

[4], controls vasopressin and oxytocin release [5] and has a peripheral antinociceptive effect [6]. It has been necessary to develop an appropriate analytical methodology to accurately analyze DALDA in maternal and fetal ovine plasma.

This manuscript describes the quantitative analysis of the mu opioid receptor agonist DALDA in those maternal plasma samples that were collected during and following a 2-h infusion of the drug. In order to optimize the molecular specificity of the analytical measurement, and to bypass the problems that had been observed with co-eluting plasma compounds, mass spectrometry (MS) is used as the post-HPLC detector. MS optimizes the molecular specificity of the detection of DALDA because the protonated molecule ion, $(M+H)^+$, of DALDA at m/z 612 is monitored. An internal standard, the deuterated DALDA analog [Tyr-D-Arg-Phe(d_5)-Lys-amide, d_5-DALDA], which is added to each ovine plasma sample before deproteinization, HPLC, and MS analysis, increases further the molecular specificity of the method. d_5-DALDA also acts as a carrier [7]. In addition to MS analysis, tandem mass spectrometry (MS–MS) [8] was used to confirm the amino acid sequence of DALDA in one of the time-course samples.

2. Experimental

2.1. Reagents

Deuterated DALDA was provided by Dr. Peter W. Schiller (Clinical Research Institute of Montreal, Canada), and its purity and amino acid sequence were confirmed by MS; DALDA and heptafluorobutyric acid (HFBA) were purchased from Sigma (St. Louis, MO, USA); trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA); and CH_3CN from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Plasma samples

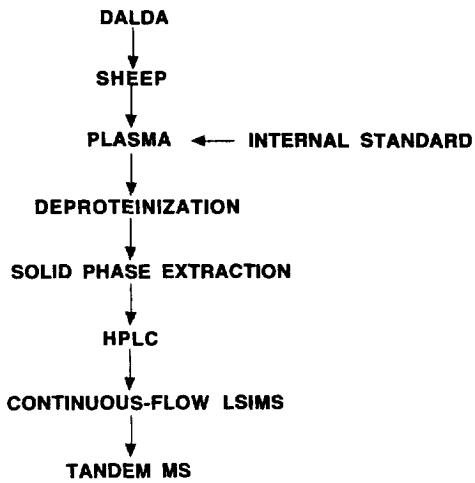
Ovine plasma (control; DALDA-infused) was provided by Dr. Hazel H. Szeto (Cornell University Medical College, New York, NY, USA) and by Dr. James Clapp (Case Western Reserve University, Cleveland, OH, USA). The sheep were infused continuously for 2 h with DALDA (0.6 mg/kg), blood was obtained at several time-points (0, 60, 90, 120, 135, 150 and 180 min), and the resulting plasma was analyzed as described in Section 2.3. Table 1 lists the three ovine plasma samples that were analyzed in this study, the volume of each plasma sample and the use of each sample.

2.3. Separation of peptides from ovine plasma

Scheme 1 illustrates the analytical procedure that was used to extract, purify and analyze DALDA from ovine plasma. Plasma (300–1200 μl) was deproteinized with a 1.5 volume of acetonitrile [9]; the mixture was centrifuged (20 min, 5600 *g*, ambient temperature), and the supernatant was evaporated under a gentle stream of nitrogen. The deproteinized plasma residue was reconstituted in TFA (0.1%, 1 ml), and was eluted through a solid-phase extraction (SPE) cartridge (Sep-Pak C_{18}, Millipore, Milford, MA, USA) that had been pre-rinsed with methanol (CH_3OH, water) and 0.1% TFA (4 ml each) [10]. After loading the sample, the cartridge was washed with TFA (0.1%, 4 ml), and

Table 1
Ovine plasma samples studied by HPLC and MS

Sheep	Plasma sample	Use
I	Control (9 ml)	Method development
II	Control (1 ml)	Method development (to demonstrate the presence of interfering compounds)
III	A. Control (1.2 ml)	Analysis of DALDA
	B. DALDA-infused (1.2 ml each) 60, 90, 120, 135, 150, 180 min	Analysis of DALDA



Scheme 1. Analytical procedure that was used to quantify DALDA in ovine plasma via the $(M+H)^+$ ion produced by LSIMS.

the peptide-enriched fraction was eluted with acetonitrile (CH_3CN, 30%, 3 ml).

2.4. RP-HPLC

A Varian 9050 variable-wavelength UV–Vis detector and a Varian 9012 solvent delivery system (Walnut Creek, CA, USA) were used for the HPLC separation of DALDA. To prepare each plasma sample for HPLC analysis, the peptide-enriched SPE fraction was dried by lyophilization, and the residue was dissolved in HFBA (2.3 m*M*, 0.5 ml). The mixture was filtered through a cellulose acetate centrifuge filter unit (SPIN-X, 0.22 μm pore size, Costar, Cambridge, MA, USA); and peptides were separated by RP-HPLC. The sample was chromatographed on an RP-analytical column (Delta Pak, 5 μ, C_{18}, 100 Å, 150×3.9 mm; Waters, Milford, MA, USA) at a flow-rate of 1.5 ml min^{-1}. UV absorption was monitored at 200 nm.

A volatile buffer is needed for the MS analysis of an HPLC-purified fraction. Because DALDA is a very polar tetrapeptide, the HFBA buffer (2.3 m*M*), which was used previously for the HPLC separation of the polar tetrapeptide tuftsin in human plasma [11], was chosen.

CH_3CN was used as the organic modifier. 1-min fractions were collected, and each fraction was lyophilized for MS analysis.

2.5. Mass spectrometry

2.5.1. MS instrument

An AutoSpecQ tandem mass spectrometer (Micromass, Altrincham, UK) was used in these experiments. The MS-1 portion consisted of E_1BE_2 (E= electric field, B=magnetic field), and MS-2 consisted [following a quadrupole collision cell (q)] of a quadrupole mass analyzer (Q). VG Opus level V3.3 software was used.

2.5.2. Continuous flow-liquid secondary ion mass spectrometry (cf-LSIMS)

For the cf-LSIMS measurement of the protonated molecule ion, $(M+H)^+$, of DALDA (molecular mass 611 Da), Cs^+ ions with an energy of 35 keV were used [8]. The ion source accelerating voltage was 8 kV. The ion source temperature was 60°C. A cf-LSIMS probe, outfitted with a 20-μl Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA), was used for sample introduction. A syringe pump (Model 22, Harvard Apparatus, South Natick, MA, USA) provided the solvent flow [water–CH_3OH_3 (1:1) with 2% (v/v) glycerol and 1% (v/v) acetic acid] through the cf-LSIMS probe at a constant flow-rate of 5 μl min^{-1} (a flow accuracy of ±1% was specified by the manufacturer). The lyophilized sample was reconstituted with the HFBA buffer (2.3 m*M*, 50 μl), and was filtered (Spin-X filter); 2 μl (4%) was used for injection. Mass resolution was ca. 1200 at *m/z* 393, the limited mass scan range was *m/z* 608–624 and the B-scan rate was 3 s/decade.

2.5.3. Quantification by cf-LSIMS

Cf-LSIMS was used to quantify DALDA in the sheep plasma samples. The $(M+H)^+$ ion current for DALDA at *m/z* 612 (±0.5 Da) was compared to the ion current from d_5-DALDA at *m/z* 617 (±0.5 Da). The total ion current at *m/z* 612 and at *m/z* 617 was accumulated during the elution of the entire volume of each injected sample. The ratio of those two ion currents was multiplied by the amount (5 μg) of d_5-DALDA internal standard that was added to each sample (see Scheme 1), and the amount of DALDA in that sample was calculated.

2.5.4. Tandem MS

In the tandem mass spectrometry (MS–MS) mode, the precursor ion was selected by MS-1 with a resolution of ca. 1200. The precursor ion was the $(M+H)^+$ ion (m/z 612) of DALDA. The precursor ion collided with helium in the collision cell (q), and MS-2 was scanned (4 s/scan over the range m/z 100–700) to obtain the product-ion spectrum of DALDA, which contained most, if not all, of the amino acid sequence-determining fragment ions [8]. The product-ion spectrum of synthetic DALDA contains a_{1-3}, b_{2-3}, c_{1-3}, y_{1-3} and z_{1-2} ions (see data in Section 3.7); the ion nomenclature is defined elsewhere [12,13]. The collision energy (10 eV) was optimized for DALDA.

3. Results and discussion

3.1. Different HPLC chromatograms for plasma controls

A linear HPLC gradient [30 min, 10 to 30% CH_3CN (0.67% min^{-1}) in HFBA] separated DALDA and d_5-DALDA from the components in the control plasma I that was used for method development. Most plasma components elute at a low percentage of organic modifier. Fig. 1a represents the HPLC chromatogram of synthetic DALDA (5 μg injected, retention time of 19.6±0.2 min, n=5), and Fig. 1b the chromatogram of control plasma I (see Table 1). No UV-absorbing plasma peaks were observed at the retention time of DALDA in the control plasma I chromatogram. But, we noticed during the course of this study that each ovine plasma had a different HPLC profile, and in many cases co-eluting (or nearly co-eluting) peaks interfered with DALDA. For example, Fig. 1c represents the chromatogram of control plasma II; a large peak is observed at 19.0 min, which is near the retention time of synthetic DALDA (19.6 min).

3.2. MS as the post-HPLC detector

An interfering peak could be separated from DALDA by an appropriate adjustment of the HPLC experimental conditions (flow-rate, molarity, gradient, etc.). However, because each ovine plasma had a different chromatogram, that adjustment process would not be time-effective. Therefore, because DALDA could not be quantified by using only HPLC due to interfering peaks, it was necessary to use a post-HPLC detector that had a very high level of molecular specificity. MS was chosen for that purpose. MS monitored the $(M+H)^+$ ion of DALDA at m/z 612 and of d_5-DALDA at m/z 617 in each HPLC-purified fraction. Thus, by scanning the mass spectrometer over a limited mass range (m/z 608–624), the presence of DALDA and d_5-DALDA in any HPLC fraction is readily detected. The limit of detection of this particular mode of MS analysis was ca. 1 ng (1.6 pmol) for synthetic DALDA.

3.3. Peptide recovery

The recovery of DALDA and d_5-DALDA, either alone or in plasma, was determined after HPLC. Synthetic DALDA and d_5-DALDA were treated as samples (see Scheme 1), and the HPLC peak area of each peptide was determined. The recovery was calculated by comparing the extracted sample peak area with the peak area of the synthetic peptides that were injected directly. The post-HPLC recovery of DALDA and of d_5-DALDA, without plasma and in the presence of plasma, was approximately the same (71–76%).

3.4. LSIMS $(M+H)^+$ data

Monitoring the $(M+H)^+$ ion of a peptide is an important piece of experimental data to demonstrate the presence of DALDA and d_5-DALDA in an ovine plasma sample. Fig. 2 represents the LSIMS B-scan mass spectrum of synthetic DALDA that was applied directly onto the LSIMS probe. The DALDA $(M+H)^+$ ion is observed at m/z 612. In this study, we conclude that any HPLC fraction that contains this m/z 612 ion above a ca. 3:1 signal-to-noise level contains DALDA.

The $(M+H)^+$ ion of DALDA and of d_5-DALDA was confirmed in spiked plasma samples. Fig. 3 shows the cf-LSIMS mass spectra, obtained over the limited mass range of m/z 608–624, of (Fig. 3a) an equimolar mixture of synthetic DALDA plus d_5-DALDA, (Fig. 3b) the HPLC fraction of control plasma I corresponding to DALDA retention time,

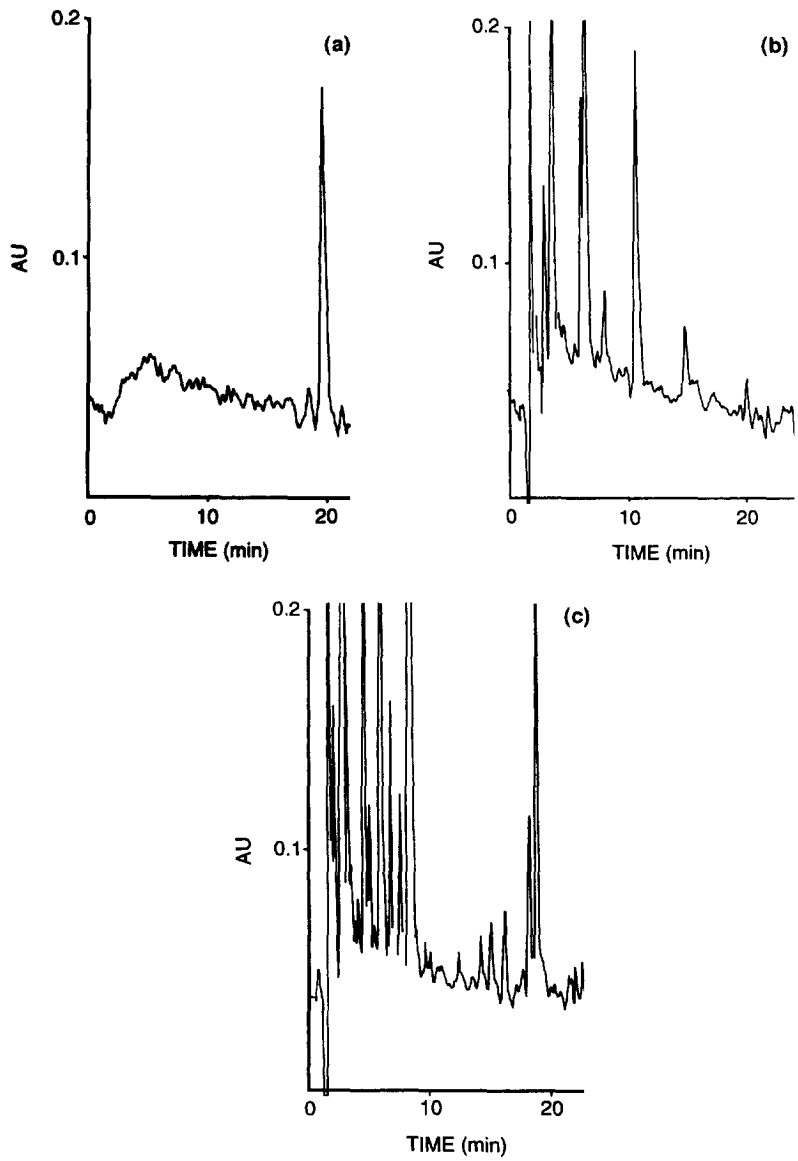


Fig. 1. HPLC chromatograms of (a) synthetic DALDA (5 μg injected), which has a retention time of 19.6 min; (b) control plasma I (600 μl) used to develop the analytical method and (c) control plasma II (600 μl) that represents ovine plasma obtained from a different sheep. See plasma nomenclature in Table 1. Data were obtained at the following experimental conditions: λ=200 nm; flow-rate=1.5 ml/min; solvent A: 2.3 m*M* HFBA in water; solvent B: 100% CH_3CN; gradient: 10 to 30% B in 30 min (0.67% min^{-1}); AUFS=0.2.

and (Fig. 3c) the HPLC fraction of control plasma I that was spiked with those two peptides. No ions (significantly above the noise level) were observed at *m/z* 612 or 617 in control plasma I (Fig. 3b). Those spectra represent measurements made on different samples, different injections, different times, different days and different instrumental conditions. Any differences observed provide an excellent reason why an internal standard is needed for each and every measurement.

The shorter amount of MS scan-time that is required to cover the limited mass range of *m/z*

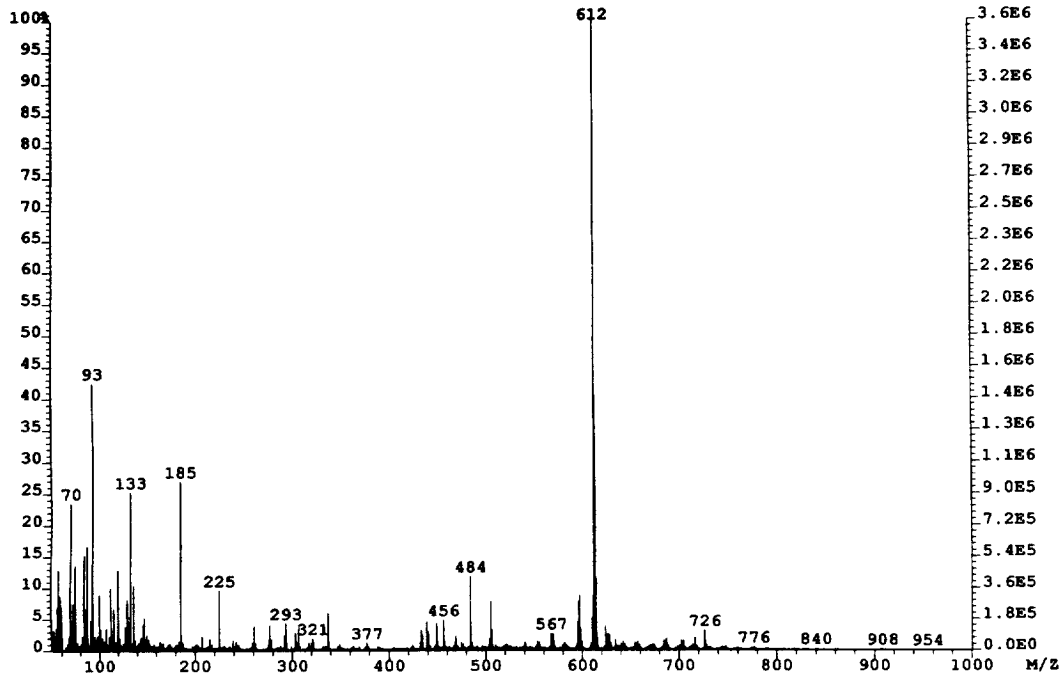


Fig. 2. LSIMS (B-scan; probe) mass spectrum of synthetic DALDA (approx. 1 μg; summation of six individual spectra). The ion at *m/z* 612 corresponds to the $(M+H)^+$ ion of DALDA (molecular mass 611 Da).

608-624 converts directly into a higher level of detection sensitivity compared to a full mass scan. The $(M+H)^+$ ion of DALDA is observed at *m/z* 612 and of d_5-DALDA at *m/z* 617 in Fig. 3a and 3c. The signal-to-noise ratio for these data is approx. 10:1.

3.5. Reproducibility of cf-LSIMS $(M+H)^+$ data

The reproducibility of the cf-LSIMS method of analyzing the $(M+H)^+$ ion of DALDA and of d_5-DALDA was determined because the ratio of the DALDA:d_5-DALDA $(M+H)^+$ ions was used to quantify DALDA in plasma samples. The reproducibility of the ratio of the DALDA:d_5-DALDA $(M+H)^+$ ions in the cf-LSIMS spectra was measured by ten consecutive injections of an equimolar solution of DALDA plus d_5-DALDA. The mean ratio of the amount of DALDA to d_5-DALDA was 0.94, with a relative standard deviation ±3.1% ($n=10$).

3.6. Analysis of DALDA in ovine plasma

This analytical system was used to determine the amount of DALDA in sample set IIIB (see Table 1), which contains ovine plasma samples obtained at six points in time (60, 90, 120, 135, 150 and 180 min) during and following a 2-h continuous infusion of DALDA. The internal standard, d_5-DALDA (5 μg), was added to each sample and to control plasma IIIA (see Scheme 1). The chromatograms of control plasma IIIA (Fig. 4a), control plasma IIIA spiked with an equimolar mixture of DALDA and d_5-DALDA (5 μg each, Fig. 4b) and DALDA-infused sample IIIB-90 min (Fig. 4c) are shown. In this particular set of ovine plasma samples, no UV-absorbing plasma components eluted at the retention time of DALDA [a new RP-HPLC analytical column was required at this stage of analysis; the retention time of synthetic DALDA shifted systematically from 19.6 min (see Fig. 1) to 20.4 min].

Cf-LSIMS monitored the $(M+H)^+$ ion of the appropriate HPLC fractions of DALDA and of d_5-DALDA, and the amount of the DALDA was calculated from the ratio between those two $(M+H)^+$ ion currents. Fig. 5 represents the profile of DALDA in sample IIIB (μg DALDA ml^{-1} plasma vs. time). A high level of confidence is associated with each data point because of the above-mentioned

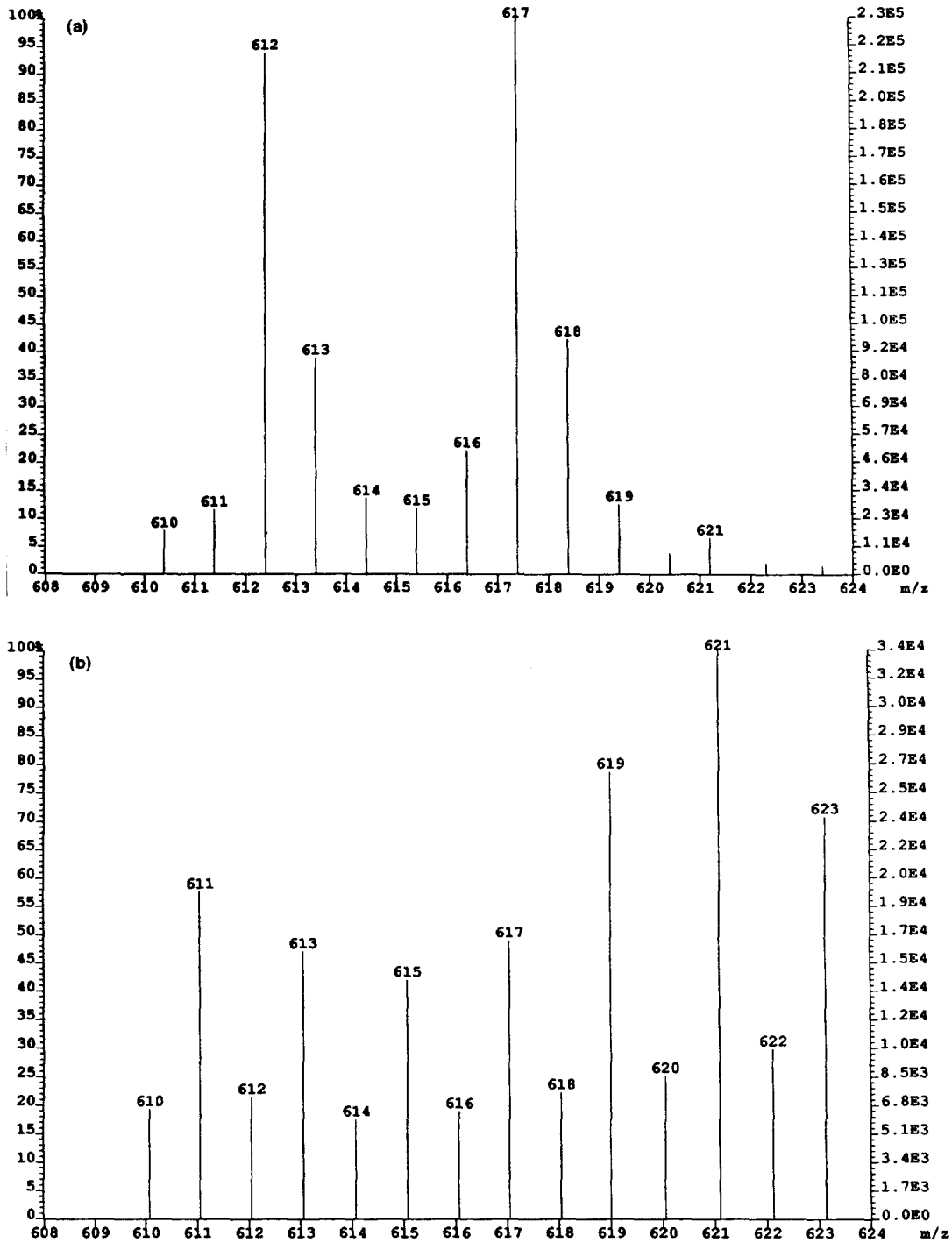


Fig. 3. Cf-LSIMS spectra, obtained over the limited mass range of m/z 608–624 of (a) an equimolar mixure of synthetic DALDA and d_5-DALDA (100 ng each); (b) HPLC-purified control plasma I (300 μl plasma after SPE and HPLC; the fraction corresponding to the retention time of DALDA was collected, dried and dissolved, as described in Section 2.4; and (c) HPLC-purified control plasma I (300 μl) that had been spiked with an equimolar mixture of DALDA and d_5-DALDA (5 μg each). The scans shown are the summation of 119, 461 and 207 individual scans, respectively.

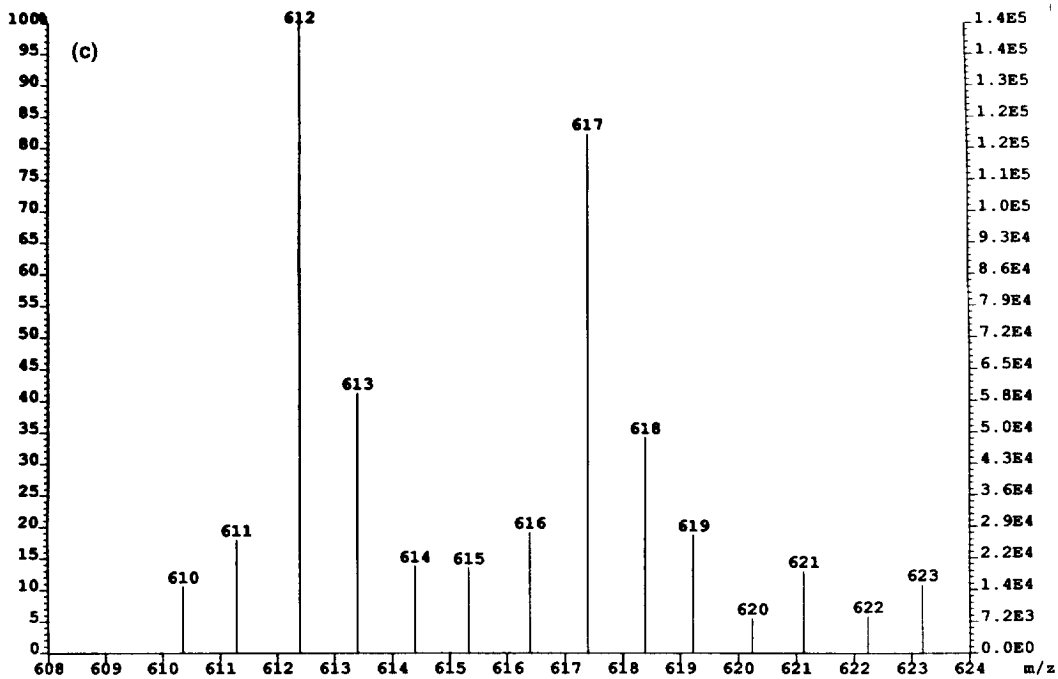


Fig. 3. (*continued*)

reproducibility of the measurement of the ratio of the $(M+H)^+$ ions of DALDA:d_5-DALDA. The concentration of DALDA in that sample increased from 0 to 120 min, and decreased to 180 min. That profile is commensurate with the pattern that would be expected for the constant infusion of a peptide drug that is metabolically stable; namely, an increase of DALDA in the plasma during the infusion (up to 120 min), followed by a decrease after the infusion was stopped. Because MS and a deuterated internal standard were used to measure the amount of DALDA in this set of ovine plasma samples (IIIB), a very high level of confidence is conveyed that only DALDA was quantified.

The development of a generalized analytical method for the measurement of peptides was not the purpose of this research, and therefore calibration curves, recoveries and method accuracy were not performed. The precision is given in the data shown.

This manuscript represents a focused attempt to measure a particular peptide in a set of ovine plasma samples because we are now in the middle of processing a large number of ovine plasma samples.

The expected range of the amount of peptide was known, a deuterated synthetic peptide analog internal standard is available, and the reproducibility of measuring the ratio of the ion currents is known. Therefore, the methodology described in this manuscript was applied and shown to work very well.

3.7. Tandem MS–MS confirmation of DALDA

The molecular specificity attained by using the $(M+H)^+$ ion for the measurement of DALDA in ovine plasma samples is higher than almost all other analytical methods. However, that molecular specificity can be increased even further by using MS–MS.

MS–MS links, in each quantitative measurement, the $(M+H)^+$ ion of DALDA with all of its product ions, which are many, if not all, of the amino acid sequence-determining fragment ions.

For example, Fig. 6a represents the low-energy product-ion mass spectrum obtained from the $(M+H)^+$ ion at m/z 612 from synthetic DALDA. Amino acid sequence-determining fragment ions are indicated on the fragmentation pattern [12,13] that is shown in Scheme 2, and by the labels on the specific

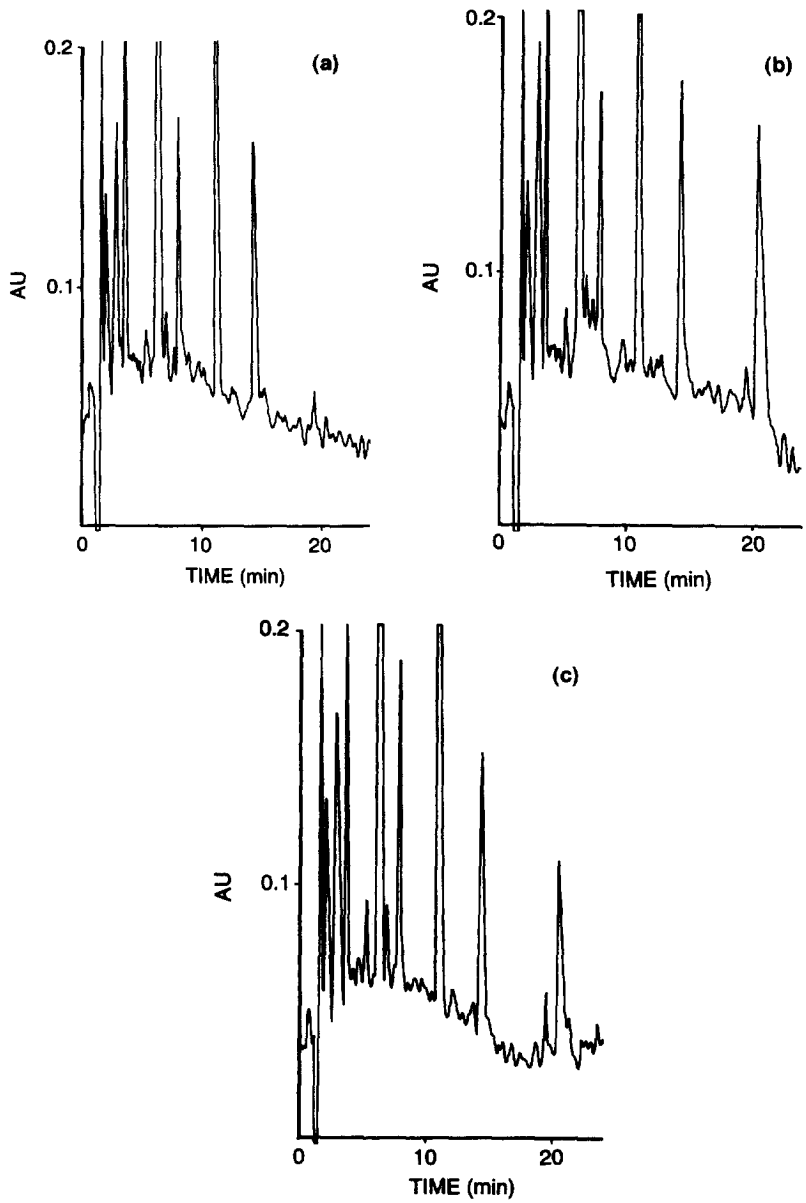


Fig. 4. RP-HPLC chromatograms from ovine plasma III (see Fig. 1 for the HPLC experimental conditions; a new RP-HPLC column was needed, and the retention time of synthetic DALDA shifted systematically from 19.6 in Fig. 1 to 20.4 min): (a) control plasma IIIA (600 μl); (b) control plasma IIIA (600 μl) spiked with an equimolar mixure of synthetic DALDA and d_5-DALDA (5 μg each) and (c) DALDA-infused sample IIIB-90 min (1.2 ml).

ions noted in the product ion spectrum (Fig. 6a). Amino acid sequence-determining fragment ions are observed at a_{1-3}, b_{2-3}, c_{2-3}, y_{1-3} and z_{1-3}. Thus, N-terminal and C-terminal amino acid sequence-determining fragment ions are observed in this product-ion spectrum. The genesis of the ions at m/z 303 and m/z 521 are discussed elsewhere [8].

The product-ion spectrum of the m/z 612 ion that occurred in the spectrum of IIIB-90 min sample is shown in Fig. 6b. That spectrum is equivalent

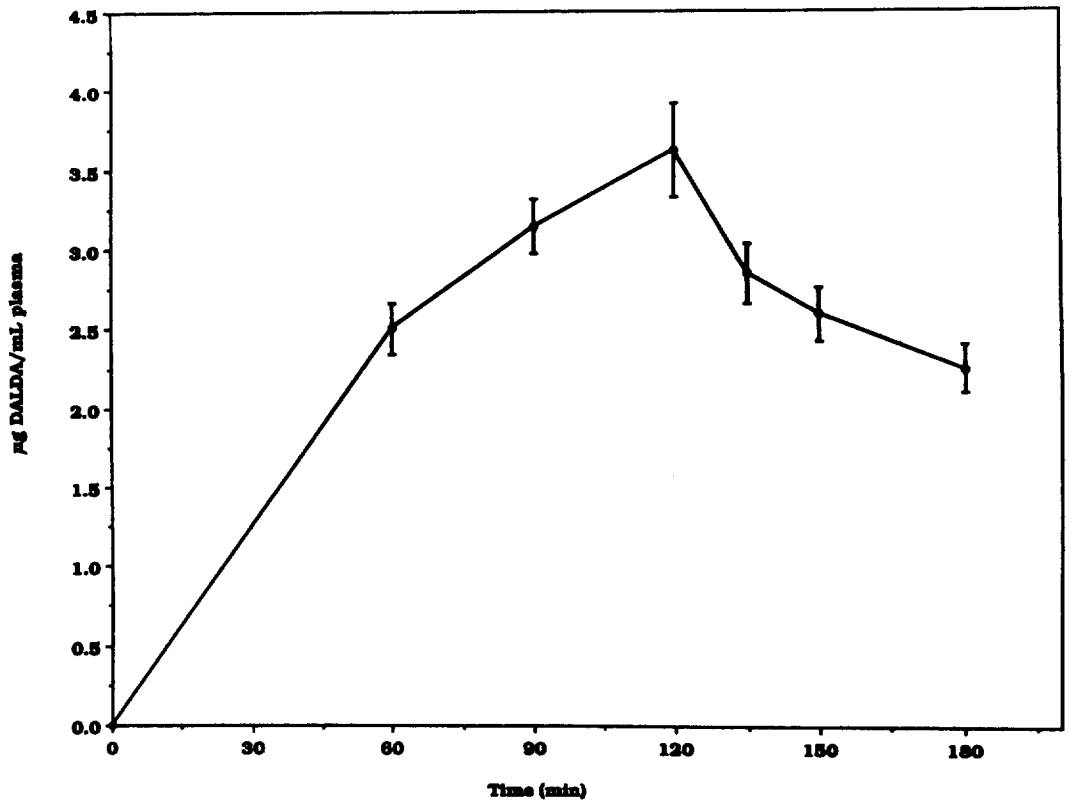


Fig. 5. Time-course profile of the DALDA that was measured in ovine plasma IIIB. The amount of DALDA (μg ml^{-1} plasma) is plotted vs. infusion time (min). Each time-point is the average of five measurements.

qualitatively (the presence or absence of amino acid sequence-determining fragment ions) and nearly quantitatively (the relative intensity of individual product ions) to the spectrum of synthetic DALDA (Fig. 6a). Thus, one can conclude unambiguously that the peak seen at *m/z* 612 for that ovine plasma sample corresponds to DALDA.

This type of time-course study will also be used to monitor the level of DALDA in ovine plasma following a bolus injection of DALDA into pregnant sheep.

4. Conclusions

The experimental data described in this manuscript have demonstrated that: (1) DALDA was quantified in ovine plasma by cf-LSIMS; (2) the amino acid sequence of DALDA was confirmed by MS–MS and (3) the amount of DALDA increased, then decreased, in the continuous infusion experiment.

These analytical data are important for pharmacokinetic studies (to be reported elsewhere) of the mu opioid receptor agonist DALDA. These data will be used to corroborate or to refute the data obtained from pharmacokinetic and physiologic studies, and will help to explain drug half-life and potency.

Acknowledgments

The authors gratefully acknowledge the scientific collaboration of Dr. Peter W. Schiller (Clinical Research Institute of Montreal, Canada), Dr. Hazel H. Szeto (Cornell University Medical College, New York, NY, USA) and Drs. James Clapp and Nicolina Olariu (Case Western Reserve University, Cleveland, OH, USA); Genevieve H. Fridland for technical

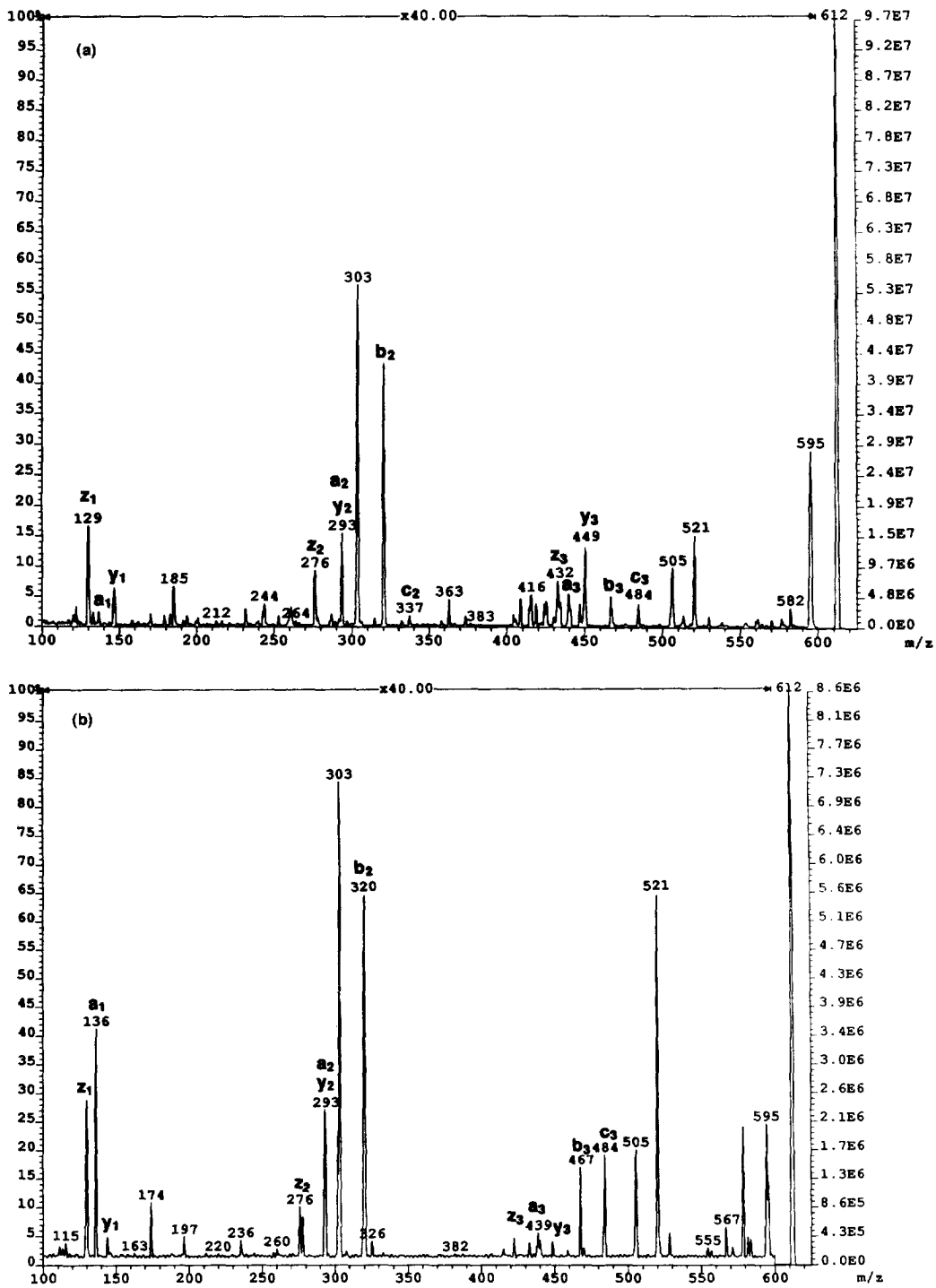
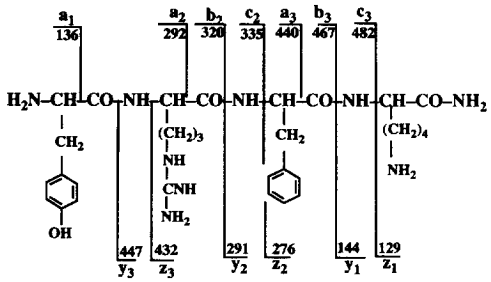


Fig. 6. The low-energy LSIMS MS–MS product-ion spectra of the $(M+H)^+$ ion of (a) synthetic DALDA (approx. 1 μg) and (b) the HPLC-purified DALDA that was extracted from the IIIB-90 min sample (see the time-course data in Fig. 5). The observed amino acid sequence-determining fragment ions are noted above their respective m/z peaks (see Scheme 2). Both spectra correspond to the summation of sixteen spectra.



Scheme 2. The low-energy MS–MS fragmentation pattern observed for synthetic DALDA [8]. Ion nomenclature follows Biemann [12] and Fohlman and Roepstorff [13].

assistance and for helpful discussions on this manuscript; Vicki Antwine for assistance in manuscript preparation; and NIDA (08924) for financial support.

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