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Journal of Chromatography B, 806 (2004) 53–57
JOURNAL OF CHROMATOGRAPHY B
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Application of liquid chromatography–tandem mass spectrometry for the determination of opioidmimetics in the brain dialysates from rats treated with opioidmimetics intraperitoneally

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Abstract

We have determined three opioidmimetics (compounds **I**–**III**) in the rat brain dialysates after intraperitoneal (i.p.) administration of compounds **I**–**III** using a liquid chromatography/mass spectrometry with tandem mass spectrometry (LC–MS/MS). The dialysate samples with methanol were directly analyzed by online column-switching liquid chromatography. Using multiple reaction monitoring (MRM, product ions *m*/*z* 421 of *m*/*z* 657 for compound **I**, *m*/*z* 421 of *m*/*z* 643 for compound **II**, and *m*/*z* 407 of *m*/*z* 629 for compound **III**) on LC–MS/MS with electrospray ionization (ESI), opioidmimetics in rat brain dialysates were determined. Calibration curves of the method showed a good linearity in the range of 10–100 ng/ml for each compound. The limit of determination was estimated to be ca. 1 ng/ml for compounds **II** and **III**, and ca. 5 ng/ml for compound **I**, respectively. The precision of analysis showed coefficients of variation ranging from 4.7 to 10.4% at compound **III** concentration (10–100 ng/ml) in Ringer's solution. As a result, the procedure proved to be very suitable for routine analysis. The method was applied to the analysis of three opioidmimetics in the brain dialysate samples from rats treated with these compounds.

Keywords: Opioidmimetics

1. Introduction

Synthetic opioidmimetics, compounds **I**–**III**, are a new class of μ-opioid receptor agonist and opioid peptides which have 3,6-bis-(2′,6′-dimethyltyrosyl-aminoalkyl)-5-methyl-2(1*H*)-pyrazinone structure (Fig. 1). These opioidmimetics have high μ-opioid receptor affinities, selectivities and pharmacological activity and exceeded morphine as an analgesic [1]. Especially, compounds **II** and **III** of these opioidmimetics have very high affinities for μ-opioid receptor (K_i = 0.04 and 0.11 nM, respectively). The in vivo study for analgesic efficacy has revealed that following intracerebroventricular (icv) administration of compound **III**, it was >50-fold and ca. 20-fold more potent than morphine in tail-flick and hot plate tests, respectively [2].

We have been interested in brain transport of synthetic opioid peptides after oral administration. In general, natural occurring and unmodified peptides are usually quite hydrophilic, and its passage across the blood–brain barrier (BBB) occurs quite poorly and at very low rates [3–6]. To elicit an analgesic response in the central nervous system (CNS) after oral administration, several physico-chemical properties of opioid peptides and the passage through intractable membrane barriers via various transport systems of opioid peptides must be considered.

In the present study, we have determined compounds **I**–**III** in the brain dialysates to confirm the brain transport of opioid peptides. Opioidmimetics in the brain dialysates from rats treated with compounds **I**–**III** intraperitoneally (i.p.) have determined using LC–MS/MS method. For quantitative analysis, a liquid chromatography/mass spectrometry (LC–MS) method has been becoming a powerful technique recently. In addition, the application of tandem mass spectrometry (MS/MS) has improved both the sensitivity and

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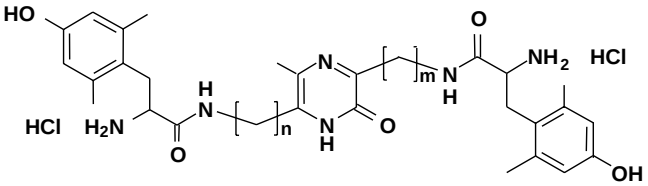
1570-0232/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2004.02.024



Compound I : n=4, m=4 (free M.W.: 634)
Compound II : n=3, m=4 (free M.W.: 620)
Compound III : n=3, m=3 (free M.W.: 606)

Fig. 1. Chemical structures of opipidmimetics, compounds **I**–**III**.

selectivity of the determination. The method using liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) has recently reported for the qualitative and quantitative analysis of opioid peptides in biological fluids [7–9]. These reports have described the analytical methods for opioid neuronal peptides in plasma, cerebrospinal fluid or brain tissue using C_{18} HPLC column with gradient elution and that it is a good procedure to determine opioid peptides. We also applied the LC–MS/MS method to determine opioidmimetics in the brain dialysates.

2. Experimental

2.1. Chemicals and reagents

Opioidmimetics (compounds **I**–**III**) (Fig. 1) were synthesized in our laboratory as described previously [10–12]. All final products were purified by semi-preparative reverse-phase HPLC; each compound exhibited a single peak on analytical HPLC. Analysis by MALDI–TOF mass spectrometry, ^{1}H and ^{13}C NMR and by HPLC revealed that they were the desired compounds with greater than 98% purity. Methanol (HPLC-grade) and high purity trifluoroacetic acid (TFA) were obtained from NACALAI Tesque Inc. (Kyoto, Japan). All other chemicals and reagents were of analytical grade from commercial sources.

2.2. In vivo brain microdialysis

Male Wistar rats (250–290 g, Japan SLC) were used in in vivo brain micro dialysis. Anesthetized (50 mg/kg, i.p., sodium pentobarbital) rats were stereotaxically implanted with 22-gauge cannulae in the left striata at anteroposterior +0.4 mm, lateral +3.0 mm from the bregma, and −3.5 mm from the skull, according to the stereotaxic atlas of Paxinos and Watson [13]. Dummy probes were then placed inside the cannulae. The rats were housed in plastic cages (35 cm × 35 cm × 40 cm) with free access to food and water, and a 20 h recover period was allotted. The microdialysis probes with dialysis area of 3 mm length were of I-shaped type reported previously [14]. The dialysis tube (0.2 mm i.d., 0.31 mm o.d.) was prepared from a polyacrylonitrile/sodium methylsulfonate membrane (Hospal, Bologna) with a molecular mass cut-off of 1100. After insertion through the guide cannulae, the probe was connected to a microinfusion pump and perfused with Ringer's solution at a flow rate of 3 μl/min for 180 min. Then, compounds (**I**–**III**) (10 mg/kg, i.p.) were administered. The dialysate samples were collected at 30 min intervals for 300 min and stored at −40 °C until analysis.

2.3. Sample preparation for LC/MS analysis

For determination of compounds (**I**–**III**), brain dialysate samples (60 μl) obtained from rat were mixed with 60 μl of methanol in the microcentrifuge tubes. It was then centrifuged at 10,000 × *g* for 10 min and the supernatant was filtrated with a 0.45 μm filter. The filtrate was transferred to the autosampler vial insert, and 100 μl of the filtrates were injected into the column-switching LC–MS/MS system.

2.4. LC–MS/MS

Column-switching LC–MS/MS system was used for determination of compounds (**I**–**III**) in the brain dialysates. LC–MS/MS was performed using a Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with electrospray ionization (ESI) and a six-port flow changeover valve. The HPLC system consisted of a Waters Alliance 2690 pump equipped with an autosampler (Waters, Milford, MA, USA) and the reversed-phase Shim-pack MAY1-ODS column (4.6 mm, i.d. × 10 mm, Shimadzu, Kyoto, Japan) for sample pretreatment. In addition, an auxiliary pump system, Shimadzu LC-10AVP (Shimadzu, Kyoto, Japan) and the reversed-phase COSMOSIL 5C8-MS column (2 mm, i.d. × 150 mm, 5 μm particle diameter, NACALAI Tesque, Kyoto, Japan) were added for LC–ESI/MS/MS analysis. A Shim-pack MAY1-ODS column was held with an eluent of water at a flow-rate of 1 ml/min for pretreatment. At 3 min after injection of sample, the changeover valve was switched and mobile phase for analysis was introduced at a flow-rate of 0.2 ml/min. Trapped target compounds were then eluted from a Shim-pack MAY1-ODS column and delivered to a COSMOSIL 5C8-MS analytical column using methanol: 0.1% (v/v) TFA solution (70:30, v/v).

Ionization conditions for LC–MS/MS were as follows: for compounds **I** and **II**, capillary voltage 3.5 kV, cone voltage 68 V, collision energy 44 eV, source temperature 85 °C, desolvation temperature 350 °C; for compound **III**, capillary voltage 3.5 kV, cone voltage 74 V, collision energy 43 eV, source temperature 85 °C, desolvation temperature 350 °C, and argon was used as collision gas. The multiplier voltage used 650 V.

3. Results and discussion

The ESI mass spectra of opioidmimetics, compounds **I**–**III** under analytical conditions were shown in Fig. 2. Compound **I** on the mass spectra showed a predominant ion

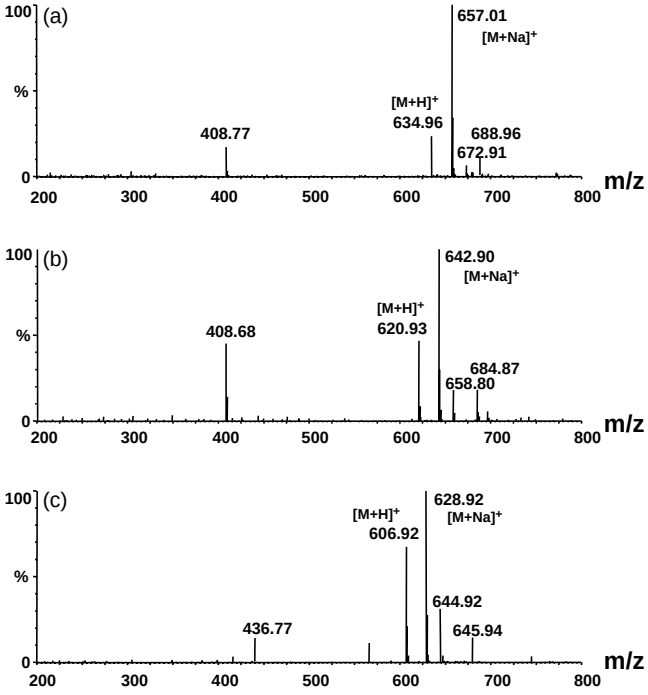


Fig. 2. LC–ESI mass spectra of opipidmimetics, compounds **I** (a), **II** (b) and **III** (c).

of *m*/*z* 657 $[M + Na]^+$. Compounds **II** and **III** also observed predominant ions of *m*/*z* 643 $[M + Na]^+$ and *m*/*z* 629 $[M + Na]^+$ on the mass spectra, respectively. The product ion profiles for opioidmimetics, compounds **I**–**III** were shown in Fig. 3. For compound **I**, the major fragment ion appeared at *m*/*z* 421. For compounds **II** and **III**, the major fragment ions appeared at *m*/*z* 421 and 407, respectively. Investigation of the MS/MS behavior of compounds **I**–**III** indicated that the product ions, *m*/*z* 421 of compound **I** (*m*/*z* 657), *m*/*z* 421 of compound **II** (*m*/*z* 643) and *m*/*z* 407 of compound **III** (*m*/*z* 629), respectively, were the most suitable ion for quantitative determination by multiple reaction monitoring.

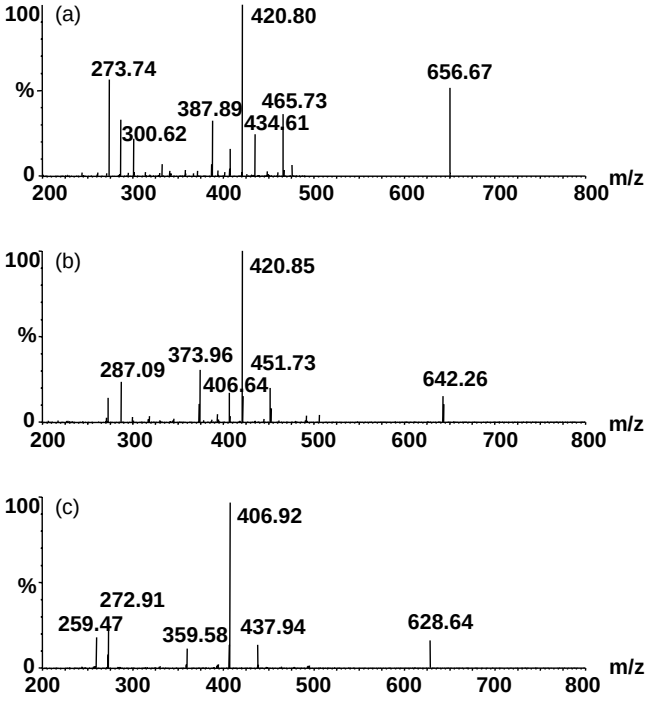


Fig. 3. Product ion spectra on LC–MS/MS of the $[M + H]^+$ ion of opipidmimetics, compounds **I** (a), **II** (b) and **III** (c).

The dialysate probe implanted into the brain is perfused with Ringer's solution and the brain dialysates contain therefore so many inorganic salts. To minimize non-specific interferences in mass spectrometry, inorganic salts in samples need to be removed using pretreatment procedures. For sample pretreatment, online column-switching procedures have been successfully developed to increase sample throughput [15–18]. We examined if online column-switching procedures were useful for de-salting of dialysate samples. The brain dialysate sample was added with methanol and the mixtures were directly injected into the online column-switching LC–MS/MS system. Fig. 4(a) shows the MRM chromatogram of compound **III** (30 ng/ml) standard solution using the product ions with *m*/*z* 407. The representative MRM chromatogram of the brain dialysates obtained from rats after injection of compound **III** (10 mg/kg, i.p.) was shown in Fig. 4(b). Elution profiles were similar to each other and no matrix interference was observed. From these results, it is suggested that this procedure is highly desirable to determine opioidmimetics in dialysate samples.

The calibration curves for compounds **I**–**III** using MRM were obtained by plotting the peak area of compounds **I**–**III** versus the amount of compounds **I**–**III**. As shown in Fig. 5, a good linearity was observed over the concentration range examined (10–100 ng/ml for standard solution). The correlation factor (r^2) of each compound was greater than 0.995. The lower limit of detection was approximately 1 ng/ml ($S/N = 4$) for compounds **II** and **III**, and 5 ng/ml ($S/N = 4$) for compound **I**, respectively.

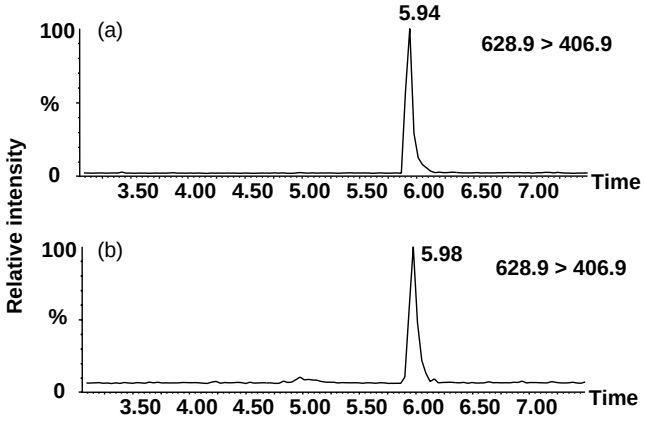


Fig. 4. MRM chromatograms on LC–MS/MS of standard solution (a) and the brain dialysate sample (b). The standard solution (a) is 30 ng/ml and the brain dialysate sample obtained from rat at 1 h after injection of compound **III** (b) is 12.2 ng/ml.

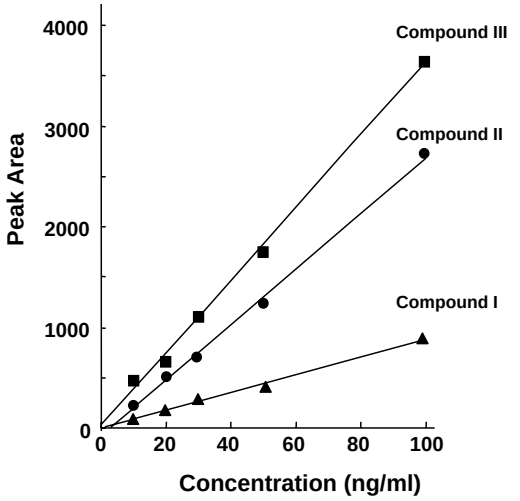


Fig. 5. Calibration curves of opipidmimetics, compounds **I–III**. The Ringer's solutions spiked with compounds were injected (100 μl). Correlation factor (r^2) of each compound was as follows; (1) compound **I**, 0.9954; (2) compound **II**, 0.9956; (3) compound **III**, 0.9975.

Experiments with spiked samples resulted in a recovery of 86.8 ± 7.4% at a concentration of 20 ng/ml of control brain dialysate sample (data not shown). Table 1 gives the intra-day and inter-day precision (CV%) of the method using brain dialysate samples spiked with compound **III**. The intra-day precision ranged from 4.7 to 10.4%, and the inter-day precision ranged from 7.1 to 10.4%. These results indicate a good reproducibility between experiments.

We examined the transport of opioidmimetics into brain using in vivo micro dialysis technique and determined the opioidmimetics in the brain dialysates using online column-switching LC–MS/MS. Fig. 6 shows the time course of the brain dialysate concentrations of compound **II** after administration of compound **II** (10 mg/kg, i.p.). The brain dialysate concentrations of compound **II** were found to reach to the maximum for 0–0.5 h following administration of compound **II** and then to decrease in an approximately linear fashion during 4 h. The time course of the brain dialysate concentrations of compounds **I–III** also give similar results (data not shown). The brain transport of compounds **I–III** following injection of opioidmimetics

Table 1
Intra-day and inter-day precision of LC–MS/MS method for the determination of spiked compound **III** in rat brain dialysates

Concentration (ng/ml)	Precision (CV%)	
	Intra-day	Inter-day
10	4.7	8.5
20	5.3	9.4
30	6.4	10.4
50	6.8	9.2
100	4.8	7.1

The data represent CV values from determination of peak area on MRM chromatogram ($n = 4$).

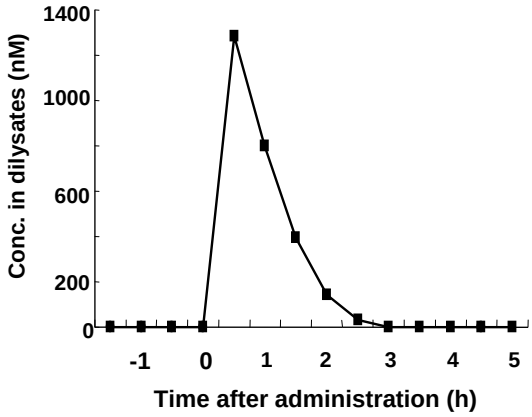


Fig. 6. Time course of the brain dialysate concentrations of compound **II** in rats after injection of compound **II** (10 mg/kg, i.p.).

Table 2
Transportation of opioidmimetics into rat brain

Compound	Transportation index	
	(pmol)[a]	(10^{-3}% of dose)[b]
I	287 ± 44	7.9 ± 1.4
II	193 ± 32	5.5 ± 1.7
III	130 ± 10	3.6 ± 0.4

The data are mean ± S.D. of three experiments.
[a] Compound levels in brain dilysates (pmol).
[b] Recoveries (%) of each compound in the brain dialysates during 0–5 h following i.p. injection of compound.

(10 mg/kg, 15.2–15.9 μmol/kg, i.p.) was shown in Table 2. The extracellular levels (pmol) of compounds **I–III** were calculated as the brain transport levels and were about 125–223 pmol. These levels were about 10 times higher than those of morphine after injection of morphine (10 mg/kg, 35.1 μmol/kg, i.p.) (about 38 pmol, data not shown). These results suggest that the opioidmimetics used in the present study are able easily to transport into the brain compared with morphine. Moreover, these results support that the present compounds have high analgesic efficacy relative to morphine.

4. Conclusion

We have developed the simple determination for opioidmimetics using online column-switching LC–MS/MS system. For pretreatment of the brain dialysate samples which are rich for inorganic salts, online column-switching system was very useful to determine opioidmimetics using LC–MS/MS. We applied the present procedure to examine the brain transport of opioidmimetics following the peripheral administration of opioidmimetics to rats. As a result, the present procedure demonstrated that this technique was simple, and had a good reproducibility and was very useful for a routine analysis of opioidmimetics.

Acknowledgements

A part of this study was supported by a Research Grant from the High Technology Center of Kobe Gakuin University.

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