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Journal of Chromatography B, 791 (2003) 291–303
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatography studies on the pharmacokinetics of phentermine and fenfluramine in brain and blood microdialysates after intraperitoneal administration to rats

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Received 4 October 2002; received in revised form 7 February 2003; accepted 12 March 2003

Abstract

A highly sensitive and simple HPLC method with fluorescence detection for the determination of phentermine (Phen), fenfluramine (Fen) and norfenfluramine (Norf, the active metabolite of Fen) in rat brain and blood microdialysates has been developed. The brain and blood microdialysates were directly subjected to derivatization with 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride (DIB-Cl) in the presence of carbonate buffer (0.1 *M*, pH 9.0) at room temperature. The chromatographic conditions consisted of an ODS column and mobile phase composition of acetonitrile and water (65:35, v/v) with flow rate set at 1.0 ml/min. The detection was performed at excitation and emission wavelengths of 325 and 430 nm, respectively. Under these conditions, the DIB-derivatives of Phen, Fen and Norf were well separated and showed good linearities in the studied ranges (5–2000 n*M* for Phen and 10–2000 n*M* for Norf and Fen) with correlation coefficients greater than 0.999. The obtained detection limits were less than 23 fmol on column (for the three compounds) in both brain and blood microdialysates at a signal-to-noise ratio of 3 ($S/N=3$). The intra- and the inter-assay precisions were lower than 10%. The method coupled with microdialysis was applied for a pharmacokinetic drug–drug interaction study of Phen and Fen following individual and combined intraperitoneal administration to rats. In addition, since the role of protein binding in drug interactions can be quite involved, the method was applied for the determination of total and free Phen and Fen in rat plasma and ultrafiltrate, respectively. The results showed that Fen and/or Norf significantly altered the pharmacokinetic parameters of Phen in both blood and brain but did not alter its protein binding. On the other hand, there was no significant difference in the pharmacokinetics of Fen when administered with Phen.

Keywords: Microdialysis; Drug interaction; Phentermine; Fenfluramine

1. Introduction

The appetite suppressants phentermine (Phen) and (±)fenfluramine (Fen) have been widely prescribed to treat obesity. Phen–Fen combination affords su-

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00231-9

perior appetite control when compared to either drug alone [1]. The anorectic properties of Fen are related to enhanced serotonergic neurotransmission [2,3]; it facilitates the release of serotonin (5-HT) and inhibits its reuptake by serotonergic nerve endings, and its metabolite norfenfluramine (Norf) releases 5-HT and stimulates postsynaptic 5-HT2 receptors [4,5]. On the other hand, Phen stimulates the release of dopamine (DA) [6–8]. Although Fen and its enantiomer dexfenfluramine were withdrawn from the US market and have not been used since 1997 due to the occurrence of valvular heart disease and primary pulmonary hypertension [9–14] and neurotoxicity in animals studies [15–21], it is still of interest to resolve the mechanism by which the combination Phen–Fen enhances weight loss at lower doses as well as the neurotoxic effect.

Several studies have been reported to elucidate the nature of interaction between Fen and Phen and clarify the mechanism by which such a combination enhances weight loss. These studies are concerned with the effect of the combination on the release of DA and 5-HT. Most investigators supposed that Phen–Fen combination enhances 5-HT activity within the synapse causing appetite suppression, hence reducing weight, and simultaneously enhances the neurotoxicity of both drugs on the 5-HT system. Studies by Balcioglu and Wurtman [6,22,23] and Shoaib et al. [8] suggested that although Phen has minimal effect on 5-HT release, it might further enhance the release of extracellular 5-HT, which is induced by Fen. Lew et al. [16] supported such mechanism and reported that the observed neurotoxicity of 5-HT induced by the combination Phen–Fen involves Phen promoting 5-HT release through DA release.

Another assumption was the role of Phen as an MAO inhibitor. Although many studies showed that Phen does not inhibit MAO at typical doses [24–26], Ulus et al. [27] and Maher et al. [28] reported that Phen is a potent inhibitor of MAO, an important enzyme for 5-HT metabolism, so the combination would inhibit 5-HT metabolism by Phen as well as inhibit its reuptake into the nerve terminal by Fen leading to an increase in 5-HT activity.

Nonetheless, pharmacokinetic interaction studies between Phen and Fen have not been reported. Balcioglu and Wurtman [22] reported the lack of evidence that Phen alters the pharmacokinetics of Fen and vice versa, while due to the similarity between amphetamine and Phen, Wellman and Maher [29] supposed the results of a previous study by Hunsinger et al. [30] could be applied in the case of Phen–Fen. In this study, the authors indicated that amphetamine altered Fen's pharmacokinetic parameters, prolonging the duration and effect of Fen.

Sampling by microdialysis has been employed for in vivo studies of drugs pharmacokinetics. This technique makes possible the investigation of drugs in the extracellular fluids of different tissues and biological fluids [31]. Hence in this work, in order to elucidate further the interaction between Phen and Fen and the effect of each drug on the pharmacokinetic parameters of the other in both blood and brain, microdialysis was coupled to HPLC–FL method for the simultaneous determination of Phen, Fen and Norf. The use of microdialysis is accompanied by many challenges including the large number of samples (especially if more than one drug has to be monitored for a long time in brain and blood simultaneously), the small microdialysate sample sizes together with the low probe recoveries for the drugs of interest in this study. To overcome these problems, the development of a simple, rapid and highly sensitive method was necessary. Previously, we reported different HPLC methods for the simultaneous determination of Phen and Fen in addition to other sympathomimetic amines [32–34] in plasma using the fluorescent reagents dansyl chloride (DNS-Cl) [34] and 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride (DIB-Cl) [32,33]. In these studies, the derivatization reaction of Phen and Fen with DIB-Cl was faster (within 10 min at room temperature) than DNS-Cl (30 min at 45 °C) and the obtained DIB-derivatives were highly fluorescent compared to DNS-Cl method. Consequently, in this study, due to the reasons mentioned above, DIB-Cl was the reagent of choice with some modifications in the reaction conditions for the determination of Phen, Fen and Norf in microdialysates and in the separation conditions to shorten the chromatographic run time. The method was then applied for the drug–drug interaction study of Phen and Fen. Phen was administered to rats at two different doses (1 and 5 mg/kg) separately or in combination with Fen (5 mg/kg), intraperitoneally (i.p.) and the brain and

blood microdialysates were collected every 20 min for 10 h. We also applied the method to examine the effect of the drug combination on the protein binding of Phen and Fen in rat plasma and plasma ultrafiltrate after i.p. administration of 1 and 5 mg/kg of Phen and Fen, respectively.

2. Experimental

2.1. Chemicals

Fen·HCl and Phen·HCl were obtained from Sigma (St. Louis, MO, USA). Fluoxetine HCl (FLX·HCl) was purchased from Tocris Cookson (Bristol, UK). DIB-Cl was synthesized in our laboratory [35] but can be obtained from Tokyo Kasei Kogyo (Tokyo, Japan).

Norf·HCl was electrochemically synthesized from Fen·HCl. In brief, Fen (200 mg) was reacted with di-*tert*.-butyl dicarbonate, which was subjected to electrochemical oxidation in a one compartment cell equipped with platinum electrodes at an applied current of 100 mA. The resulting crude Norf was purified via Boc-Norf followed by hydrolysis with methanolic HCl to afford a white solid (yield 10 mg). Elemental analysis for $C_{10}H_{12}NF_3$·HCl was: calculated: C, 50.11%; H, 5.47%; N, 5.84%; found: C, 49.61%; H, 5.15%; N, 5.68%.

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

2.2. HPLC system and chromatographic conditions

The simultaneous separation of the DIB-derivatives of Phen, Norf and Fen in brain and blood microdialysates was performed using an isocratic HPLC system (Shimadzu, Kyoto, Japan) consisting of a pump (LC-6A), a recorder (R-112) and a fluorescence detector (RF-550) set at an excitation wavelength of 325 nm and an emission wavelength of 430 nm. A Rheodyne 7125 injector (Cotati, CA, USA) with a 20-μl sample loop was used. The column was a Daisopak SP-120-5-ODS-BP (250× 4.6 mm I.D., 5 μm, Daiso, Osaka, Japan). The mobile phase consisted of a mixture of acetonitrile–water (65:35, v/v). The flow rate was 1.0 ml/min.

For the protein binding study, a gradient HPLC system was used. To the above system a second pump (LC-6A) was connected with a system controller (SCL-6A). Mobile phase A was kept the same and mobile phase B was acetonitrile. The program started after the elution of DIB-Fen as follows: from 28 to 30 min mobile phase B increased from 0 to 45%, held for 10 min and stopped at 40 min.

2.3. Brain and blood microdialysis

Male Wistar rats were used in the experiments (270–390 g; Otsubo Experimental Animals, Nagasaki). The animals were group-housed in wire-top clear Plexiglas cages (26×42×15 cm) with woodchips for bedding and were provided with standard laboratory food (Oriental Yeast, Tokyo) and water ad libitum. The animals were housed under controlled conditions with an ambient temperature of 24±1 °C and a 12-h light/dark cycle. All animal procedures involving animal care were approved by the Nagasaki University Animal Care and Use Committee.

A CMA microdialysis system (Carnegie Medicine, Stockholm, Sweden) was used. A blood probe (PC 10, 4-mm membrane length, 20 000 Da) was implanted within the jugular vein. A brain probe (PC 12, 2-mm membrane length, 20 000 Da) was implanted in the frontal cortex [36] with the coordinates: A 3.5 mm, L 1.3 mm, V 5.6 mm relative to bregma [37]. Both probes were perfused with an artificial cerebrospinal fluid (CSF) having the following composition: 145 m*M* NaCl, 1.5 m*M* KCl, 1.5 m*M* $MgCl_2$, 1.25 m*M* $CaCl_2$, 10 m*M* glucose, 1.5 m*M* K_2HPO_4, adjusted to pH 7.0, with a flow rate of 1 μl/min [36]. Rats were divided into three groups. Group 1 was administered a single dose of Phen (1 or 5 mg/kg, i.p., $n=6$ for each dose), group 2 a single dose of Fen (5 mg/kg, i.p., $n=6$) and group 3 a single dose of a Phen and Fen combination (1 or 5 mg/kg of Phen and 5 mg/kg of Fen, $n=6$ for each combination). Drugs were dissolved in saline before administration. Brain and blood microdialysates were collected every 20 min for 10 h and stored at −20 °C until analysis.

2.4. Protein binding study

For this experiment, rats were also divided into three groups and administered single i.p. doses of Phen (1 mg/kg, $n=3$), Fen (5 mg/kg, $n=3$) or Phen (1 mg/kg) and Fen (5 mg/kg) in combination ($n=4$). Blood samples (400 μl) were collected before (0 time) and at 15, 30, 45, 60, 90, 120, 150, 180, 240, 300 and 360 min following drug administration in tubes containing EDTA and centrifuged. Plasma was then separated and stored at −20 °C until analysis.

Separation of unbound Phen, Fen and Norf was achieved by ultrafiltration using disposable Ultrafree-MC centrifugal filter devices with 10 000 molecular mass cut-off (Millipore, Bedford, USA). To these devices, 80 μl of plasma sample were transferred and centrifuged at 5000 *g* for 60 min at 25 °C.

2.5. Extraction of plasma and ultrafiltrate samples

Plasma and ultrafiltrate samples were extracted in the same manner as described previously [32]. In brief, to 50 μl of plasma FLX (2 μ*M*, 10 μl as IS), borate buffer (0.1 *M*, 100 μl, pH 10.6) and ethyl acetate were added. Samples were centrifuged and the organic layer was pipetted and evaporated. The residues were then derivatized with DIB-Cl as described below.

2.6. Derivatization with DIB-Cl

Microdialysate samples were directly derivatized. To the dialysates (20 μl), 5 μl of 0.1 *M* carbonate buffer (pH 9.0) were added followed by 75 μl of 2 m*M* DIB-Cl suspension in acetonitrile. Samples were vortex mixed and incubated at room temperature for 10 min. The reaction was stopped by adding 5 μl of 25% ammonia solution.

Plasma and ultrafiltrate samples were derivatized as above except the volumes of carbonate buffer and DIB-Cl added were 10 and 150 μl, respectively. From the reaction mixtures, 20 μl were injected onto the column.

2.7. Method validation

The calibration curves were prepared over the ranges of 5–2000 n*M* for Phen (0.75–298 ng/ml), and 10–2000 n*M* for Fen (2.3–461 ng/ml) and Norf (2.0–407 ng/ml) in both brain and blood microdialysates, while for plasma and ultrafiltrate samples, calibration curves were constructed in the ranges of 10–2000 n*M* for Phen (1.5–298 ng/ml) and Norf (2.0–407 ng/ml) and 20–4000 n*M* for Fen (4.6–921 ng/ml). The precision was calculated as the relative standard deviation (RSD) within a single run (intra-assay) and between different assays (inter-assays). The limit of detection (LOD) was calculated as the peak height at a signal-to-noise ratio of 3 ($S/N=3$) on column. The recovery was also evaluated.

2.8. Pharmacokinetics and statistical analysis

The concentrations of Phen, Norf and Fen in brain and blood microdialysates were calculated from the corresponding calibration curves. The concentrations obtained in brain and blood microdialysates were corrected to the in vivo loss for the three compounds, and the pharmacokinetic parameters were performed using the corrected data.

Pharmacokinetic calculations were processed by the non-compartmental method [38]. The peak concentrations (C_{max}) and concentration peak times (T_{max}) were obtained directly from the original data. The area under the curve for concentrations versus time (AUC) was calculated using the linear trapezoidal rule. The terminal elimination rate constant (k_{el}) was calculated as the negative slope of the non-weighted least squares curve fit to logarithmically transformed concentration versus time. The elimination half-life ($t_{1/2}$) and the apparent clearance (Cl) were determined by the equations $\ln 2/k_{el}$ and dose/AUC, respectively. Finally the mean residence time (MRT) was calculated from the equation area under the moment curve (AUMC)/AUC. All data are presented as means±standard error of mean (S.E.M.). Statistical analysis was assessed by Student's *t*-test with $P<0.05$ being considered significant.

3. Results

At the beginning of this study, the derivatizing conditions of our previous report [32] used for plasma-evaporated residues (1.5 m*M* DIB-Cl, 150 μl and 0.01 *M* carbonate buffer, 50 μl) were applied for direct derivatization of the microdialysates. Due to

the high salts concentration present in the CSF and the increase in the aqueous portion (microdialysate and carbonate buffer), inconsistent results were obtained. This problem could not be solved by evaporating the samples to dryness, which led us to modify the derivatizing conditions to be 2 m*M* DIB-Cl, 75 μl and 0.1 *M* carbonate buffer, 5 μl. Under these conditions, consistent as well as reproducible results were obtained. Also, to simplify the method, the same derivatizing conditions as used for microdialysates were applied for plasma and ultrafiltrate samples except that the volumes of DIB-Cl and carbonate buffer were doubled.

3.1. Chromatographic conditions

3.1.1. HPLC separation of the DIB-derivatives

A good separation of the three DIB-derivatives was achieved within 30 min with retention times of 18, 20 and 27 min for the DIB-derivatives of Norf, Phen and Fen, respectively. Since an extraction step was required for plasma and ultrafiltrate samples, FLX was added as IS. This caused us to use a gradient system to speed up the elution of FLX at 37 min. Typical chromatograms are shown in Figs. 1 and 2. Fig. 1 illustrates chromatograms obtained from rat brain microdialysates, before (A) and at 40 min (second interval) following drug co-administration (B) and blood microdialysate (C) at 60 min (third interval) following administration. Fig. 2 shows chromatograms obtained from rat plasma, before (A) and at 45 min (B) following co-administration and from rat plasma ultrafiltrate (C) at 120 min following administration.

3.1.2. Method validation

3.1.2.1. Calibration curves and LODs. The calibration curves of both brain and blood microdialysates were linear in the ranges studied with *r*-values

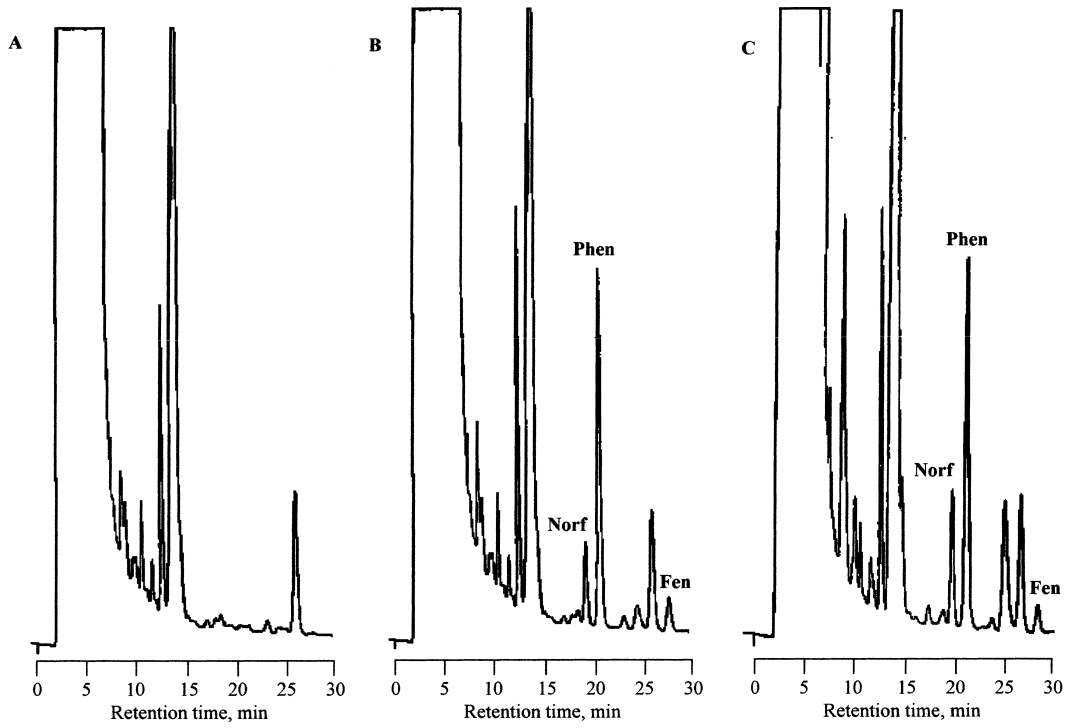

Fig. 1. Chromatograms obtained from rat brain microdialysates (A) before drug administration, (B) at 40 min following Phen and Fen co-administration (5 mg/kg each, i.p.) and (C) from rat blood microdialysate at 60 min following Phen and Fen co-administration (5 mg/kg each, i.p.).

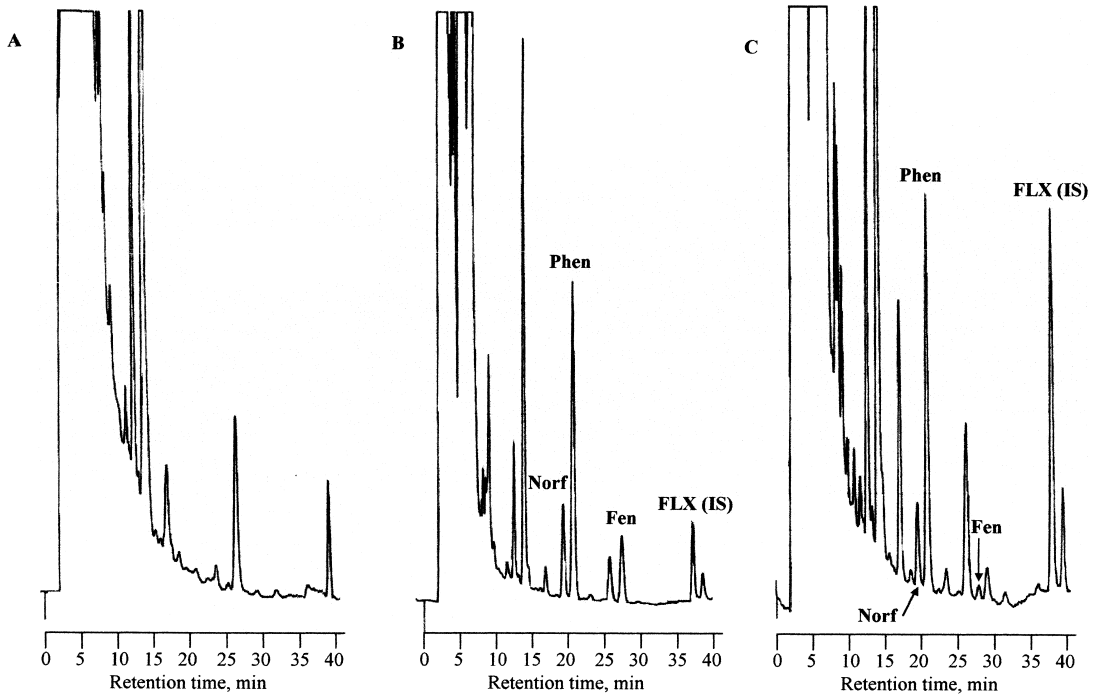

Fig. 2. Chromatograms obtained from rat plasma (A) before drug administration, (B) at 45 min following Phen (1 mg/kg, i.p.) and Fen co-administration (5 mg/kg, i.p.) and (C) from rat plasma ultrafiltrate at 120 min following Phen (1 mg/kg, i.p.) and Fen co-administration (5 mg/kg, i.p.).

greater than 0.999 for the three compounds. The LODs on column at $S/N=3$ were 19 fmol for Phen and 23 fmol for Fen in both microdialysates, while for Norf the LODs were 14 and 17 fmol in brain and blood microdialysates, respectively.

For the protein binding study, the calibration curves prepared in spiked rat plasma and ultrafiltrate were linear with *r*-values of 0.999 for the compounds. The LODs ranged from 18 to 27 fmol for Phen and Norf, while for Fen the LODs were 65 and 61 fmol on column in rat plasma and ultrafiltrate, respectively. Calibration ranges and LODs are shown in Tables 1 and 2 for the microdialysates and plasma and ultrafiltrate, respectively. Compared to our previous report [32], in spite of the low recoveries of Fen and Phen and the small sample size (20 μl of dialysate compared to 100 μl plasma), the LODs were almost unchanged under the modified derivatization conditions for the microdialysates. In contrast, the LODs of Phen and Fen for plasma samples were increased, since in this study 50 μl were used compared to 100 μl for the previous report.

3.1.2.2. Precisions and recoveries. The precision of the method was evaluated by analyzing four replicates of spiked rat brain and blood microdialysates with known concentrations of Phen, Fen and Norf at two levels. The intra-day RSDs ranged from 3.1 to 7.9% for brain microdialysates and from 0.0 to 9.1% for blood microdialysates, while the inter-day RSDs ranged from 2.0 to 8.3% and from 1.3 to 10.0% for brain and blood microdialysates, respectively (Table 1). The inter-day RSDs for Phen, Fen and Norf in spiked plasma and ultrafiltrate ranged from 1.1 to 7.5% for both plasma and ultrafiltrate (Table 2).

The in vitro and the in vivo recoveries of the brain and blood probes for the three compounds are shown in Table 3. The in vitro recoveries for Phen, Fen and Norf ranged from 21.4 to 25.4% and from 34.9 to 35.5% for brain and blood probes, respectively. The in vivo recoveries ranged from 20.0 to 23.7 for the

Table 1
Studied ranges, LODs and method precision for Phen, Norf and Fen in spiked rat brain and blood microdialysates

Compound	Range, n*M*	LOD (*S*/*N*=3), n*M* (fmol/injection)	Spiked, n*M*	Precision, RSD% (*n*=4)	
				Intra-day	Inter-day
Brain microdialysate					
Phen	5–2000	5	10	7.9	6.0
	(*r*=1.000)	(19)	500	4.3	2.7
Norf	10–2000	4	50	5.0	5.7
	(*r*=1.000)	(14)	500	4.3	2.7
Fen	10–2000	6	50	6.0	8.3
	(*r*=0.999)	(23)	1000	3.1	2.0
Blood microdialysate					
Phen	5–2000	5	10	9.1	10.0
	(*r*=0.999)	(19)	500	3.7	1.5
Norf	10–2000	4.4	50	4.2	6.4
	(*r*=1.000)	(17)	500	4.0	2.1
Fen	10–2000	6	50	0.0	7.5
	(*r*=0.999)	(23)	1000	2.7	1.3

brain probe and from 28.8 to 36.7% for the blood probe for the three compounds.

Following liquid–liquid extraction of spiked plasma and ultrafiltrate with Phen, Fen and Norf, the recoveries were comparable with previous reports [32–34] and ranged from 95 to 104% (Table 2).

3.2. Pharmacokinetics of Phen and Fen in rats brain and blood microdialysates

3.2.1. Phen pharmacokinetics

To study the effect of the combination of Phen and Fen on DA and 5-HT release and on food intake, the

Table 2
Studied ranges, LODs and method precision for Phen, Norf and Fen in spiked rat plasma and ultrafiltrate

Compound	Range, n*M*	LOD (*S*/*N*=3), n*M* (fmol/injection)	Spiked, n*M*	Precision RSD% (*n*=4)
Plasma				
Phen	10–2000	2.9	50	5.5
	(*r*=0.999)	(18)	1000	3.2
Norf	10–2000	4.5	50	7.0
	(*r*=0.999)	(27)	1000	7.5
Fen	20–4000	10.7	125	4.4
	(*r*=0.999)	(65)	2000	3.5
Ultrafiltrate				
Phen	10–2000	3.1	50	6.6
	(*r*=0.999)	(19)	1000	1.1
Norf	10–2000	3.1	50	3.7
	(*r*=0.999)	(19)	1000	7.8
Fen	20–4000	10.0	125	5.6
	(*r*=0.999)	(61)	2000	4.7

Table 3
In vitro and in vivo recoveries (%) of brain and blood microdialysis probes for Phen, Norf and Fen

Compound	Brain probe		Blood probe	
	In vitro	In vivo	In vitro	In vivo
Phen	21.4±5.3	20.0±6.4	34.9±7.1	28.8±12.7
Norf	22.7±6.5	23.7±5.5	35.0±5.0	36.7±8.0
Fen	25.4±3.0	23.0±6.6	35.5±6.4	31.2±14.9

available animal studies have utilized widely varying dose ranges of Phen and Fen [8,15,16,22,23,36,39], ranging from 1 to 20 mg/kg for Phen and from 0.5 to 16 mg/kg for Fen. Hence in this study Phen was administered i.p. in two different single doses (1 and 5 mg/kg) individually or in combination with Fen (5 mg/kg).

The profiles of brain and blood microdialysate concentrations versus time of the two doses of Phen in the absence and presence of Fen are shown in Fig. 3A,B and their corresponding pharmacokinetic parameters are listed in Table 4. The effect of Fen on

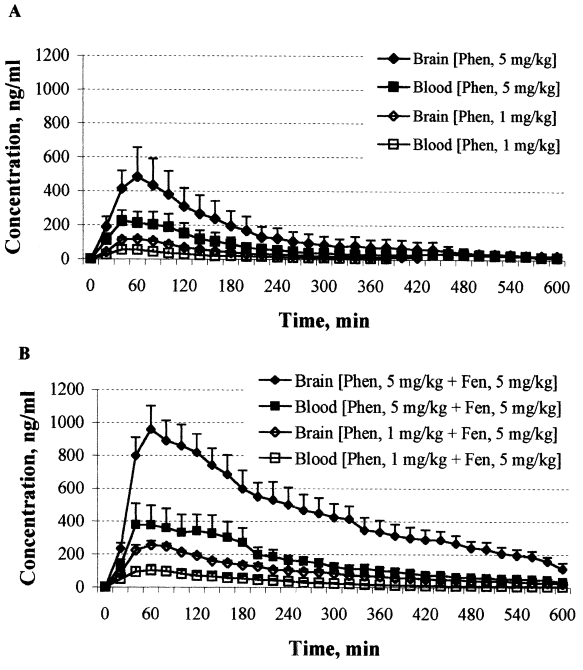

Fig. 3. Mean concentrations of Phen in rat brain and blood microdialysates after (A) individual administration of Phen (1 and 5 mg/kg, i.p.) and (B) simultaneous administration of Phen (1 and 5 mg/kg, i.p.) and Fen (5 mg/kg, i.p.).

the two doses of Phen was the same. In brain, Fen significantly increased Phen levels. C_{max} of Phen increased from 124±12 (in the absence of Fen) to 268±25 ng/ml (in the presence of Fen) and from 507±170 (in the absence of Fen) to 993±127 ng/ml (in the presence of Fen) for the 1 and 5 mg/kg doses, respectively ($P<0.05$). Although the T_{max} of Phen (1 and 5 mg/kg) was longer in the presence of Fen (70±9 and 73±10 min for 1 and 5 mg/kg, respectively), it was not significantly different from rats treated with Phen alone (57±10 and 53±16 min for 1 and 5 mg/kg, respectively, $P>0.2$).

Also, Fen significantly increased the AUC of Phen 3.4-fold equally for the two doses (from 19 543±2154 to 66 689±7184 ng.min per ml and from 93 049±39 766 to 320 270±49 617 ng.min per ml for 1 and 5 mg/kg Phen, respectively, $P<0.01$). The $t_{1/2}$ of Phen was considerably prolonged from 100±5 and 115±18 for 1 and 5 mg/kg, respectively, in the absence of Fen to 178±25 and 225±27 in the presence of Fen ($P<0.02$). Other parameters including the elimination rate and clearance were also altered by Fen. Following the combined administration of Phen with Fen, the Cl of Phen was reduced to 16±2 and 18±3 compared to 55±7 and 94±26 ml/min per kg in the absence of Fen ($P<0.02$) and k_{el} was decreased to 0.0042±0.0005 and 0.0033±0.0004 min^{-1} compared to 0.007±0.0003 and 0.0068±0.0013 min^{-1} in the absence of Fen ($P<0.03$) for 1 and 5 mg/kg doses of Phen, respectively. Although the apparent Cl of Phen following 5 mg/kg individual administration was higher (94 ml/min per kg) than the results obtained from its 1 mg/kg individual administration (55 ml/min per kg), the results were not significantly different ($P=0.46$).

In blood, the AUC and Cl of Phen were the only parameters that were significantly altered by Fen co-administration. The AUC of Phen increased 2.6-fold (from 8891±1747 to 22 874±4955 ng.min per ml) and 2.2-fold (from 48 980±17 748 to 107 951±25 682 ng.min per ml) for the 1 and 5 mg/kg doses of Phen, respectively, when combined with Fen in comparison with Phen alone ($P<0.05$). Clearance of Phen from blood was decreased from 146±38 and 148±29 ml/min per kg for 1 and 5 mg/kg, respectively, in the absence of Fen to 55±10 and 57±10 ml/min per kg in the presence of Fen

Table 4
Pharmacokinetics of Phen (1 and 5 mg/kg) in brain and blood microdialysates after single i.p. administration to rats alone and in combination with Fen (5 mg/kg)

Treatment	C_{max} (ng/ml)	T_{max} (min)	$t_{1/2}$ (min)	AUC (ng.min per ml)	k_{el} (min^{-1})	Cl (ml/min per kg)	MRT (min)
Brain microdialysate							
I	124±12	57±10	100±5	19 543±2154	0.0070±0.0003	55±7	162±6
II	268±25*	70±9	178±25*	66 689±7184*	0.0042±0.0005*	16±2*	272±32*
III	507±170	53±16	115±18	93 049±39 766	0.0068±0.0013	94±26	180±29
IV	993±127‡	73±10	225±27‡	320 270±49 617‡	0.0033±0.0004‡	18±3‡	347±28‡
Blood microdialysate							
I	57±14	50±4	117±24	8891±1747	0.0071±0.0011	146±38	182±34
II	104±30	63±3	159±24	22 874±4955*	0.0050±0.0008	55±10*	247±30
III	243±67	47±10	185±37	48 980±17 748	0.0042±0.0010	148±29	310±96
IV	395±125	63±12	179±22	107 951±25 682‡	0.0041±0.0004	57±10‡	289±28

Data are expressed as mean±S.E.M. I: Individual administration of Phen (1 mg/kg) to rats (n=6). II: Simultaneous administration of Phen (1 mg/kg) and Fen (5 mg/kg) to rats (n=6). III: Individual administration of Phen (5 mg/kg) to rats (n=6). IV: Simultaneous administration of Phen (5 mg/kg) and Fen (5 mg/kg) to rats (n=6).
*$P<0.05$, significantly different from group I.
‡$P<0.05$, significantly different from group III.

($P<0.05$). The results of other parameters are shown in Table 4. Although the C_{max} and T_{max} of Phen resulting from the combined administration were higher and longer, respectively, compared to its individual administration, but there was no significant difference. This could be related to inter-variation between rats as can be observed by the large S.E.M.

3.2.2. Fen pharmacokinetics

The profiles of brain and blood microdialysate concentrations versus time of Fen and its metabolite Norf in the absence and presence of Phen (1 and 5 mg/kg doses) are shown in Figs. 4 and 5 and the corresponding pharmacokinetic parameters of Fen are listed in Table 5. The pharmacokinetic parameters of Fen were not significantly affected by the co-administration of 1 and 5 mg/kg Phen compared to the individual administration of Fen ($P>0.05$ for the three groups). The pharmacokinetic parameters of Norf were not calculated because the microdialysates' collection interval (10 h) did not cover its elimination phase due to its long half-life (more than 12 h) in rats [40,41]. However, to assess the effect of Phen on the kinetics of Norf, the AUC_{0-10h} obtained from brain and blood microdialysates after the individual and co-administration of Fen with Phen were compared. The AUC_{0-10h} of Norf after in-

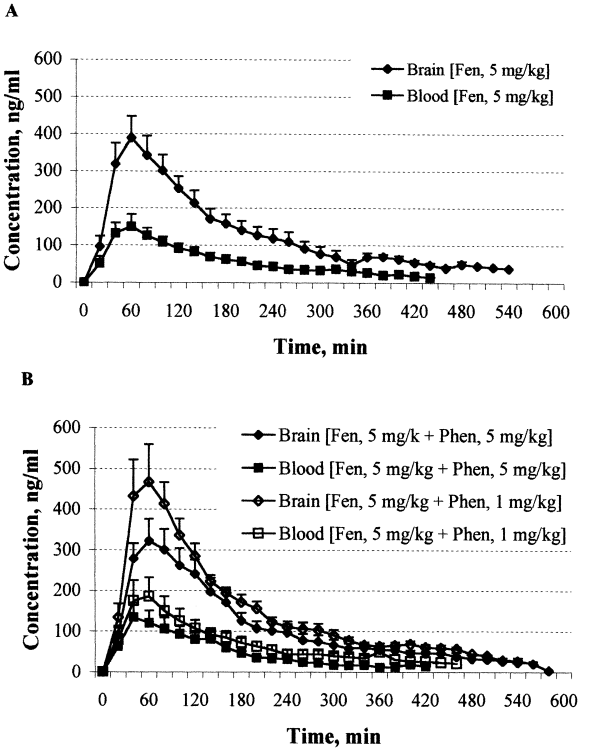

Fig. 4. Mean concentrations of Fen in rat brain and blood microdialysates after (A) individual administration of Fen (5 mg/kg) and (B) simultaneous administration of Phen (1 and 5 mg/kg, i.p.) and Fen (5 mg/kg, i.p.).

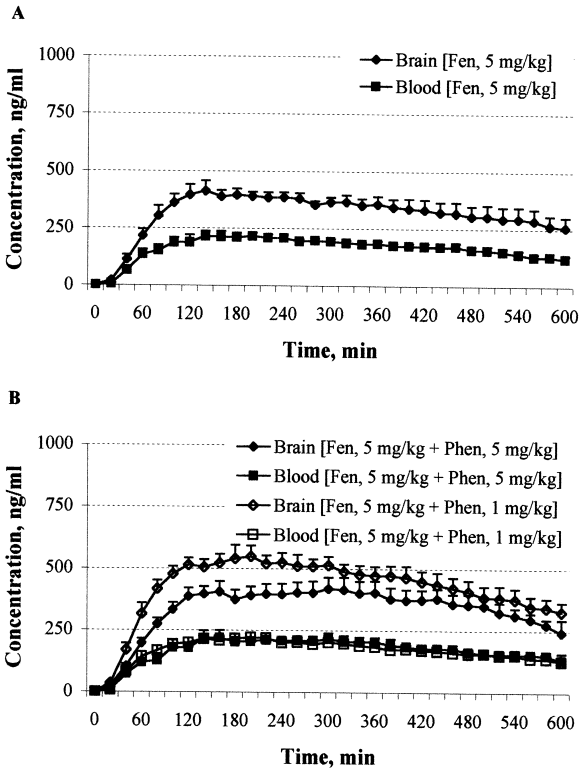

Fig. 5. Mean concentrations of Norf in rat brain and blood microdialysates after (A) individual administration of Fen (5 mg/kg) and (B) simultaneous administration of Phen (1 and 5 mg/kg, i.p.) and Fen (5 mg/kg, i.p.).

dividual administration of Fen (5 mg/kg) were 190 004±13 372 and 99 256±6199 ng.min per ml in brain and blood microdialysates, respectively, which were comparable to those after the simultaneous administration of Fen with 1 mg/kg dose of Phen (254 483±18 568 and 101 011±4145 ng.min per ml in brain and blood microdialysates, respectively) and with 5 mg/kg dose of Phen (200 435±19 141 and 102 168±9788 ng.min per ml in brain and blood microdialysates, respectively).

3.3. Determination of total and free Phen, Fen and Norf in rat plasma

While microdialysis allowed the in vivo determination of unbound drug concentrations in blood, total (bound and unbound) concentration in plasma was also determined. The AUC values were used to assess the protein binding of Phen and Fen. Protein binding displacement drug–drug interaction tend to produce an increase in free drug concentration, which, however, causes an increase in elimination and thus an overall reduction in total drug concentration, potentially maintaining the free concentrations unchanged [42]. However, this was not the case with Phen–Fen combination. As shown in Table 6, the AUC of total Phen in plasma increased 2.2-fold after simultaneous administration with Fen. This is comparable to the increase in the free levels of Phen in plasma (2.4-fold). Although Fen showed higher AUC values after the individual administration in comparison to the combination, they were not significantly different ($P>0.2$), and showed high inter-variation between the rats as indicated by the high S.E.M.

Table 5
Pharmacokinetics of Fen (5 mg/kg) in brain and blood microdialysates after single i.p. administration to rats alone and in combination with Phen (1 and 5 mg/kg)

Treatment	C_{max} (ng/ml)	T_{max} (min)	$t_{1/2}$ (min)	AUC (ng.min per ml)	k_{el} (min^{-1})	Cl (ml/min per kg)	MRT (min)
Brain microdialysate							
I and III	396±57	60±4	103±13	68 449±10 625	0.0075±0.0011	87±16	208±21
II	480±88	70±4	123±8	83 378±10 959	0.0058±0.0004	65±7	196±10
IV	347±48	70±11	126±23	62 888±9230	0.0070±0.0021	88±12	179±18
Blood microdialysate							
I and III	157±32	69±6	104±14	26 413±4057	0.0081±0.0052	225±41	180±18
II	188±50	57±3	92±6	30 379±7511	0.0078±0.0005	217±47	157±7
IV	140±35	47±8	114±20	22 757±2671	0.0076±0.0018	233±24	194±26

Data are expressed as mean±S.E.M. I and III: Individual administration of Fen (5 mg/kg) to rats ($n=6$). II: Simultaneous administration of Fen (5 mg/kg) and Phen (1 mg/kg) to rats ($n=6$). IV: Simultaneous administration of Fen (5 mg/kg) and Phen (5 mg/kg) to rats ($n=6$).

Table 6
Protein binding % of Phen and Fen after their individual and simultaneous administration to rats

Treatment	Phen			Fen		
	AUC (ng.min per ml)	AUC(II)/ AUC(I)	Protein binding %	AUC (ng.min per ml)	AUC(II)/ AUC(I)	Protein binding %
Total						
I	11 860±3888	2.2	35	49 171±12 555	0.74	24
II	25 767±5683*		28[#]	36 558±4409		25
Free						
I	7677±3216	2.4		37 590±13 761	0.73	
II	18 519±4398*			27 404±3066		

Data are expressed as mean±S.E.M. I: Individual administration of Phen (1 mg/kg) or Fen (5 mg/kg) to rats ($n=3$). II: Simultaneous administration of Phen (1 mg/kg) and Fen (5 mg/kg) to rats ($n=4$).
*$P<0.05$, significantly different from group I.
[#]$P>0.2$, not significantly different from group I.

Phen and Fen after individual administration to rats ($n=3$) showed protein binding of 35 and 24%, respectively, compared to 28 and 25% for Phen and Fen after their simultaneous administration ($n=4$). In spite of the small number of animals, the results showed no significant difference in Phen and Fen protein binding ($P>0.2$). Also, there was no significant difference ($P>0.2$) in the protein binding % of Norf following the individual administration of Fen (40%) compared to the simultaneous administration of Fen and Phen (43%).

4. Discussion

The above results clearly showed that Fen and/or Norf altered the pharmacokinetic parameters of Phen in blood and brain. In brain, Phen, Fen and Norf levels are much higher than in blood with AUC ratios (AUC_{brain}/AUC_{blood}) of 1.9, 2.6 and 1.9, respectively, after the individual administration. Such result indicates the ease of diffusion of these compounds across the blood–brain barrier (BBB) that could be related to their hydrophobic nature as well as their small molecular mass. On the other hand, after the simultaneous administration of Phen and Fen, as the AUC ratios of Fen and Norf were kept almost the same (2.8 and 2.0, respectively), AUC ratio of Phen increased to 3.0 ($P<0.05$). In blood, the co-administration of Fen caused a 2.2-fold increase in the levels of Phen compared to 3.4-fold in the brain, which may indicate a mechanism other than increased blood levels of Phen is involved in such elevation in the brain.

Like eliminating organs such as liver, kidney and intestine, the BBB contains multiple efflux transporters including P-glycoproteins (P-gp), which transport cationic and zwitterionic compounds, and multidrug resistance proteins (MRP) that preferentially transport anionic compounds but can also transport neutral compounds [43–45]. These transporters are responsible for the active efflux of drugs from the brain limiting their accumulation. Many interactions between substrates and/or inhibitors of P-gp have been reported [46,47]. Wu et al. [48] reported evidence for the expression of the organic cation transporter OCT3 in the brain. This transporter in addition to other cationic transporters participates principally in the elimination of cationic endobiotics and xenobiotics in tissues such as kidney and liver [49]. The authors conducted competitive experiments and found that the drugs of abuse amphetamine and methamphetamine and the neurotransmitters DA and 5-HT interact with OCT3 with significant affinity and among many neurotoxins and neurotransmitters tested; amphetamine was the most potent in inhibiting OCT3-mediated uptake. However, in the literature there are no reports concerning the elimination of Fen or Phen from the brain. Although further studies are required, the obtained results in this study suggest that Fen and/or its metabolite Norf may have a similar inhibiting effect on the transporters responsible for Phen elimination thus inhibiting its efflux from brain to the blood leading to higher brain

distribution, and hence altering its pharmacokinetic parameters.

However, the increase in brain levels of Phen when co-administered with Fen will prolong its action and may explain the enhanced weight loss with this combination even at lower doses compared to each drug alone. Consequently, Phen will enhance the release of extracellular DA, which is in agreement with other studies which reported the enhanced release of DA when the combination Phen–Fen was administered to rats compared to Phen alone [8,16,22,50]. On the other hand, the reported enhanced release of 5-HT by the combination [8,16,22,50] appears not to be related to Fen and Norf levels in the brain, since, as shown here, the pharmacokinetic parameters of both compounds were not altered by Phen. This may lead to consideration of the effect of Phen on 5-HT release when combined with Fen either as an MAO inhibitor [27,28] or by promoting 5-HT release through the enhanced release of DA [16].

In blood, Fen did not cause any significant changes in the plasma levels (C_{max}) or the peak time (T_{max}) of Phen, but significantly affected its AUC and clearance. Although in this study the main metabolite of Phen in rats, *p*-hydroxyphentermine, was not determined, the mechanism by which Fen and/or Norf altered Phen's pharmacokinetics is possibly related to metabolism inhibition of Phen caused by Fen and/or Norf.

Alternatively, at physiological pH, organic cations such as protonated primary, secondary, tertiary, and quaternary amine compounds undergo renal tubular transport by OCTs and P-gp [51,52]. In clearance studies, renal elimination of organic cations was saturable and reduced by the presence of other organic cations where the drug with higher affinity for the transporter will be a more potent inhibitor of the transport of a similar chemical substance for the same transporter [42,53]. Thus, since Phen clearance was significantly affected by Fen, the possibility that Fen altered the renal excretion of Phen by inhibiting its renal transportation should be considered.

Arends et al. [54] reported an interesting study concerning the effect of Fen on enhancing the antinociceptive action of morphine and inhibiting the development of tolerance to morphine in rats. When both drugs were administered in combination, Fen increased morphine levels in both plasma and brain. Such increase was not related to the inhibition of morphine metabolism by Fen because the results showed that the morphine metabolite, morphine-3-glucuronide, was also increased with Fen co-administration. Morphine is a P-gp substrate where the inhibition of P-gp may result in higher brain uptake of morphine. It has been reported that its distribution in the brain tissue of mdr1a gene-knockout mice was increased compared to wild mice or in mice co-treated with verapamil, which is a P-gp inhibitor [55]. Although further investigations are required, these results may elucidate the role of Fen as an inhibitor or a competitive substrate of P-gp leading to significant increase in brain penetration.

5. Conclusion

In this work, a highly sensitive and simple HPLC method with fluorescence detection has been developed for the determination of the DIB-derivatives of Phen, Fen and Norf in rat microdialysates. The method was applied for the analysis of the in vivo pharmacokinetic interaction between the two anti-obesity drugs Phen and Fen. Fen remarkably altered the pharmacokinetics of Phen in both brain and blood, the effect being more significant in the brain. The consequences of such alteration in the pharmacokinetics of a CNS drug may play in part in the enhanced effect as well as neurotoxicity of Phen and Fen.

Furthermore, the mechanism by which Fen increased Phen levels, especially in the brain, requires further investigation in order to elucidate and clarify the increased levels and CNS accumulation of Phen. The role of multidrug-transporters such as OCTs and P-gp which are responsible for drug efflux from different tissues including brain and eliminating tissues should also be considered.

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