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Determination of donepezil hydrochloride in human and rat plasma, blood and brain microdialysates by HPLC with a short C_{30} column

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Abstract

A simple and sensitive HPLC method with fluorescence (FL) detection for determination of donepezil (DP) in plasma and microdialysate samples was developed. A rapid isocratic separation of DP could be achieved by a short C_{30} column using mobile phases of 25 mM citric acid/50 mM Na_2HPO_4 (pH 6.0)–CH_3CN (73:27%, v/v) containing 3.5 mM sodium 1-octanesulfonate for plasma and H_2O–CH_3CN–CH_3OH (80:17:3%, v/v/v) containing 0.01% acetic acid for microdialysate. The eluate was monitored at 390 nm with an excitation at 325 nm. The detection limits (S/N = 3) of DP for human plasma, rat plasma and rat brain or blood microdialysates were 0.2, 1.0 and 2.1 ng/ml, respectively. Reproducible results could be obtained by using (±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimethoxyindan-1-one hydrochloride as an internal standard (IS).

The method was successfully applied for monitoring of DP levels in rat plasma, blood and brain microdialysates and patient plasma.

Keywords: Donepezil hydrochloride; HPLC; Plasma; Microdialysate

1. Introduction

Donepezil hydrochloride ((±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimethoxyindan-1-one hydrochloride, DP), which is commercially available as Aricept®, is a potent, selective and reversible acetylcholinesterase inhibitor and has been prescribed worldwide for the treatment of Alzheimer's disease [1]. More recently, DP treatment for Down syndrome showed potential improvement of the symptom in a non-randomized-controlled trial [2]. In addition, some reviews on the pharmacokinetics, pharmacodynamics and clinical profiles of DP have been published [3–5]. Many piperidine derivatives with effectiveness as DP have been synthesized and compared their inhibitory effects [6,7]. In these views, the determination of DP in biological fluids has become significant, and thus the development of a simple and sensitive method for determining DP is required.

DP determination has been performed by many methods, such as HPLC with ultra violet (UV) [8–14] and fluorescence (FL) [15] detection, LC–MS [16], LC–MS/MS [17] and CE-UV detection [18]. Since the UV detection methods need simple apparatus, a number of studies have been reported for determination of DP in blood sample [8–13] and tablets [14]. However, these are less sensitive and require a relatively large blood sample (≥1 ml) for pharmacokinetic study of DP. Although LC–MS is selective and sensitive and has been successfully applied to enantio-selective analysis of DP by using an avidin-conjugated column [16,17], it requires expensive instrumentation. On the other hand, DP has native fluorescence and utilizing this nature, Haginaka and Seyama developed a sensitive enantio-selective HPLC-FL method for DP in human plasma [15].

Microdialysis is a powerful tool to collect low molecular weight substances, which are present in the extracellular tissue fluids and it has been applied to pharmacokinetic studies of many drugs. We have reported the pharmacokinetic profiles of drugs of abuse [19], medicines [20–22] and environmental pollutants [23] by using this tool.

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In this study, a simple HPLC-FL method for determination of DP in different biological fluids was investigated. The isocratic separation condition of DP was optimized using a short C_{30} column. Few DP determination methods applicable to various matrices have been reported. To evaluate the applicability of the proposed method, determination of DP in plasma samples with different time were examined. The proposed method was also applied to the analysis of DP in rat plasma, blood and brain microdialysates after administration of a single dose.

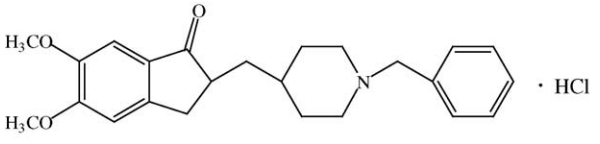
2. Experimental

2.1. Chemicals

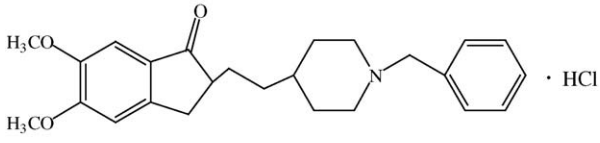
The structures of DP and (±)-2-[(1-benzyl-piperidine-4-yl)ethyl]-5,6-dimethoxyindan-1-one hydrochloride as an internal standard (IS) are shown in Fig. 1 and were kindly gifted by Eisai Co. (Tokyo, Japan). Stock solutions of these compounds were prepared at 1 mM in water and stored at 4 °C. Sodium 1-octanesulphonate, CH_3CN and CH_3OH were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Other reagents used were of analytical grade. Water was deionized and distilled by an Aquarius GSR-500 automatic water distillation apparatus (Advantec, Tokyo, Japan).

2.2. HPLC system

The HPLC system consisted of an LC-10AT *VP* liquid chromatographic pump (Shimadzu, Kyoto, Japan), a 7125 injector with a 100-μl sample loop (Rheodyne, Cotati, CA, USA), a Develosil Combi-RP-5 packed column (50 mm × 4.6 mm, i.d., 5 μm, Nomura Chemical Co., Aichi, Japan), a CTO-6AS column oven set at 32 °C (Shimadzu), an RF-550 spectrofluorometric detector set at (λ_{ex} = 325 nm) and (λ_{em} = 390 nm) (Shimadzu), a signal cleaner SC-77 (System Instrument Co., Tokyo, Japan) and an R-111 recorder (Shimadzu). DP and IS were isocratically separated with 25 mM citric acid/50 mM Na_2HPO_4 (pH 6.0)–CH_3CN (73:27%, v/v) containing 3.5 mM sodium 1-octanesulfonate for plasma and H_2O–CH_3CN–CH_3OH (80:17:3%, v/v/v) containing 0.01% acetic acid for blood and brain microdialysates. The flow rate of eluent was set at 1.0 ml/min.



Donepezil hydrochloride



(±)-2-[(1-benzyl-piperidine-4-yl)-ethyl]5,6-dimethoxyindan-1-one hydrochloride, IS

Fig. 1. Structures of donepezil and IS.

2.3. Human plasma sample

Normal control human blood samples were obtained from healthy volunteers in our laboratory. Blood samples from Alzheimer's patients were obtained 2.5–8.0 h after the daily dose of 5 mg DP. Four female patients aged from 79 to 90 were enrolled in this study, and informed consent was obtained from all subjects. Blood samples were collected in EDTA tubes and centrifuged at 1000 × *g* for 10 min. The plasma samples were stored at −20 °C until analysis.

2.4. Blood sampling from rats administered donepezil

Wistar male rats (285–360 g, Otsubo experimental animals, Nagasaki, Japan) were used for experiments. Rats were administered i.p. with 1 or 5 mg/kg DP and then anesthetized with ethyl carbamate (1.5 g/kg), waiting 5 min before start of sampling. Blood samples were collected via arteria femoralis, transferred to EDTA tubes and centrifuged for 10 min at 4 °C, 1000 × *g* to separate the plasma. Sampling of blood was performed before administration of DP and then at 5, 10, 20, 30, 45, 60, 90, 120, 180, 210, 240, 300, 360, 480 and 600 min after administration of DP. All animal procedures and care in this experiment were approved by the Nagasaki University Animal Care and Use Committee (no. 0204300134).

2.5. Extraction of plasma sample

To 100 μl of rat plasma [or 500 μl of human plasma], 15 μl of IS (1 or 3 μM) and 100 μl [or 500 μl for human] of borate buffer (50 mM, pH 10) were added. DP was extracted with 500 μl [or 2.5 ml for human plasma] of 5% isopropanol in *n*-hexane. The mixture was vortex-mixed for 1 min and centrifuged (1000 × *g*) for 10 min at 10 °C. Four hundred microlitres [2 ml for human] of the organic layer were taken and evaporated to dryness with a CE1 centrifugal evaporator (Hitachi, Tokyo, Japan). The residue was reconstituted with 200 μl [300 μl for human plasma] of mobile phase and subjected to HPLC analysis.

2.6. Microdialysis samples

A CMA microdialysis system (Carnegie Medicine, Stockholm, Sweden) was used. The PC10 membrane microdialysis probes (0.5 mm i.d., cut-off 20,000 Da) used for blood and brain microdialysis sampling were of length 4 and 3 mm, respectively. The probes were implanted within the jugular vein for blood samples and hippocampus (AP, +5.3 mm; ML, −4.7 mm; DV, −7.5 mm; according to the atlas of Paxinos and Watson) for brain samples [24,25]. The artificial cerebrospinal fluid (CSF) consisted of 145 mM NaCl, 2.3 mM $CaCl_2$ and 1.5 mM KCl, which was adjusted to pH 7.4 [26] and perfused through both probes at a flow rate of 1.0 μl/min. Blood and brain microdialysates were collected before and after i.p. administration of a single dose of

DP (5 mg/kg). Twenty microlitres of each microdialysate were collected for 6 h and stored at −20 °C until analysis.

In vivo recovery of microdialysis probe for DP was calculated by Eq. (1) according to our previous report [23]. A standard solution of DP in CSF at 500 nM was used for measurement of recovery and delivery.

$$\text{Recovery}_{\text{in vivo}} = \frac{\text{delivery}_{\text{in vivo}} \times \text{recovery}_{\text{in vitro}}}{\text{delivery}_{\text{in vitro}}} \quad (1)$$

2.7. Pharmacokinetics of donepezil

Pharmacokinetic parameters were estimated using the DP concentration data obtained in this study. The peak concentration (C_{max}) and the time occurrence of C_{max} (t_{max}) were direct experimental observations. The moment parameters (AUC, MRT) for the concentration profile of rat plasma, blood and brain microdialysates were calculated by numeral integration using a linear trapezoidal [27]. The elimination rate constant (K_{el}) was calculated as the native slope of non-weighted least squares curve fit to logarithmically transformed concentration versus time. The elimination half-life ($t_{1/2}$) was determined by the equation $\ln 2/K_{el}$. The apparent clearance (CL_{app}) was estimated as the ratio of administered dose to AUC.

3. Results and discussions

3.1. Separation conditions

An isocratic separation of DP in plasma sample was examined using some commercially available reversed-phase columns. Among these, the short C_{30} column with 25 mM citric acid/50 mM Na_2HPO_4 (pH 6.0)–CH_3CN (73:27%, v/v) containing 3.5 mM sodium 1-octanesulfonate gave acceptable separation of DP and IS from the plasma components. Typical chromatograms of DP spiked in human plasma are shown in Fig. 2 and retention times of DP and IS were 12.4 and 17.6 min, respectively. On the other hand, the same eluent could not give sharp and well-separated peaks of DP and IS from those due to components of microdialysates. As a result, H_2O–CH_3CN–CH_3OH (80:17:3%, v/v/v) containing 0.01% acetic acid was selected as the optimum eluent for the separation of DP in blood and brain microdialysates. A good separation of DP and IS was achieved within 16 min with retention times of 8.8 and 14.0 min, respectively. Fig. 3 shows chromatograms obtained from rat blood (A) and brain (B) microdialysates before and at 80 min after administration of DP. Therefore, the separation conditions are acceptable for DP separation in biological samples.

3.2. Method validation

Parameters of calibration curves for the proposed method are summarized in Table 1. Calibration curves obtained with spiked biological matrices showed good linearities ($r \geq 0.999$) between the concentration and peak height ratio. The detection limits of DP for rat plasma, microdialysate and human plasma at S/N ratio of 3 were 2.5 (1.0), 5.0 (2.1) and 0.5 nM (0.2 ng/ml), respectively.

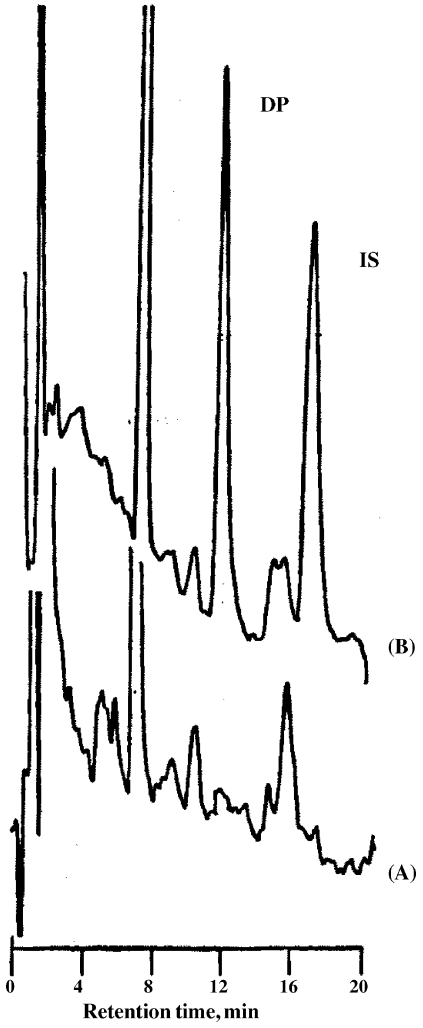


Fig. 2. Chromatograms obtained from human plasma (A) and spiked with 25 nM of DP (B). The detector sensitivity in (A) is two times higher than in (B).

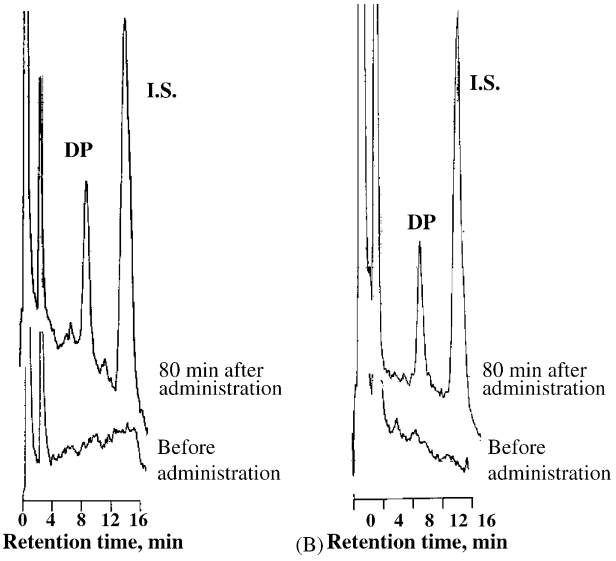


Fig. 3. Chromatograms obtained from rat brain microdialysate (A) and blood microdialysate (B).

Table 1
Parameters of calibration curves for the proposed method

Matrix	Linear range (nM)	Equation[a]	r[b]	Detection limit (nM)[c,d]
Rat plasma	5–500	$y = 0.011x - 0.02$	0.999	2.5 (1.0)
Brain microdialysate	10–500	$y = 0.0033x + 0.0123$	0.999	
Blood microdialysate	10–500	$y = 0.0034x + 0.0252$	1.000	5.0 (2.1)
Human plasma	1–50	$y = 0.005x + 0.03$	0.999	0.5 (0.2)

[a] y, the ratio of DP peak height to IS peak height; x, the concentration of DP in nM.
[b] Correlation coefficient.
[c] Detection limit at signal–noise ratio of 3.
[d] Values in parenthesis in ng/ml.

In comparison with other methods for analysis of DP in human plasma, the proposed method is 10 times more sensitive than the methods using UV detection [7–11], comparable with fluorescence detection [15] and 10 times less sensitive than LC–MS [17].

Intra- and inter-day precision of the proposed method was evaluated by using each biological fluid spiked with DP standard. Table 2 shows the relative standard deviation (R.S.D.) and recovery of spiked DP. For intra-day measurement, R.S.D. ranged from 0.9 to 7.8% ($n \geq 4$), while the inter-day precision ranged from 2.5 to 9.3%. The recoveries obtained for rat and human plasma and were 91.0 and 101.7%, respectively.

3.3. Donepezil in patient plasma

Stability of DP in plasma was examined with normal human plasma spiked with 2.5 and 25 nM DP. No decomposition of DP in plasma stored at room temperature or 4 °C was observed within 48 h (data not shown). Additionally, the stability of DP for freeze–thaw cycle was also satisfactory shown in Fig. 4. Consequently, DP in plasma can be stored in the refrigerator without degradation.

The proposed method was also applied to the determination of DP levels in plasma from four patients taken 2.5–8.0 h after the daily dose (Table 3). The levels of DP were in the range 14.4–49.2 ng/ml. From this result, all subjects were considered to be in the steady state of DP concentration. However, patient number 2 showed higher DP concentration than the others. In the previous reports, the mean DP plasma concentrations at steady state in healthy male volunteers were 17.2 ± 1.7 for C^{ss}_{min}, 22.8 ± 2.2 for C^{ss} and 30.8 ± 4.2 ng/ml for C^{ss}_{min}, respectively [8]. Although the pharmacokinetic parameters, such as K_{el} and t_{max} were significantly longer in the aged than in the young, there were no significant differences in C_{max} and AUC between the two groups [28]. The hepatically impaired patients received a single 5 mg oral dose of DP and showed a statistically significant higher C_{max} value compared with the control group [10]. The pharmacokinetics of DP in patient with impaired renal function did not change compared to healthy subjects [11]. Considering

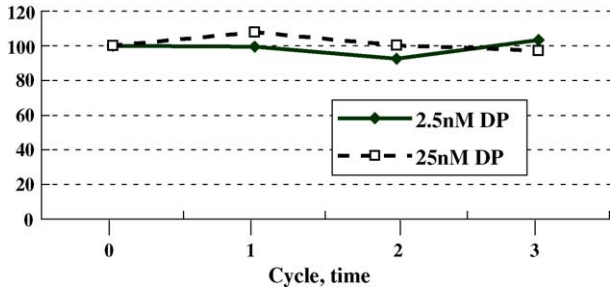


Fig. 4. Stability of DP in human plasma for freeze–thaw cycle.

Table 3
Determination of DP in patient's plasma samples

Number	Gender	Age	Withdrawal time (h)	Found concentration (ng/ml)
1	F	79	8	14.4
2	F	90	8	49.2
3	F	87	2.5	22.1
4	F	81	4	31.4

Table 2
Intra- and inter-day assay precision and recovery for the proposed methods

Matrix	Added DP (nM)	n	Precision (R.S.D.%)		Recovery (%)
			Intra-day	Inter-day	
Rat plasma	5	4	4.4	6.4	91.0
	250		5.9	3.9	
Brain microdialysate	25	4	7.8	9.3	–
	100		7.5	6.7	
Blood microdialysate	25	4	5.2	5.9	–
	100		0.9	2.5	
Human plasma	2.5	5	5.4	6.7	101.7
	50		2.8	3.0	

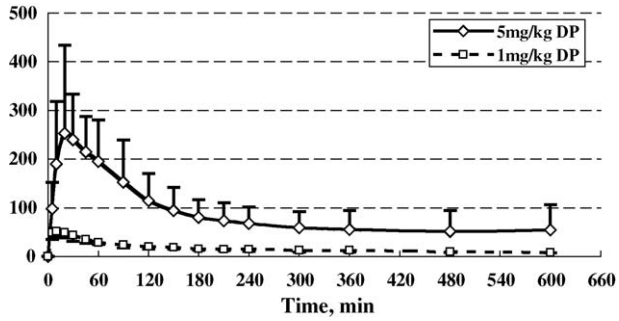


Fig. 5. Time course of DP concentration in rat plasma after i.p. administration to rat. Data are expressed as mean ± S.D., $n = 5$.

Table 4
Pharmacokinetic parameters of DP in plasma following DP administration

	Dose of DP (mg/kg)	
	1	5
C_{max} (ng/ml)	52.0 ± 12.6	308.2. ± 146.8
T_{max} (min)	13.0 ± 6.7	32.0 ± 16.4
$T_{1/2}$ (min)	369.0 ± 78.9	355.2 ± 211.5
AUC (ng/ml min)	$1.4 \times 10^4 \pm 1.9 \times 10^3$	$8.0 \times 10^4 \pm 5.5 \times 10^4$
MRT (min)	479.6 ± 109.5	489.8 ± 332.9
K_{el} (min^{-1})	$2.0 \times 10^{-3} \pm 4.9 \times 10^{-4}$	$2.6 \times 10^{-3} \pm 1.3 \times 10^{-3}$
CL_{app} (ml/min/kg)	70.9 ± 10.6	94.5 ± 66.7

Data are expressed as mean ± S.D., $n = 5$.

these facts, the overdose of DP for patient number 2 should be noted to avoid the cholinergic side effect which might be caused by DP.

3.4. Donepezil monitoring in rat plasma

Fig. 5 shows the time course of DP concentration in plasma after i.p. administration (1 and 5 mg/kg) to rats. The peak concentration of DP in plasma appeared at 13 min for 1 mg/kg and 32 min after administration for 5 mg/kg. DP could be detected after 10 h of administration. The pharmacokinetic parameters for DP after single i.p. administration are summarized in Table 4. The C_{max} and AUC were dose-dependent, while $t_{1/2}$, MRT and CL_{app} were dose-independent. In the previous report, the absorption, disposition and excretion of ^{14}C-labeled DP after a single administration (1.0 mg/kg) to rat were studied in pharmacokinetic profiling of DP [29]. After i.v. administration, over 90% of DP was absorbed rapidly, and the $t_{1/2}$ and CL_{app} were 3 h and 78.6 ml/(min kg), respectively. These parameters are well in agreement with the results obtained by the proposed method.

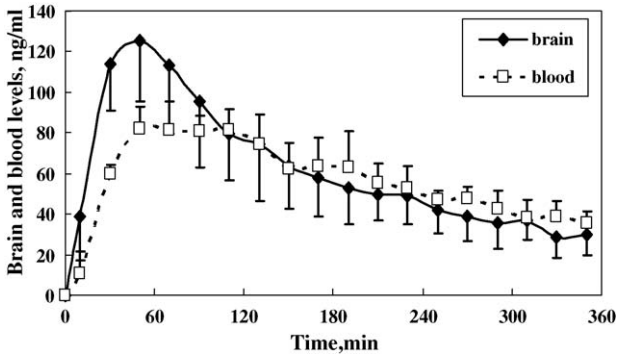


Fig. 6. Time course of DP concentration in rat blood and brain dialysates after i.p. administration to rat. Data are expressed as mean ± S.D., $n = 6$.

3.5. Donepezil monitoring in rat blood and brain microdialysate

The recovery and delivery factors to calculate the $recovery_{in\ vivo}$ of microdialysis probe are shown in Table 5. The ratios for brain and blood microdialysates were 28.4 ± 5.6 and 35.7 ± 4.7% ($n = 3$), respectively.

In a small amount of microdialysate (20 μl) spiked with 10 μl of IS, DP could be determined by the proposed method. Fig. 6 shows the time courses of DP concentration in rat blain and blood microdialysates after a single administration (5 mg/kg). The DP concentration in both microdialysates gradually increased and the peak concentration appeared within 60 min of administration. The C_{max} of brain microdialysate of 133.9 ± 25.8 ng/ml was 1.5 times higher than that of blood microdialysate (87.3 ± 10.5 ng/ml). The AUC and $t_{1/2}$ values in brain and blood were $2.9 \times 10^4 \pm 7.6 \times 10^3$ and $3.1 \times 10^4 \pm 4.8 \times 10^3$, and 171.4 ± 37.1 and 215.8 ± 48.9 min, respectively. In general, hydrophobic and small molecular mass compounds can pass through BBB. DP might easily diffuse into brain due to its hydrophobicity (log $P = 4.3$). However, the AUC ratio (AUC_{brain}/AUC_{blood}) which indicates transitivity of DP to brain was low at 0.94. Matsui et al. reported that the concentration and AUC in brain was two and seven times higher than in plasma [29]. One of the possible reasons is as follows: DP concentration in hippocampus was determined in this study, but Matsui et al. determined DP in whole brain. Pharmacokinetic parameters of DP in rat blood and brain microdialysates are summarized in Table 6.

In conclusion, we have developed a simple and sensitive HPLC-FL method for the determination of DP in some biological fluids and successfully applied it to plasma samples from Alzheimer's patients or monitoring of DP levels in rat brain

Table 5
In vivo recovery of 500 nM DP for rat brain and blood microdialysate

	$Delivery_{in\ vitro}$ (%)	$Delivery_{in\ vivo}$ (%)	$Recovery_{in\ vitro}$ (%)	$Recovery_{in\ vivo}$ (%)
Brain	24.3 ± 1.1	31.8 ± 4.2	37.3	28.4 ± 5.6
Blood	34.9 ± 3.0	42.3 ± 14.0[a]	43.2	35.7 ± 4.7

Data are expressed as mean ± S.D. ($n = 3$).
[a] $n = 5$.

Table 6
Pharmacokinetic parameters of DP in brain and blood microdialysates following DP administration

	Microdialysate	
	Brain	Blood
C_{max} (ng/ml)	133.9 ± 25.8	87.3 ± 10.5
T_{max} (min)	46.7 ± 8.2	60.0 ± 16.7
$T_{1/2}$ (min)	171.4 ± 37.1	215.8 ± 48.9
AUC ng/(ml min)	$2.9 \times 10^4 \pm 7.6 \times 10^3$	$3.1 \times 10^4 \pm 4.8 \times 10^3$
MRT (min)	251.6 ± 49.9	339.0 ± 64.6
K_{el} (min^{-1})	$4.2 \times 10^{-3} \pm 8.8 \times 10^{-4}$	$3.4 \times 10^{-3} \pm 7.5 \times 10^{-4}$

Data are expressed as mean ± S.D., $n = 6$.

and blood microdialysates after a single administration of DP. The proposed method might be applicable to therapeutic drug monitoring of DP and drug–drug interaction studies of DP with concomitant drugs.

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