Capillary reversed-phase high-performance liquid chromatographic determination of acetyl-methylprednisolone in feline spinal cord

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Abstract

Capillary reversed-phase high-performance liquid chromatography (RP-HPLC) was used to determine acetyl-methylprednisolone (A-MP) that had been administered to feline spinal cord tissue. The method used a 300 mm × 0.32 mm I.D. packed capillary octadecylsilyl (ODS) column and an isocratic mobile phase of 40 mM triethylamine formate (TEAF, pH 3.2)–acetonitrile (50:50, v:v). The chromatographic behavior of A-MP was evaluated with respect to peak-area and peak-height by varying the A-MP concentration (12–190 μM) with a fixed injection volume (1 μl), and by varying the injection volume (1–10 μl) with a fixed concentration (12 μM) of A-MP. The limit of detection (signal-to-noise ratio, 3:1) was 250 pg (600 fmol) of synthetic A-MP. Various amounts of A-MP directly spiked into feline spinal cord segments were solvent extracted, separated, and plotted against peak-area ($r^2 = 1.00$). Background tissue without A-MP gives minimal (< 1%) interference at 243 nm. The method also detects exogenous A-MP that was administered into feline spinal cord via an intrathecal injection. Furthermore, the presence of A-MP was confirmed via its molecular ion and corresponding product ions that were obtained by fast-atom bombardment tandem mass spectrometry (FAB-MS–MS).

1. Introduction

Compared to conventional standard-bore (4.6 mm I.D.) high-performance liquid chromatography (HPLC), packed capillary (0.2–0.35 mm I.D.) HPLC offers the advantages of increased separation efficiency and mass sensitivity, de-creased peak volume, low reagent/sample consumption, and the ease of interfacing to a mass spectrometer without any post-column flow split [1–6]. Despite the conventional hardware limitations for low flow-rate (< 10 μl/min), most researchers [1–3,6–8] use a pre-column split-flow technique (T-split system) to regulate the desired flow velocity of solvent traveling through the packed capillary column. Furthermore, a fused-

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silica capillary "on-column" detector (e.g., UV) or a specially designed low-volume (<1 μl) detector cell is now commercially available. This micro-scale HPLC technique has been used to separate and to analyze biomolecules [1,3,6–8,10–12] and small molecules [2,4,5,9], and was chosen here for the analysis of A-MP in feline spinal cord.

Methylprednisolone (MP), a synthetic anti-inflammatory glucocorticoid, is used clinically for many applications. In our area of interest, acute spinal cord injury, current clinical protocols use methylprednisolone sodium succinate (MPSS) [13–15]. Free MP, the biologically active metabolite that crosses the blood–brain barrier, is released from MPSS by liver esterases [13]. High doses of MPSS, 30 mg/kg bolus followed by 5.4 mg/(kg h) for 23 h, are injected intravenously following acute spinal cord injury [15,16]. The second National Acute Spinal Cord Injury Study (NASCIS) has shown that this treatment regimen using MPSS improves neurologic outcome following an acute spinal cord injury [14,16]. The neuroprotective effect of MP is believed to be mediated through an inhibition of free radical formation and lipid peroxidation [13]. These protective effects have been shown to be optimum in vitro at an MP level of 30 μg/ml [17].

We believe that we can avoid the high plasma levels of MP that are associated with the established intravenous route, and that we can provide more beneficial tissue levels by intrathecal administration. Previous work with radiolabeled A-MP has indicated that the central nervous system (CNS) uptake of A-MP is five to ten times higher than what could be attained by systemic injection [18]. That research suggests that the lipophilicity of A-MP allows its adequate absorption into CNS tissues when A-MP is injected into the intrathecal space. Therefore, because of its lipophilicity and biological activity [19], A-MP was selected over MPSS for our study. Accordingly, this study was designed to provide an analytical method for determining the spinal cord tissue concentration of A-MP following intrathecal administration into the cat. Tissue concentrations of A-MP determined by this method will be used to formulate the proper dosage and dosing schedule to study the clinical efficacy of intrathecal administration of A-MP following acute spinal cord injury.

A normal-phase HPLC method has been developed to quantify and to correlate the uptake of MP into the injured cat spinal cord [20]. However, because A-MP but not MP was our analyte of interest, the use of that normal-phase method without extensive further investigation was precluded. RP-HPLC has also been described for the analysis of MP in polar biological fluids [21,22], but not in the non-polar CNS tissues. Therefore, in this paper, we describe a novel, rapid, and sensitive chromatographic assay for determining A-MP in feline spinal cord tissue using isocratic capillary RP-HPLC with UV detection at 243 nm, fast atom bombardment (FAB) mass spectrometry (MS) to produce the protonated molecule ion, $(M + H)^+$, at m/z 417, of A-MP, and MS/MS to produce the product ions from $(M + H)^+$.

2. Experimental

2.1. Reagents and materials

Synthetic A-MP (DEPO-MEDROL), (IUPAC): 11β,17α,21-trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione-21-acetate, was obtained from The Upjohn Company, Kalamazoo, MI, USA. The volatile TEAF ion-pairing buffer (40 mM) was prepared from formic acid (J.T. Baker, Phillipsburg, NJ, USA), which was titrated to pH 3.2 with triethylamine (Pierce, Rockford, IL, USA). HPLC-grade acetonitrile and glacial acetic acid were also obtained from J.T. Baker. A capillary (300 mm × 320 μm I.D.) packed with 5-μm ODS was purchased from LC Packings, San Francisco, CA, USA. Fused-silica capillary (50 μm I.D., 360 μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Instrumentation

HPLC

The HPLC system was composed of two Waters Chromatography (Millipore, Milford, MA, USA) pumps (Models M-45 and 6000A

Solvent Delivery Systems) and a Waters Chromatography Model 660 Solvent Programmer. The Model 6000A pump is equipped with a dynamic mixer, enabling a thorough mixing of solvents from the two pumps. A Rheodyne Model 7125 injector (Alltech Associates, Deerfield, IL, USA) with a 10-μl injection loop was used. With the exception of the stainless-steel tubing for the direct connection to the pumps, all other tubing used fused-silica capillaries to minimize the volume external to the column. The design of the splitter-T to regulate the flow of the mobile phase traveling through the HPLC column is similar to that described by Huang and Henion [1]. In our case, a flow-split capillary (63 cm × 50 μm I.D.) was empirically determined to provide a capillary HPLC flow-rate of 5–6 μl/min. This flow-split was ca. 2% of the combined pump flow-rate (0.3 ml/min). The post-column tubing is a fused-silica capillary (2.7 μl internal volume, 140 cm long, 50 μm I.D.). The polyimide on the capillary exterior was burned off at 115 ± 0.2 cm from the column outlet to provide a window for UV detection. The variable wavelength (190–360 nm) UV detector in the ISCO Model 3140 Electropherograph (ISCO, Lincoln, NE, USA) was used. The electropherograph was independently controlled via an IBM Personal System/2 Model 30 286 computer (IBM, Armonk, NY, USA) and ISCO ICE 3.1.0 level software.

Mass spectrometry

An AutoSpecQ hybrid (E_1BE_2qQ) mass spectrometer (VG-Fisons, Altrincham, U.K.), consisting of E_1BE_2 for MS-1 and qQ for MS-2, was used to optimize the molecular specificity for detecting A-MP. The $(M + H)^-$ at m/z 417 of A-MP was produced in MS-1, and structure-elucidating product ions from that $(M + H)^-$ precursor ion were collected in MS-2. Operation of this instrument and data collection/analysis were controlled by the VG Opus level 1.7f software.

2.3. Methods

Animal and tissue preparation

Anesthetized adult felines underwent T12 to L2 bilateral laminectomies to expose the dura-

covered spinal cord. Using a weight-drop injury technique similar to that described by Hall and Braughler [23], a 600 g/cm force was administered to each animal. After (30 min) the injury, 4.35 mg/kg A-MP was injected into the intrathecal space. Based on the relationship of brain weight to body weight in felines [24], the dose of A-MP was estimated to provide a CNS tissue level of 30 μg/g. The animal was sacrificed with a pentobarbital overdose 30 min after A-MP injection. The spinal cord from T12 to L2 (ca. 4 cm) was removed, sectioned into 5 equal segments, and frozen in liquid nitrogen. The tissue samples were weighed, and prepared by the lipophilic extraction method described by McGinley et al. [20].

Capillary high-performance liquid chromatography

The isocratic mobile-phase was TEAF (buffer A) and acetonitrile (buffer B) in a ratio of 50:50 (v:v). The flow-rate was ca. 5 μl /min. The UV absorption of A-MP was monitored at 243 nm (λ_{max} for MP) [25]. Whenever necessary (deterioration of peak parameters), the ODS capillary column was cleaned with isopropanol and acetonitrile; also, 10 μl of sample solvent (see below) was injected between tissue samples to avoid memory effects. Injection volumes ranged between 1–10 μl, as indicated. Synthetic A-MP (250 pg to 80 ng) was dissolved in 35% acetonitrile containing 2% acetic acid, and each processed tissue sample (see Table 1) was dissolved in 150 μl of this sample solvent. For each tissue sample, a volume of 100 μl was transferred to a

Table 1
Determination of A-MP in cat spinal cord tissue segments

Sample	Weight (g)	μg A-MP/g tissue[a]
Distant cephalad	0.124	2
2 Segments cephalad	0.133	107
1 Segment cephalad	0.141	3755
Injury site	0.201	291
1 Segment caudad	0.148	50
2 Segments caudad	0.193	15
Distant caudad	0.492	3

[a] Average of two determinations; average %difference is < 8%.

0.45-μm Nylon membrane (Spin-X 8170, Cambridge, MA, USA), which was centrifuged in a microcentrifuge (Compact 10/Compac 10V Centrifuge, Fisher Scientific, Pittsburgh, PA, USA). The filtrate (10 μl) was injected into the capillary column. Further dilution of some samples was necessary. A-MP fractions from tissue samples and non-tissue samples were collected and lyophilized. Those lyophilized samples were dissolved in a solvent [5 μl, CH$_3$OH–H$_2$O, 1:1 (v:v), containing 1% acetic acid]. Aliquots of 1 μl of each solution was used for MS analysis, and the remaining portion for MS/MS analysis.

Chromatographic peak-height and peak-area (peak-height $\times$ peak-width at 1/2 peak-height) were calculated manually from the computer traces. Peak-height was correlated to absorbance units.

Mass spectrometry

The Cs$^+$ ion gun was operated at 35 kV for sample ionization. The ion source acceleration voltage was 8 kV. The glycerol matrix (ca. 1 μl) was deposited onto the stainless-steel probe tip, which was rinsed with methanol between sample applications. In the MS mode, resolution was ca. 1000 at m/z 393, the scan range was from 398 to 419, and the B-scan rate was 3 s/decade. In the MS/MS mode, the precursor ion was selected by MS-1, with a resolving power of ca. 1000. The precursor ion collided with helium in the collision cell (q), and the quadrupole field (Q) was used to scan the product ion spectrum. The collision energy was optimized at 40 eV. The collision gas pressure was adjusted to reduce the intensity of the precursor ion by ca. 50%, and the scan speed of Q was 5 s/scan (50–450 u). The resolution of MS-2 was ca. 5, which is a lower than normal value to increase sensitivity.

3. Results and discussion

Fig. 1 shows the structure of A-MP; the acetyl group is on C21. A-MP is practically insoluble in water. Again, this distinct lipophilic property of A-MP plus its biological activity [19] allow for the direct and favorable absorption of A-MP into
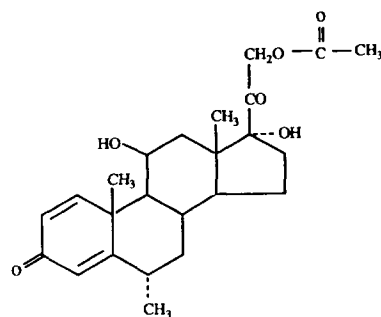

Fig. 1. Chemical structure of A-MP. The acetyl group is on C21. The elemental composition of A-MP is C$_{24}$H$_{32}$O$_6$, corresponding to a molecular mass of 416.220.

CNS tissue. This anti-inflammatory drug is therefore suitable to be administered intrathecally for treating and targeting the acute spinal injury site.

Acetonitrile (35%) in the mobile phase was used in the analysis of MP in human serum by RP-HPLC [21]. A similar percentage of acetonitrile in our study also gave an MP retention time of < 10 min, but a significantly longer retention time (> 20 min) for A-MP. Increasing the acetonitrile concentration to 50% decreased the retention time of A-MP to ca. 11 min. The acetonitrile content was not increased further in an effort to minimize the risk of encountering any interference from the HPLC solvent front.

Capillary HPLC was chosen over conventional HPLC because of the advantages mentioned above. Although the capillary HPLC technique is already a more sensitive method, it is imperative that we explore the "loadability" of sample because of the significantly lower injection volume (≤ 1 μl) [1,2,5,7,8–11] typically used in a capillary system. This information is important, especially when a relatively large volume of sample must be injected. Therefore, the dose-response of synthetic A-MP in our system was examined via two methods: (a) a fixed injection volume (1 μl) of different concentrations of A-MP; (b) different injection volumes (1–10 μl) of a fixed sample concentration (12 μM).

These two variations for increasing the amount of injected A-MP were examined with respect to chromatographic peak-area. A linear ($r^2 = 1.00$; equation: $y = 13.24x + 9.45 \cdot 10^{-5}$) relationship
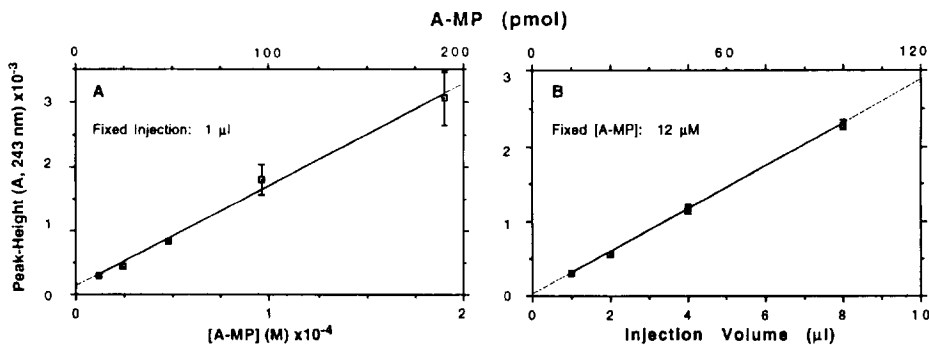
Fig. 2. Peak-height versus (A) [A-MP] and (B) injection volume. In Fig. 2A, the amount (pmol, top x-axis) of A-MP was varied by injecting 1 μl of five different concentrations (12, 24, 48, 96, and 192 μM) of A-MP. In Fig. 2B, the injection volume was increased from 1 to 8 μl, using the least concentrated sample (12 μM). Error bars (± S.D.; $n = 3$) are shown. (A) (y-Axis) = absorbance.

between peak-area and amount of A-MP over the range of 12–192 pmol was obtained using a fixed 1-μl injection of different concentrations (12–192 μM) of A-MP. The least concentrated sample was 12 μM; that sample was used for the further study where the injection volume was increased (1, 2, 4, and 8 μl). Increasing the injection volume to 8 μl using that 12 μM sample maintained the high linear correlation coefficient ($r^2 = 1.00$; equation: $y = 2.15 \cdot 10^{-4}x - 9.83 \cdot 10^{-6}$) for peak-area vs. amount of A-MP (12–96 pmol) injected. The average relative standard deviation (R.S.D.; $n = 3$) was < 4% for these two standard curves (peak-area vs. pmol of A-MP).

Fig. 2A and B shows the plots when the above data were analyzed for peak-height vs. A-MP. Because peak-height is an indicator of peak-shape integrity and thus detectability, it is also an important chromatographic parameter. In Fig. 2A, although an excellent linear correlation coefficient was obtained ($r^2 = 0.99$; equation: $y = 6.29 \cdot 10^{-2}x - 7.12 \cdot 10^{-6}$), the length of the error bars revealed that the fixed 1-μl injection of the samples of the two highest concentrations (96 and 192 μM) had a relatively high R.S.D. ($n = 3$) value of 12.7 and 13.3%, respectively. This fluctuation suggests that the system cannot provide a consistent peak-height using the combined conditions of low injection volume and more concentrated samples. However, one could

readily dilute the sample to avoid that potentially adverse fluctuation. Furthermore, a comparison of Fig. 2A and the above standard curves (R.S.D. < 4%) for peak-area vs. pmol of A-MP indicates that the magnitude of peak-height fluctuation did not perturb significantly the magnitude of peak-area.

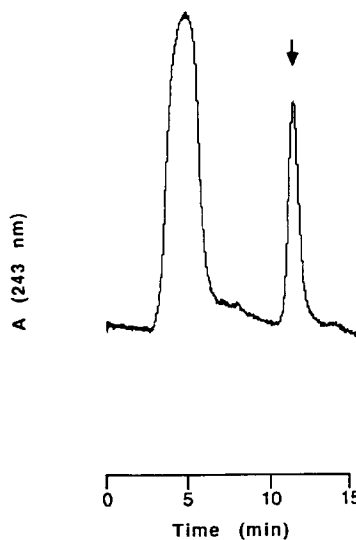Fig. 2B demonstrates that relatively high injection volumes (1–8 μl) of a highly diluted sample

Fig. 3. Chromatogram of A-MP. Synthetic A-MP was injected (10 μl × 4.8 μM = 48 pmol). The peak at ca. 5 min is due to solvent. A-MP elutes at 11.5 ± 0.4 min ($n = 3$). AUFS = 0.002.

(12 μM) may be applied to the system without the loss of peak integrity. A similar phenomenon has been mentioned [3]. This observation is most likely due to the fact that the sample solvent contained less acetonitrile (35%) than the mobile-phase solvent (50%). The best-fit line has an r^2 value of 1.00 (y = $2.86 \cdot 10^{-4}$x + $1.22 \cdot 10^{-5}$), and the average R.S.D. ($n = 3$) is <4%. In fact, a comparison of Figs. 2A and 2B suggests that a large injection volume of a diluted sample is more favorable than the low fixed injection volume of a more concentrated sample.

Fig. 3 is a chromatogram of synthetic A-MP from the 10-μl injection of a 4.8 μM sample (48 pmol). The retention time is 11.5 min. The large peak at ca. 5 min is due to solvent. Note the symmetry of the A-MP peak, though injecting a large volume (10 μl). Our limit of UV detection (signal-to-noise ratio, 3:1) for A-MP was 250 pg

(600 fmol). Therefore, A-MP could be detected from a 10-μl injection of a 60 nM sample in our system.

Collectively, the chromatographic behavior of A-MP in these standard curves [peak-area and peak-height (Fig. 2) vs. pmol of A-MP] and in Fig. 3 indicates that this HPLC method is a highly sensitive and reproducible method for an injection volume of 10 μl. However, to quantify A-MP in a spinal cord tissue sample, a background sample-tissue without A-MP must give minimal interference at 243 nm under these chromatographic conditions. Fig. 4A shows the chromatogram of such a control feline spinal cord tissue sample. (Note the minimal interference at ca. 11 min.) The presence of the A-MP that was added to the tissue prior to the lipophilic extraction is clearly shown in Fig. 4B.

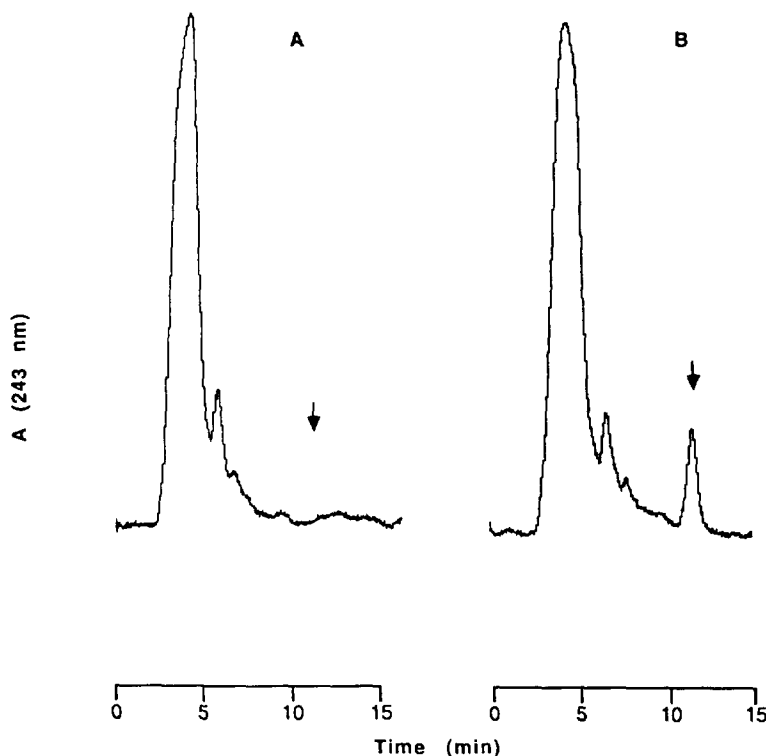Three different amounts (2.1, 4.5, and 6.5 μg)

Fig. 4. Chromatograms of (A) control tissue and (B) tissue spiked with A-MP. Spinal cord tissues were processed and prepared as described in the Experimental section. In B, A-MP (30 μg/g tissue) was added to the tissue prior to the extraction. Tissue weights were 0.102 and 0.151 g for (A) control and (B) spiked sample, respectively. Injection volume was 10 μl, and AUFS = 0.002.

of A-MP were added to three different spinal cord tissue segments (0.139, 0.151, and 0.145 g, respectively). Triplicate chromatograms were obtained for each of the tissue samples, and each A-MP peak-area was calculated and was plotted against the amount (mol) of A-MP added (data not shown). The excellent linear correlation coefficient ($r^2 = 1.00$; equation: $y = 2.33 \cdot 10^4 x + 3.46 \cdot 10^{-6}$) for the plot clearly demonstrates the high accuracy of the method for the quantification of A-MP in the presence of CNS tissue.

The recovery of A-MP from the lipophilic extraction was also investigated. Compared to the peak-area of a known amount of A-MP that did not undergo the tissue processing, the peak-area for A-MP after tissue processing was determined to be $16 \pm 1\%$ ($n = 9$). This recovery is lower than the 70% reported for MP [20]. However, the current method is sufficiently sensitive to readily detect that percentage of recovered A-MP. Therefore, by using the same tissue extraction and post-extraction sample processing procedures, the equation ($y = 2.33 \cdot 10^4 x + 3.46 \cdot 10^{-6}$) of the straight line obtained from the plot of peak-area vs. mol of A-MP added to tissue was used to quantify the amount of A-MP in tissue samples from the intrathecal injection study.

Fig. 5 shows a typical chromatogram that was obtained from a tissue sample that contained A-MP from the intrathecal administration of A-MP to the cat. The high volume (10 μl) injected gives a significant background before 10 min, but a nearly complete lack of interference near the elution of A-MP. Based on the equation of the standard curve obtained for varying amount of A-MP extracted from tissue, this A-MP peak at 11.5 min in Fig. 5 corresponds to 2.4 μg (5.8 nmol) of A-MP in that tissue (0.218 g), or 11 μg (26 nmol) of A-MP/g of tissue.

A feline with an acute spinal cord injury was studied. Quantitative HPLC results are summarized in Table 1. This method of detection shows that the intrathecal administration of A-MP results in an uptake of A-MP into the injured spinal cord. Table 1 shows a sample distribution of A-MP in the injured spinal cord after the intrathecal injection of 4.35 mg/kg, with the
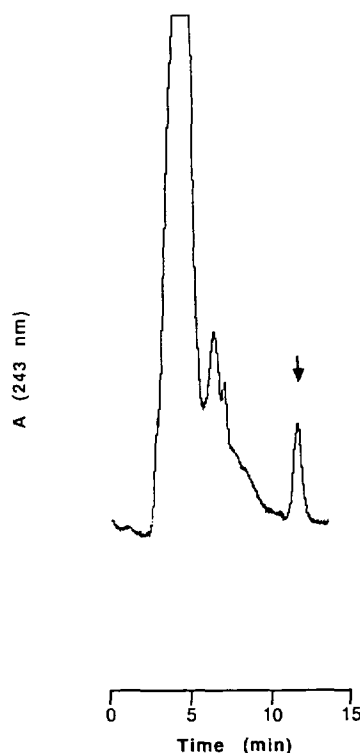
Fig. 5. Chromatogram of A-MP administered to tissue via intrathecal injection. Weight of this tissue was 0.218 g, injection volume was 10 μl, and AUFS = 0.002.

placement of the injection catheter 1 segment above the injury site. These data corroborate the findings of Lehrer et al. [18], who showed that the concentration of A-MP after intrathecal administration is highest at the site of injection. Further studies will allow us to determine the optimum catheter placement and dosage.

Finally, Fig. 6A and B shows the MS/MS spectra of synthetic A-MP and of an HPLC fraction collected from an intrathecally injected tissue sample, respectively. The $(M + H)^+$ ion of A-MP (m/z 417) is observed in both Figs. 6A and 6B. That $(M + H)^+$ ion was also detected in these two samples by the MS mode only (i.e., the B-scan) (data not shown). Furthermore, the $(M + H)^+$ ion of A-MP was the dominant ion in that fraction (all other ions were <10% compared to A-MP). The product ion spectra shown in these two Figs. indicate the fragment peaks II–V, which correlate to the loss of H_2O, 2 ×
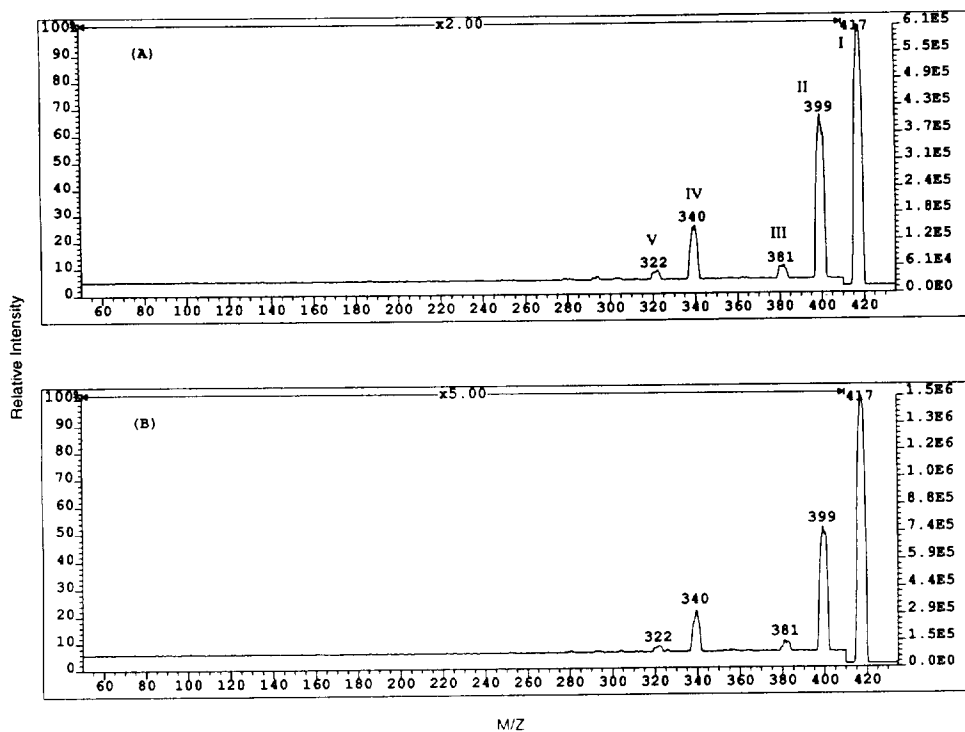
Fig. 6. MS/MS spectra of (A) synthetic A-MP and of (B) the A-MP fraction collected from intrathecally injected tissue sample. In A, $I = (M + H)^+$; $II = (M + H)^+ - H_2O$; $III = (M + H)' - 2H_2O$; $IV = (M + H)^- - H_2O -$ acetyloxy $(CH_3COO -)$; and $V = (M + H)^+ - 2H_2O -$ acetyloxy $(CH_3COO -)$. Fig. 6A was obtained from 4 μg of A-MP. Based on the peak-area of the A-MP fraction collected, ca. 70 ng (168 pmol) was used to obtain the product ion spectrum shown in Fig. 6B.

H_2O, $-H_2O - CH_3COO$, and $-2 \times H_2O -$ CH_3COO, respectively. Because the synthetic and tissue product ion spectra are equivalent, the molecular identity of the A-MP in the tissue sample is confirmed.

4. Conclusion

A simple, rapid, and sensitive capillary RP-HPLC assay for quantifying A-MP in feline spinal cord tissue is described. The use of 50% acetonitrile in the mobile phase resulted in a considerably earlier elution of potentially interfering material in the tissue, and also gave minimal background interference for the UV detection of A-MP (Fig. 4A) at 243 nm. An optimal level of confidence for the detection of A-MP by UV in the system was obtained from

the MS and MS/MS analysis of the collected A-MP. The investigation of maximizing the injection volume increased further the detectability of a sample with a potentially low concentration. For example, 600 fmol of A-MP could be detected in our capillary HPLC system. This high detection sensitivity precluded any need to improve the extraction recovery of A-MP. The linear correlation coefficient ($r^2 = 1.00$) obtained in the plot of peak-area vs. mol of A-MP in tissue demonstrates the precision and accuracy of the technique. An experimentally injured feline was studied. The intrathecally injected A-MP was quantified in various spinal cord tissue segments (Table 1) using the equation of the standard curve obtained also with a known amount of A-MP added to and extracted from tissue. The results obtained in Table 1 will be used to formulate the proper dosage and dosing

schedule to study the clinical efficacy of intrathecal administration of A-MP following acute spinal cord injury.

Acknowledgment

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