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High performance liquid chromatography with fluorescence detection for the determination of phenylpropanolamine in human plasma and rat's blood and brain microdialysates using DIB-Cl as a label

Amal Kaddoumi, Toyomi Mori, Mihoko N. Nakashima, Mitsuhiro Wada, Kenichiro Nakashima*

Division of Analytical Research for Pharmacoinformatics, Department of Clinical Pharmacy, Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

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Abstract

A high performance liquid chromatographic method for the determination of phenylpropanolamine (PPA) in human plasma and rat's brain and blood microdialysates using fluorescence (FL) detection after precolumn derivatization with 4-(4,5-diphenyl-1*H*-imidazole-2-yl)benzoyl chloride (DIB-Cl) is described. PPA was extracted from plasma samples by a liquid–liquid extraction method with ethyl acetate followed by derivatization with DIB-Cl, while the blood and brain microdialysates were directly subjected for derivatization. The DIB-derivatives of PPA and the internal standard, ephedrine (EP), were then separated using an isocratic HPLC-FL set at excitation and emission wavelengths of 325 and 430 nm, respectively, on an ODS column. Calibration curves of PPA in spiked human plasma were linear over the concentration range of 5–5000 nM (0.755–755 ng/ml) and those in spiked blood and brain microdialysates were linear over the range of 25–5000 nM (3.775–755 ng/ml) with limits of detection of 17, 48 and 40 fmol on column in plasma and blood and brain microdialysates, respectively. As well, the intra- and the inter-assay precisions were lower than 12% for human plasma and the microdialysates.

The method was successfully applied for the monitoring of PPA levels in rat's brain and blood microdialysates administered with a single oral dose of PPA (2.5 mg/kg).

Keywords: PPA; Plasma; DIB-Cl; HPLC-FL; Microdialysis

1. Introduction

Phenylpropanolamine (PPA) is a sympathomimetic compound has been widely used in over-the-counter (OTC) and prescription medications for cough and

* Corresponding author. Tel.: +81-95-819-2450; fax: +81-95-819-2450.
E-mail address: naka-ken@net.nagasaki-u.ac.jp (K. Nakashima).

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cold, nasal decongestant and appetite suppressant. Recently, considerable interest in PPA has arisen due to the serious side effects accompanied its use including hemorrhagic stroke, arrhythmias and hypertension [1–6]. This caused the FDA of the United States to ask the OTC manufacturers voluntarily to reformulate products containing PPA and to exclude it from these products in a process to remove PPA from the market and also to issue a public advisory warning about the risks linked to PPA [7]. However, the withdrawal of PPA is not applicable everywhere and still available and being in use.

In human, the reported plasma levels of PPA as maximum concentrations after oral dose ranged from 69 to 440 ng/ml [8] making the need for sensitive analytical methods essential for its determination. The current methods utilize either GC [9,10] or HPLC [11–15]. In these methods the used plasma or serum volumes were not less than 0.5 ml with reported quantitation limit of 2 ng/ml [13], while another study used 3 ml plasma to achieve quantitation limit of 1 ng/ml [10].

Yamashita et al. [14] reported an HPLC method with column switching for the determination of PPA in plasma. Although the authors could provide limit of detection (LOD) of 0.4 ng/ml, the method was rather complicated and required 1.0 ml plasma.

In many cases the availability of such volumes is not feasible, either for PPA monitoring in human plasma or for animal pharmacokinetic studies where volumes in few microliters are available. Hence, in this paper, a detailed HPLC method is described for the determination of PPA with fluorescence (FL) detection following its derivatization with 4-(4,5-diphenyl-1*H*-imidazole-2-yl)benzoyl chloride (DIB-Cl). The applicability of this method to human plasma was demonstrated using small volumes (100 μl) of spiked plasma samples with known concentrations of PPA. The separation was achieved isocratically by using an ODS column. Furthermore, the method was used for the determination of PPA in blood and brain microdialysates with volumes did not exceed 15 μl. The method was applied for PPA monitoring in brain and blood microdialysates of rats administered orally with a single dose of 2.5 mg/kg PPA.

2. Experimental

2.1. Chemicals

(+)-Norephedrine hydrochloride was obtained from Sigma-Aldrich (Tokyo, Japan). Ephedrine (EP) was obtained from Dainippon Pharmacy (Osaka, Japan) and used as the internal standard (IS). DIB-Cl was synthesized in our laboratory [16] and can be obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan).

Acetonitrile, methanol and ethyl acetate of HPLC grade were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Water was deionized and passed through an automatic water distillation apparatus (Aquarius GSR-500, Advantec, Tokyo). Other reagents were of analytical grade.

A CMA microdialysis system (Carnegie Medicine, Stockholm, Sweden) was used. The probes used for blood and brain microdialysis sampling were of the same membrane length PC 10 (4 mm membrane length, 20,000 Da).

2.2. HPLC system and chromatographic conditions

The separation of the DIB-derivative of PPA and EP (as IS) in human plasma and brain and blood microdialysates was performed using an isocratic HPLC system consisting of a pump (LC-10Ad*vp*, Shimadzu, Kyoto, Japan) and fluorescence detector (Hewlett Packard series 1100, Tokyo) set at an excitation wavelength of 325 nm and an emission of 430 nm and interfaced to a PC where data acquisition was achieved by HP ChemStation software (Tokyo, Japan). A Rheodyne 7125 injector (Cotati, CA, USA) with a 20-μl sample loop. The column was Daisopak SP-120-5-ODS-BP (250 mm × 4.6 mm i.d., 5 μm, Daiso, Osaka). The mobile phase consisted of a mixture of acetonitrile–sodium acetate (pH 6.5; 0.1 M) (55:45, v/v). The flow rate was 1.0 ml/min.

2.3. Derivatization conditions with DIB-Cl

To choose the optimal conditions for the derivatization of PPA with DIB-Cl, the effects of DIB-Cl concentration, carbonate buffer concentration and pH, reaction time as well as the reaction temperature were

investigated. Duplicate samples of PPA were derivatized at five different carbonate buffer pHs from 8.5 to 11 and concentrations ranged from 0.01 to 0.1 M. The examined DIB-Cl concentrations ranged from 0.05 to 1.0 mM in acetonitrile. The reaction time was evaluated from 5 to 60 min at room temperature, 45 and 60 °C. The concentration of PPA used for examining the optimum conditions was 50 μM, where 20 μl of PPA in methanol were pipetted into a vial, evaporated under a stream of nitrogen gas and then derivatized with DIB-Cl as described in Section 2.6.

2.4. Plasma samples and extraction procedure

Human blood samples were drawn from healthy volunteers in our laboratory into EDTA tubes. Blood samples were centrifuged at 2000 × *g* for 10 min, and the resultant plasma samples were separated and kept frozen at −20 °C prior to use.

The extraction was done as follows: to 100 μl of plasma sample, 10 μl of 250 nM (for low standard concentrations) and 1000 nM (for high standard concentrations) of EP in methanol as IS were added and mixed, followed by the addition of 200 μl of 0.1 M borate buffer (pH 10.6) and 750 μl of ethyl acetate. Samples were vortex mixed for 1 min and centrifuged at 20 °C for 10 min at 2000 × *g*. To a 600 μl of the organic layer in a vial, 10 μl of acetic acid were added and evaporated to dryness by a centrifugal evaporator (Hitachi, CE1 model, Tokyo) for 15 min at 45 °C. Samples were then derivatized with DIB-Cl as described in Section 2.6.

2.5. Microdialysis samples

Blood and brain microdialysate samples were collected as described in a previous study [17]. The artificial cerebrospinal fluid consisted of 145 mM NaCl, 1.5 mM KCl, 1.5 mM $MgCl_2$, 1.25 mM $CaCl_2$, 10 mM glucose and 1.5 mM K_2HPO_4, which was adjusted to pH 7.0 and pumped through CMA/PC 10 probes for both blood and brain at a flow rate of 0.5 μl/min. Blood and brain microdialysates were collected before and after oral administration of single dose of PPA (2.5 mg/kg) to Wistar rats with sampling interval of 30 min allowing the collection of 15 μl microdialysis samples from both blood and brain. Sampling was continued for 9 h. All samples were stored at −20 °C until analysis.

2.6. Derivatization with DIB-Cl

The residues of the evaporated plasma samples were derivatized as follows: 75 μl of 0.5 mM DIB-Cl solution in acetonitrile and 25 μl of 0.025 M carbonate buffer (pH 9.5) were added to the residue, vortex mixed and then incubated at room temperature for 10 min. The reaction was stopped by adding 5 μl of aqueous ammonia (25%) from which 20 μl of the resultant solution were injected onto the column.

Microdialysates were derivatized directly without any further treatment in the same manner of plasma samples, with slight modification for the addition of the internal standard. To the microdialysates, 5 μl of 500 nM EP (IS) in acetonitrile were added followed by the addition of 75 μl of 0.5 mM DIB-Cl solution and 25 μl of 0.025 M carbonate buffer. Other conditions were similar to those for plasma samples.

2.7. Method validation

Quantitation of PPA in human plasma and in rat's blood and brain microdialysates was performed via the internal standard method using the peak height ratios of the PPA to that of the internal standard. The calibration curves were prepared over the ranges of 5–5000 nM (0.755–755 ng/ml) in plasma and 25–5000 nM (3.775–755 ng/ml) in brain and blood microdialysates. For the validation of the method, spiked plasma and microdialysate samples with known concentrations of PPA were used. The precision was calculated as the relative standard deviation (RSD) within a single run (intra-day) and between different assays (inter-day). The LOD was calculated according to the equation:

$$\mathrm{LOD} = 3.3 \times \frac{\delta}{S}$$

where δ is the standard deviation of intercepts of the calibration curves and S is the slopes average. The recovery as well as the accuracy of the method was also evaluated.

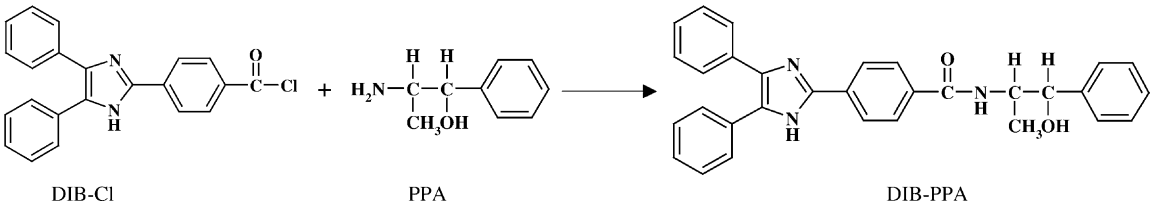

Fig. 1. The reaction scheme of PPA with DIB-Cl.

3. Results and discussion

3.1. Optimal conditions for derivatization with DIB-Cl

Previously, we developed different methods for the simultaneous determination of sympathomimetic amines including PPA in human and rat plasma. In these methods, two derivatizing reagents were used, dansyl chloride (DNS-Cl) [18] and DIB-Cl [19,20]. DIB-Cl method [20] provided higher sensitivity compared to DNS-Cl considering all the studied compounds, which make us continue further studies for the determination of PPA using DIB-Cl as the fluorescence derivatizing reagent. Fig. 1 shows the derivatizing reaction of PPA with DIB-Cl.

The effects of DIB-Cl and carbonate buffer concentrations on the reaction yield were studied. PPA showed maximum peak height at DIB-Cl concentration of 0.5 mM and kept constant to 1.0 mM while carbonate buffer showed maximum yield at 0.025 M with 0.5 mM of DIB-Cl, which were then used for the following experiments. Since alkaline conditions are required for derivatization with DIB-Cl, the pH of carbonate buffer was adjusted at 8.5, 9, 9.5, 10, 10.5 and 11. The peak heights of the DIB-derivatives were almost the same in the studied pH range; hence pH 9.5 was chosen as the optimum. In addition, the effects of temperature and the reaction time were examined. The yield of DIB-derivative of PPA (DIB–PPA) at room temperature, 45 and 60 °C were almost the same as well the reaction was almost complete within 5 min, accordingly reaction time for 10 min at room temperature were selected.

The stability of DIB–PPA was examined at different intervals, 0 time (directly after the reaction was stopped), 2, 4, 6, 24 and 48 h after the reaction was stopped. The deviation in DIB–PPA peak heights at the intervals examined compared to its value at 0 time ranged from +2.4 to +3.5%. These results indicate that DIB–PPA is stable for at least 48 h when left at room temperature in dark which permits the use of an autosampler.

3.2. Separation of DIB–PPA in plasma and microdialysates

Typical chromatograms of plasma and microdialysates are shown in Figs. 2 and 3, respectively.

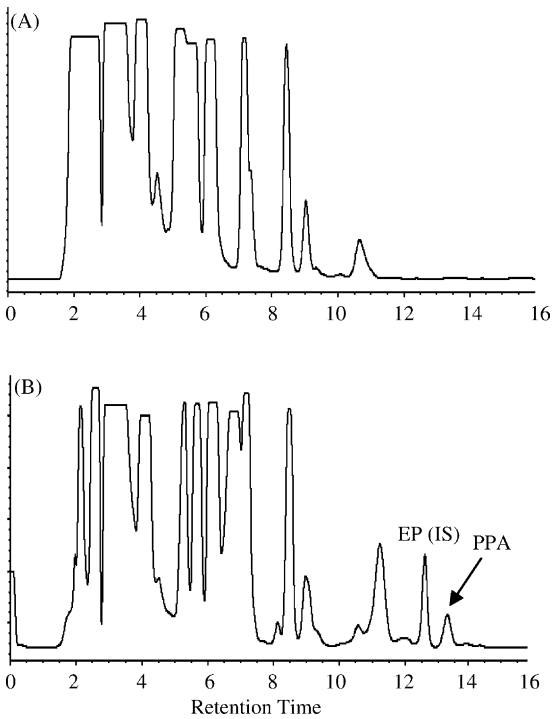

Fig. 2. Typical chromatograms obtained from human plasma. (A) Non-spiked and (B) spiked with 15 ng/ml PPA and 41 ng/ml EP as IS.

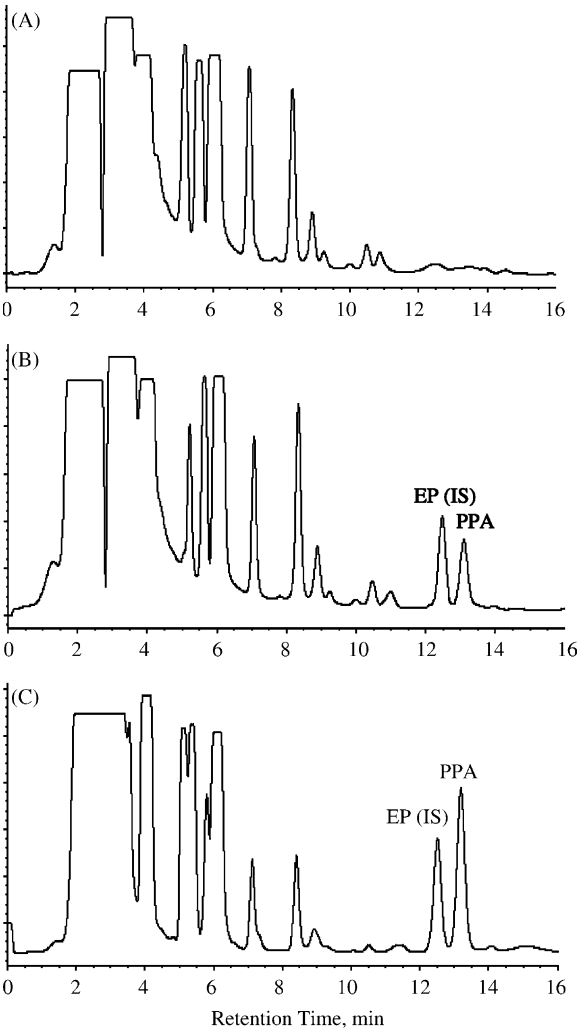

Fig. 3. Typical chromatograms obtained from rats. (A) Non-spiked brain microdialysate, (B) spiked with 37.75 ng/ml PPA and 41 ng/ml EP as IS, and (C) spiked blood microdialysate with 377.5 ng/ml PPA and 165 ng/ml EP as IS. The detector sensitivity in *C* is lower by four-times than A and B.

Fig. 2A represents a non-spiked human plasma sample and Fig. 2B represents a sample spiked with PPA at a concentration of 15 ng/ml. Fig. 3A illustrates a non-spiked brain microdialysates obtained before PPA administration to rats, Fig 3B represents brain microdialysate spiked with 37.75 ng/ml of PPA, and Fig. 3C represents a chromatogram obtained from blood microdialysate spiked with 377.5 ng/ml PPA. The retention times for the DIB-EP (as IS) and DIB–PPA were 12.5 and 13.2 min, respectively. These derivatives were well separated from the reagent peaks as well as from the endogenous compounds where no interfering peaks were co-eluted.

3.3. Method validation

The calibration curves of PPA in human plasma and both brain and blood microdialysates were linear in the ranges 0.755–755 ng/ml plasma and 3.775–755 ng/ml brain and blood microdialysates with r values $= 0.999$. The regression equations for PPA in plasma, brain and blood microdialysates were $y = 0.108x - 0.055$, $y = 0.013x + 0.048$ and $y = 0.015x - 0.034$, respectively, where y represents the peak heights ratio of PPA to the IS and x is PPA concentration in ng/ml. The calculated LOD's on column were 17, 40 and 48 fmol in plasma, brain and blood microdialysates, respectively, which correspond to 0.14, 2.4 and 2.9 ng/ml, respectively. Calibration ranges and LODs are summarized in Table 1. The proposed method proved to be highly sensitive compared to other reported methods for PPA determination in biological fluids including plasma and serum. The reported LODs using GC [9,10] ranged from 1 to 30 ng/ml while by HPLC [11–15] ranged from 0.4 to 25 ng/ml. In these studies, the used volumes ranged from 0.5 to 3 ml, making this proposed method more practical were volumes of 100 μl and less where sufficient. Furthermore, compared to the available methods utilized mass spectroscopy (MS) the present method continues to be more sensitive. Among those methods, Bogusz et al. [21] reported an LC-APCI-MS method for the simultaneous determination of amphetamines in addition to PPA in serum with LOD ranged from 1 to 5 ng/ml for all the compounds. Others reported LOD's for PPA in urine by GC-MS of less than 10 ng/ml [22] and 30 ng/ml [23] using 1 and 5 ml urine, respectively.

The precision of the method was evaluated by analyzing four replicates of spiked human plasma and rat brain and blood microdialysates with known concentrations of PPA at three levels for both plasma samples and the mirodialysates. The intra-day RSD ranged from 2.1 to 6.7% for spiked human plasma, from 1.7 to 5.4% for brain microdialysates and from 2.9 to 7.6% for blood microdialysates, while the inter-day RSD ranged from 6.6 to 9.0% for spiked human plasma,

Table 1
Studied ranges, LODs, method precision and accuracy of PPA in spiked human plasma and brain and blood microdialysates

Matrix	Range (r) (ng/ml)	LOD (ng/ml) (fmol on column)	Spiked (ng/ml)	Precision, RSD% ($n = 4$)		Accuracy[a]
				Intra-day	Inter-day	
Plasma						
	0.755–755	0.14	1.51	6.7	7.7	100
	(0.999)	(17)	15.1	2.1	6.6	94
			150	2.2	9.0	104
Brain microdialysate						
	3.775–755	2.4	15.1	5.4	11.6	101
	(1.000)	(40)	75.5	5.0	5.0	106
			151	1.7	7.8	101
Blood microdialysate						
	3.775–755	2.9	15.1	2.9	4.9	102
	(0.999)	(48)	75.5	4.7	4.2	106
			151	7.6	7.3	100

[a] Accuracy% = (found concentration/nominal concentration) × 100.

from 5.0 to 11.6% for brain microdialysates and from 4.2 to 7.3% for blood microdialysates. The results are listed in Table 1.

Following the liquid–liquid extraction of spiked plasma, the obtained recovery of PPA was comparable with the previous reports [18–20] and was 104%.

The in vitro and in vivo recoveries of the brain and blood probes for PPA were 44.6 and 17.1%, respectively, for brain probe and 56.4 and 31.1%, respectively, for blood probe.

The accuracy deviations from the nominal concentrations ranged from −6 to +4% for spiked plasma and from 0 to +6% for both brain and blood microdialysates (Table 1).

3.4. Method application

Microdialysis experiments, in most cases, are accompanied with small available sample size as well as the large number of samples especially for long periods of simultaneous monitoring of more than one tissue. The present method overcame these two disadvantages of microdialysis. The use of DIB-Cl as the derivatizing reagent allowed the high sensitive determination of PPA in few microliters (15 μl) of microdialysates. Also, the short chromatographic run (15 min), made the method suitable for the simultaneous monitoring of PPA in brain and blood for 9 h with 30 min sampling intervals within a reasonable time.

As an application for the proposed method, PPA was orally administered to Wistar rats with a single dose of 2.5 mg/kg. PPA levels in brain and blood were monitored for 9 h every 30 min. Fig. 4 shows typical chromatograms obtained from rat after 300 min of PPA administration (A) brain microdialysate and (B) blood microdialysate. DIB–PPA peaks in A and B represent the concentrations 8.6 and 10.1 ng/ml (not corrected to the in vivo recovery). Fig. 5 shows the profiles of brain and blood microdialysates concentrations of PPA (corrected to the in vivo probes recoveries) versus the time ($n = 3$).

To the best of our knowledge, this is the first time for the simultaneous monitoring of PPA levels in rat's brain and blood. The pharmacokinetic parameters are summarized in Table 2. Following the oral administration of PPA, the obtained C_{max} in blood and brain

Table 2
Pharmacokinetics of PPA in rats' brain and blood microdialysates administered with single oral dose of PPA, 2.5 mg/kg ($n = 3$)

Parameter	Brain	Blood
C_{max} (ng/ml)	143 ± 48	129 ± 64
T_{max} (min)	280 ± 122	180 ± 121
$T_{0.5}$ (min)	773 ± 513	363 ± 51
AUC_{10h} (μg min/ml)	48 ± 18	47 ± 27
Cl (ml/min/kg)	38 ± 22	62 ± 24
MRT (min)	1251 ± 809	576 ± 141

Data are expressed as mean ± S.E.M..

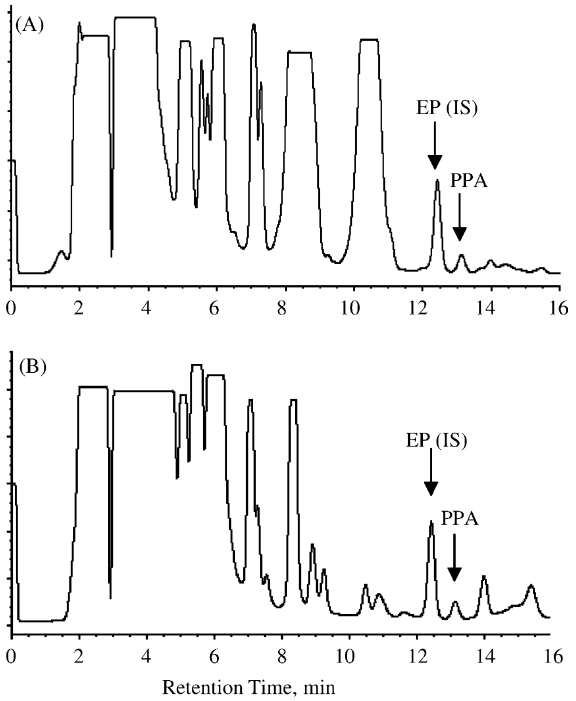

Fig. 4. Chromatograms obtained from rat (A) brain microdialysate and (B) blood microdialysate after 300 min of single oral administration of PPA (2.5 mg/kg). PPA concentrations are 8.6 and 10.1 ng/ml in brain and blood microdialysates, respectively.

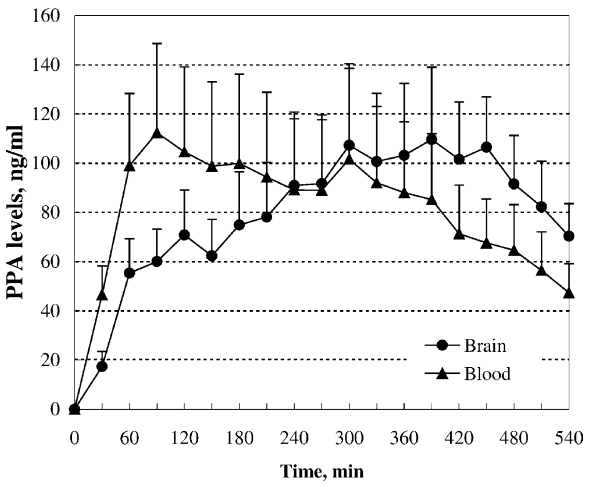

Fig. 5. Mean concentrations of PPA in rat brain and blood microdialysates after single oral administration of PPA (2.5 mg/kg), $n = 3$.

were 129 ± 64 ng/ml and 143 ± 48 ng/ml, respectively, at 180 min for the former and 280 min for the later. Though the inter-variation was large, no significant difference between the AUCs of PPA in blood (47 ± 27 μg min/ml) and brain (48 ± 18 μg min/ml) was obtained ($P > 0.05$, Student's *t*-test). PPA levels in both brain and blood are comparable, however, unlike other amphetamines, PPA brain-to-blood ratio (as AUCs) is 1.0, while the reported brain-to-blood ratios for amphetamine [24], methamphetamine [24], fenfluramine and phentermine [17] were 8.5, 9.7, 1.9 and 2.6, respectively. This could be related to the lower lipophilicity of PPA, due to the hydroxyl group present on the β-carbon, which may caused a decrease in its diffusion from the blood to the brain through the blood brain barrier compared to the other mentioned amphetamines.

In conclusion, we have described a method for the sensitive and simple determination of PPA in biological fluids including human plasma and rat's blood and brain microdialysates. The method showed high sensitivity with detection limits of 0.14 ng/ml in plasma using only 100 μl sample, and 2.4 and 2.9 ng/ml in brain and blood microdialysates, respectively, using 15 μl dialysates. As well, the method showed its simplicity where the derivatization reaction with DIB-Cl did not exceed 10 min at room temperature with chromatographic run time of 15 min per sample. The method could be useful for PPA monitoring in human plasma for clinical and forensic purposes. As well, the proposed method could be used for pharmacokinetic animal's studies including drug interactions that are required and essential to understand and elucidate the serious side effects accompanied by PPA ingestion with other drugs.

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