A sensitive HPLC-fluorescence detection of morphine labeled with DIB-Cl in rat brain and blood microdialysates and its application to preliminarily study on pharmacokinetic interaction between morphine and diclofenac

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Abstract

A sensitive HPLC-fluorescence method for determination of morphine (Mor) in rat brain and blood microdialysates was developed using 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl) as a label. Mor was labeled with DIB-Cl under the mild reaction conditions (at room temperature for 10 min). The separation of DIB-Mor was carried out by an ODS column with CH_3CN/0.1 M acetate buffer (pH 5.4) within 14 min. The detection limits of Mor in brain and blood microdialysates at a signal-to-noise ratio of 3 were 0.4 and 0.6 ng/ml, respectively. The proposed method was successfully applied to preliminarily study of potential pharmacokinetic interaction of Mor with diclofenac.

1. Introduction

Morphine (Mor) is most frequently used as an analgesic drug for both post-operative and cancer pain. The World Health Organization proposed a three-stage approach for the treatment of chronic pain, where opioids including Mor are used to be combined with non-steroidal anti-inflammatory drugs (NSAIDs). Several studies have demonstrated the benefits of the combination use of Mor with NSAIDs in comparison to Mor alone [1-3]. These reported the additive effect on pain relief caused by the different pharmacodynamics of Mor and NSAIDs. On the other hand, although pharmacokinetic interactions between Mor and NSAIDs cannot be neglected, only limited knowledge is available [4-6]. This might be caused by a lack of simple and sensitive method for Mor determination.

Microdialysis is powerful technique to collect free drug in any tissue including the brain. Collection of drug molecule across the semipermeable microdialysis membrane makes it possible to estimate the free drug concentration in tissue. Furthermore, the clean-up procedure of the sample could be often omitted. However, since the sample size of microdialysis is generally in the microlitter range with low sample concentrations, a sensitive analytical method is required. Bengtsson et al., achieved sensitive determination of Mor and its glucuronides by using a direct injection HPLC-MS/MS [20].

For the determination of Mor, high performance liquid chromatography (HPLC) methods with UV or DAD detection [7,8], electrochemical detection (ECD) [9,10], mass spectrometry (MS) detection [11,12] and fluorescence detection (FL) [13-16] are

widely used. Quite recently, HPLC methods combined with tandem MS/MS for very low-level quantification of Mor in biological samples have been described with sub-nanogram per milliliter of quantitation limit [17-20]. In the HPLC-FL detection methods, either native FL of Mor [13-15] or FL of labeled Mor [16] is used. The native FL detection method with 10-50 ng/ml of detection limits is not enough sensitive to detect very small amount of Mor. On the other hand, FL labeling is powerful technique to determine analyte sensitively and many FL labeling reagents have been utilized [21]. However, few studies were performed for determination of labeled Mor by HPLC-FL detection because few labeling reagents for Mor were available. 4-(4,5-Diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl) could selectively label the compounds having hydroxyl group under mild conditions and produce intense fluorescence compounds. In our previous report, sensitive determination of pentazocine, a short-acting narcotic-antagonist analgesic drug, was achieved [22].

In this study, an HPLC-fluorescence detection method using DIB-Cl as a labeling reagent was developed for determination of Mor in rat brain and blood microdialysates. Moreover, the proposed method was applied for the study of pharmacokinetic interaction of Mor with diclofenac (Dic) after a single administration of Mor with/without Dic.

2. Experimentals

2.1. Chemicals

Mor hydrochloride was purchased from Takeda Pharmaceutical Co., Ltd. (Osaka,

Japan). Dic sodium, ethyl carbamate, CH_3CN and MeOH were obtained from Wako

Pure Chemical Industries, Ltd. (Osaka). DIB-Cl was synthesized in our laboratory as

reported previously [23]. Other reagents used were of analytical reagent grade. Water

was deionized and distilled by an Aquarius GSR-500 automatic water distillation

apparatus (Advantec Mfs, Inc., Tokyo, Japan). Chemical structures of Mor, Dic and

DIB-Cl are shown in Fig. 1.

Fig. 1

2.2. HPLC system

An HPLC system consisted of an LC-10AT$_{VP}$ pump (Shimadzu Co., Ltd., Kyoto,

Japan), a CCPD pump (Tosoh Co., Tokyo), a 7725 injector with a 20-µl sample loop

(Rheodyne Inc., CA, USA), a Wakopak Handy-ODS column (250 x 4.6 mm, i.d.; 6 µm,

Wako Pure Chemical Industries, Ltd.), a CD-8010 column oven (Tosoh), an RF-10A$_{XL}$

spectrofluorometric detector (Shimadzu), and an FBR-2 recorder (Tosoh). DIB-Mor was

separated with CH_3CN/0.1 M acetate buffer (pH 5.4) 45:55 (v/v %) as the mobile phase

and the flow rate of the mobile phase was set at 1.2 ml/min. Additional washing of the

separation column with CH_3CN (total flow rate: 2.2 ml/min) was performed for 5 min to

wash out the latent peaks after elution of DIB-Mor (ca 16.5 min). The DIB-Mor was

monitored at excitation and emission wavelengths of 355 and 486 nm, respectively.

2.3. Microdialysis

Wistar male rats (290-340 g, Otsubo experimental animals, Nagasaki, Japan)

were used for experiments. Rats were anesthetized with ethyl carbamate (1.5 g/kg)

before implanting a probe. A CMA microdialysis system (Carnegie Medicine, Stockholm, Sweden) was used. The probe used for blood and brain microdialysis was TP-20-04 (4x0.2 mm, i.d., Eicom Co., Kyoto) and A-I-8-04 (4x0.2 mm, i.d., Eicom) cellulose membrane, respectively. The artificial cerebrospinal fluid (CSF) consisted of 140 mM NaCl, 2 mM $CaCl_2$, 3 mM KCl, 2 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 25 mM $NaHCO_3$ and 6 mM glucose, which was adjusted to pH 7.4 with 0.1 M HCl, and perfused through both probes at a flow rate of 0.5 µl/min. The CSF was stored at 4°C until analysis and used after filtration with a membrane (JGWP04700, 0.22 µm, Millipore Co., MA, USA). The probe was implanted within jugular vein for blood and frontal cortex (A: +2.7 mm, L: +0.8 mm, V: +4.8 mm; according to the atlas of Pakinos and Watson) for brain. Blood and brain microdialysates were collected before and after i.p. administration of Mor with/without Dic. Collection of 15 µl each of microdialysate was performed and continued for 5 h. All samples were stored at –30°C until analysis.

2.4. Labeling of Mor with DIB-Cl

To the 15 µl of blood or brain microdialysate, 5 µl of 0.4 M carbonate buffer (pH 10) and 100 µl of 0.5 mM DIB-Cl suspension in CH_3CN were added. The mixture was vortex-mixed and allowed to stand for 10 min at room temperature. The reaction was stopped by addition of 10 µl of 1 M HCl.

2.5. Method validation

Calibration curves of Mor were prepared by adding Mor standard to blood or brain

microdialysate to give final concentrations ranged of 5-500 ng/ml. Detection limit of Mor was defined as the concentration at a signal-to-noise (S/N) ratio of 3.

Within- and between-day assay precisions for the proposed method were assessed by using blood and brain microdialysate spiked with 25 and 250 ng/ml of Mor (n=10). Precision was represented as a relative standard deviation (RSD, n=10).

The robustness of the method was examined by changing chromatographic parameters such as column temperature, flow rate of mobile phase and pH of acetate buffer. The retention time and peak height obtained by the optimal conditions were taken as 100. The robustness of the method was presented as percentage recovery to the retention time and peak height obtained by the optimal conditions (n=5)

2.6. Coadministration of Mor with Dic

This experiment was performed with an approval of Nagasaki University Animal Care and Use Committee (No. 030620285). Rats were divided into two groups as follow; one (n=3) was administered with a single i.p. dose of Mor (10 mg/kg) and another (n=3) with a single i.p. dose of Mor (10 mg/kg) after 1 min of a single i.p. dose of Dic (5 mg/kg). The medicaments were administrated to rat after perfusing CSF for 1 h. Sampling of microdialysate was performed at 0, 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min after administration of Mor. The pharmacokinetic parameters of Mor for blood and brain microdialysates were calculated by moment analysis.

3. Results and Discussion

3.1. Labeling and HPLC separation conditions

The labeling conditions such as concentration and pH of carbonate buffer, DIB-Cl concentration, reaction time and temperature were examined. At first, the effects of concentration (0.1-0.5 M) and pH (pH 9-11) of carbonate buffer on the fluorescence intensity (FI) of DIB-Mor were examined. DIB-Mor gave constant FI with more than 0.25 M and pH 10, thus the following experiments were done using 0.4 M carbonate buffer (pH 10) to obtain reproducible result. Next, DIB-Cl concentration was examined ranged from 0.5 to 7.5 mM, and constant and maximum FI was achieved with any concentration of DIB-Cl, thus 0.5 mM was selected due to economical reason. Finally, reaction time and temperature were studied in range of 1-30 min and 4-60°C, respectively. Although decomposition of DIB-Mor was observed at 60°C, FI of DIB-Mor at 4 °C and room temperature was constant after a reaction of more than 10 min. The following experiments were done at room temperature for 10 min. To stop labeling reaction, addition of 10 µl of 25% NH_3 or 1 M HCl solution was examined. As decomposition of DIB-Mor was observed with NH_3 aqueous solution, 1 M HCl was selected for this purpose.

The isocratic separation of DIB-Mor was performed by a Wakopak Handy-ODS column with CH_3CN/0.1 M acetate buffer (pH 5.4) 45:55 (v/v %) as the mobile phase. DIB-Mor could be well separated from the interfering peaks (13.3 min). After eluting of DIB-Mor, the separation column was washed with CH_3CN for 5 min as a clean-up step: Typical chromatograms obtained from brain (A) and blood (B) microdialysates are shown in Fig. 2.

Fig. 2

3.2. Method validation

Calibration curves obtained with the spiked brain and blood microdialysate were linear with r-values greater than 0.998 in the range of 5-500 ng/ml. Detection limits of Mor for brain and blood dialysate at S/N ratio of 3 were 0.4 ng/ml (2.7 fmol on column) and 0.6 ng/ml (3.8 fmol on column), respectively. The proposed method could improve the sensitivity of Mor by more than 20 times compared to HPLC-DAD method [7,8] and native FL method [13-15]. Furthermore, it was comparable to those of HPLC-ECD [10] and –MS/MS method [20] published recently for determination of Mor in microdialysate.

Within- and between-day precisions of the proposed method were evaluated by analyzing microdialysate spiked with known concentrations of Mor (25 and 250 ng/ml). The within-day RSDs for brain and blood microdialysate were 7.9 (25 ng/ml) and 3.9 % (250 ng/ml), and 8.0 (25 ng/ml) and 4.3 % (250 ng/ml), respectively. Between-day precision (RSDs) of 8.4 (25 ng/ml) and 6.3 % (250 ng/ml) for brain microdialysate were obtained, while those for blood microdialysate were 8.5 (25 ng/ml) and 6.5 % (250 ng/ml).

The robustness of the proposed method was examined by changing chromatographic parameters such as column temperature ($\pm 1℃$), flow rate of mobile phase (± 0.05 ml/min) and pH of acetate buffer (± 0.1 unit). Each of parameters varied in the range of $\pm 2.5\%$, $\pm 4.2\%$ and $\pm 1.8\%$ compared to their optimal conditions ($40℃$, 1.2 ml/min and pH 5.4). The percent recoveries of retention time were in the range of 97-102%. The percent recoveries of

peak height were in the range of 99-104%. Since the obtained values were within the acceptable limits (95-105%) in all cases, the robustness of this HPLC method could be elucidated (Table 1).

3.3. Recovery of microdialysis probe

The probes used for blood and brain microdialysis were TP-20-04 (4x0.2 mm, i.d.) and A-I-8-04 (4x0.2 mm, i.d.) cellulose membranes, respectively. *In vivo* recoveries of these probes for DP were calculated by using standard solutions of Mor in CSF at 50 and 500 ng/ml. Recovery and loss were calculated by following equations according to our previous report [24].

Recovery % = $C_{out}/C_{in} \times 100$

Loss % = $[(C_{in}-C_{out})/C_{in}] \times 100$

C_{in}: Mor concentration in perfusate, ng/ml

C_{out}: Mor concentration in dialysate, ng/ml

Recovery $_{in\ vivo}$=Loss $_{in\ vivo} \times$ (Recovery $_{in\ vitro}$/Loss $_{in\ vitro}$)

The recovery and loss factors for calculating recovery *in vivo* of microdialysis probe were shown in Table 2. Subsequently, the ratios for brain and blood microdialysates were 18±6 (n=4) and 11±4 % (n=5), respectively.

Table 2

3.4. Pharmacokinetic interaction between Mor and Dic

The applicability of the proposed method was confirmed by preliminarily evaluating of potential pharmacokinetic interaction of Mor and Dic after a single administration of Mor (10 mg/kg) with/without Dic (5 mg/kg) which is an effective and frequently prescribed NSAID as described in our previous report [25]. Concentration-time profiles of Mor in rat brain and blood microdialysates after a single administration of Mor with/without Dic are shown in Fig. 3 and 4, respectively. Mor in very small amount of microdialysate (15 µl) could be determined until 300 min after administration of medicaments by the proposed method. The pharmacokinetic parameters of Mor in brain and blood calculated by these data were summarized in Table 3. Significant difference for any parameters could not be found between two groups in both brain and blood.

Mor was a low-transitive compound for a blood-brain-barrier, since AUC ratio (AUC_{brain}/AUC_{blood}), which indicates transitivity of Mor to brain, was 0.38 in this study. And also, 2.7 times longer $T_{1/2}$ of Mor in brain than that in blood indicated the difficulty of Mor efflux from brain. In the previous report, the AUC ratio of Mor in rat striatum was 0.22-0.28 [26]. And it was also reported that the $T_{1/2}$ of Mor in brain was significantly prolonged compared with that in blood. These results were discussed as follow: Mor was actively effluxed at the blood-brain barrier by P-glycoprotein and elimination from the central nervous system was rate-limited by redistribution of Mor from brain tissue. These were well agreed with our results.

The combination of Mor with Dic produced the benefit to the patient such as reduction of Mor consumption [27] and enhancing analgesic effect to severe pain after

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operation [2]. These additive effects between Mor and NSAIDs were caused by different pharmacodynamic mechanisms; opioids display their analgesic activity in the central nervous system via opioids receptors, and NSAIDs reduce the synthesis of inflammatory prostaglandins via inhibition of the enzyme cyclooxygenase [1-3]. Ammon et al. reported that NSAIDs inhibit UDP-glucurosyltransferase *in vitro* study [4]. Furthermore, inhibition of renal clearance of Dic was expected as a pharmacokinetic mechanism of additive effect between Mor and Dic [3]. On the other hand, some reports were concluded that Dic did not interact pharmacokinetic of Mor in human [6]. In our condition no significant difference to pharmacokinetic parameters of Mor could found, however, applicability of the proposed method for preliminarily study of drug interaction of Mor could be confirmed.

Fig. 3

Fig. 4

Table 3

In conclusions, an HPLC-FL detection method coupling with a microdialysis technique for determination of Mor in brain and blood microdialysates was developed. It is confirmed that the proposed method is enough sensitive to monitor Mor in these biological fluids. Furthermore, pharmacokinetic interaction of Mor with Dic was preliminarily studied. No significant difference of almost pharmacokinetic parameters of Mor between rats administered Mor with/without Dic. However, these basic findings may help clinical inference when Mor is co-administered with Dic to human.

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Morphine (Mor)

Diclofenac (Dic)

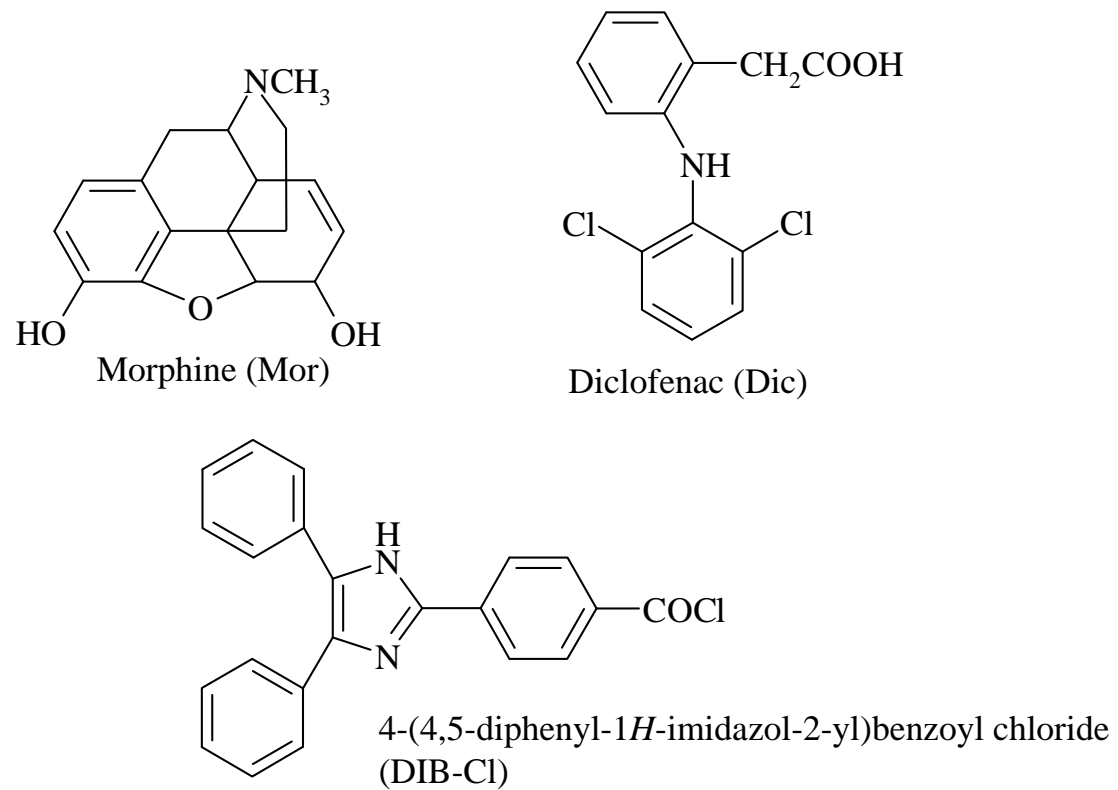4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl)

Fig. 1 K. Nakashima et al.
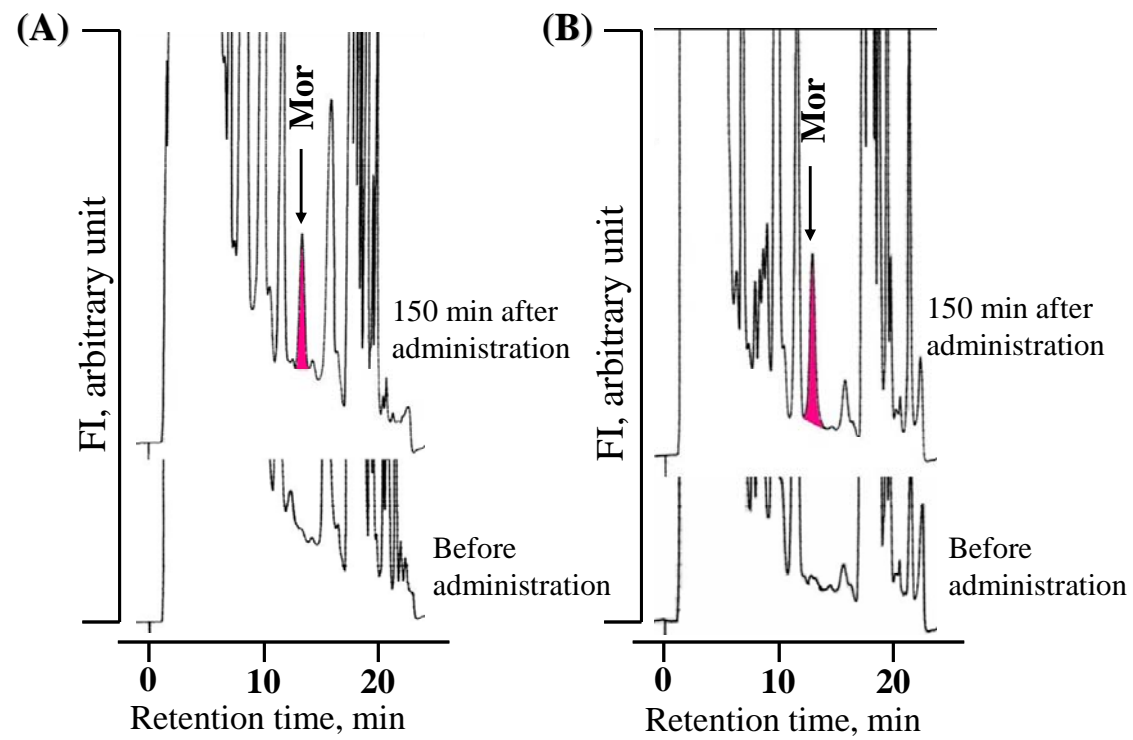
Fig. 1 Structures of Mor, Dic and DIB-Cl.

Fig. 2 K. Nakashima et al.

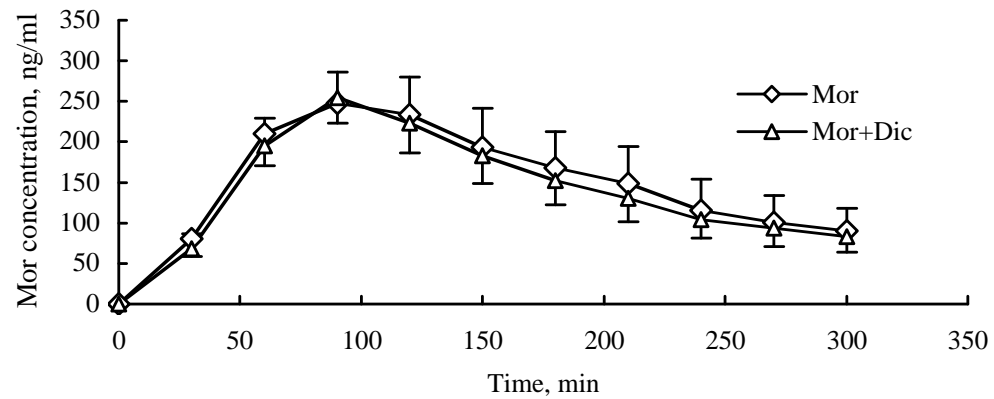Fig. 2 Chromatograms of Mor in rat (A) brain and (B) blood dialysates.

Fig. 3 K. Nakashima et al.

Fig. 3 Concentration-time profiles of Mor in rat brain dialysate after a single administration of Mor with/without Dic.
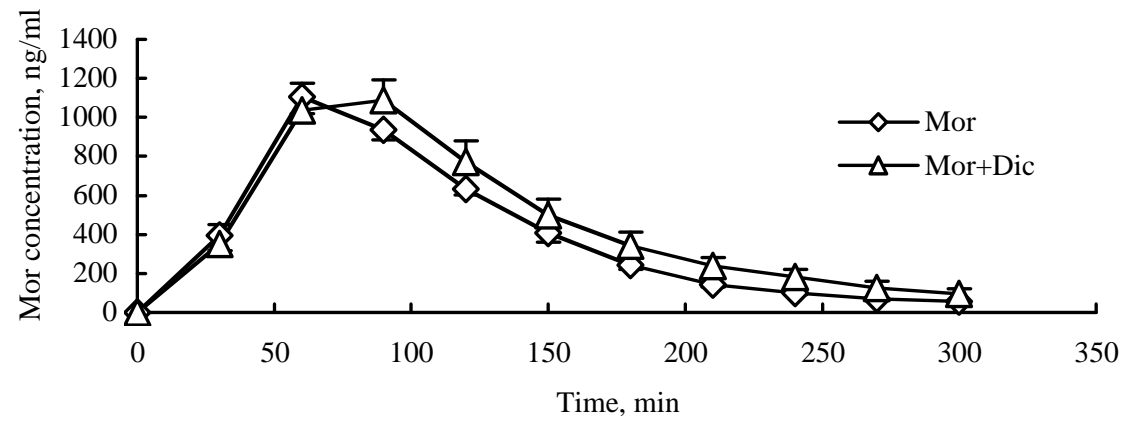Data are expressed as mean±SEM (n=3).

Fig. 4 K. Nakashima et al.

Fig. 4 Concentration-time profiles of Mor in rat blood dialysate after a single administration of Mor with/without Dic.
Data are expressed as mean±SEM (n=4).

Table 1 The robustness of the proposed method

Chromatographic parameters	Percent recovery (mean±SD, n=5) %	
	Retention time	Peak height
Column temperature 39℃	101±2	99±1
40℃	102±2	99±2
Flow rate of mobile phase 1.15 ml/min	104±0.3	100±0.4
1.25 ml/min	97±1	99±1
pH of acetate buffer 5.3	98±2	101±1
5.5	101±2	100±1

Table 2 In vivo recovery of Mor in rat brain and blood microdialysates.

Dialysate	Recovery $_{in\ vitro}$ %	Loss $_{in\ vitro}$ %	Loss $_{in\ vivo}$ %	Recovery $_{in\ vivo}$ %
Brain	30±4	37±4	22±7	18±6
Blood	30±4	37±4	14±5	11±4

Data are expressed as mean±SD (brain: n=4; blood: n=5).

Table 3 Pharmacokinetic parameters of Mor in rat brain and blood dialysates after a single administration of Mor (10 mg/kg) with/without Dic (5 mg/kg) to rats.

	Mor	Mor+Dic
Brain dialysate (n=3)		
C_{max}, ng/ml	260±46	260±38
T_{max}, min	90±17	90±0
$T_{1/2}$, min	130±29	130±17
AUC_{0-300}, ng・min/ml	46000±11000	46000±11000
k_{el}, min^{-1}	0.0061±0.0017	0.0057±0.0008
CL_{app}, ml/min/kg	37±12	36±8
Blood dialysate (n=4)		
C_{max}, ng/ml	1100±100	1100±120
T_{max}, min	68±8	75±9
$T_{1/2}$, min	47±3	58±5
AUC_{0-300}, ng・min/ml	120000±6900	140000±22000
k_{el}, min^{-1}	0.015±0.009	0.012±0.001
CL_{app}, ml/min/kg	150±37	99±5

Data are expressed as mean±SEM.