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Fluorometric determination of DL-fenfluramine, DL-norfenfluramine and phentermine in plasma by achiral and chiral high-performance liquid chromatography

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Abstract

High-performance liquid chromatography (HPLC) with fluorescence detection has been developed for the simultaneous determination of sympathomimetic amines including ephedrine, norephedrine, 2-phenylethylamine, 4-bromo-2,5-dimethoxy-phenylethylamine, phentermine (Phen) and DL-fenfluramine (Fen) in spiked human plasma. Furthermore, an enantioselective HPLC method for the separation of D-Fen (dexfenfluramine) and L-Fen (levofenfluramine) in addition to their active metabolites D- and L-norfenfluramine (Norf) is described. The detection was achieved at emission wavelength of 430 nm with excitation wavelength of 325 nm for both methods. The analytes were extracted from plasma (100 μl) at pH 10.6 with ethyl acetate using fluoxetine as the internal standard. The extracts were evaporated and derivatized with the fluorescence reagent 4-(4,5-diphenyl-1*H*-imidazole-2-yl)benzoyl chloride in the presence of carbonate buffer (pH 9.0). A gradient separation was achieved on a C_{18} column for the achiral separation or on a Chiralcel OD-R column for the chiral separation. The methods were fully validated, and shown to have excellent linearity, sensitivity and precision. The chiral method has been applied for the determination of D- and L-enantiomers of Fen and Norf, in addition to Phen in rat plasma after an intraperitoneal administration of DL-Fen and Phen, simultaneously. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Fenfluramine; Norfenfluramine; Phentermine

1. Introduction

Fenfluramine (Fen) and phentermine (Phen) have been widely prescribed, off-label, in combination as appetite suppressants for the treatment of obesity in conjunction to dietary restriction following the novel approach of Weintraub et al. [1,2] in promoting and maintaining weight reduction than either drug alone. However, DL-Fen and the active enantiomer dexfenfluramine (D-Fen) were withdrawn from the US market in September 1997 after many cases reported the development of serious sides effects including valvular heart disease and primary pulmonary hypertension (PPH) [3–8], either after the administration of Fen alone or in combination with Phen. Yet, in spite of the withdrawal, Fen and Phen are still being prescribed and widely abused [9–11], suggesting the development of a method for their simultaneous determination in biological fluids will be helpful in monitoring their levels.

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To our knowledge, no reports are available regarding the simultaneous determination of Phen and Fen in plasma. Most of the methods determined Phen and Fen in biological fluids, independently [12–22]. Therefore, an analytical method for their simultaneous determination in plasma may represent a useful tool in therapeutic drug monitoring (TDM). Palmer et al. [9] described a gas chromatography–mass spectrometry (GC–MS) method for the simultaneous determination of Phen and Fen in urine. The authors reported a detection limit of 500 ng/ml for both drugs (3.35 μ*M* for Phen and 2.2 μ*M* for Fen), which makes it not sensitive enough for their determination in plasma, since the therapeutic concentrations of Phen and Fen in human plasma are in the low ng/ml. At usual dosage of Fen (20 mg three times daily), the therapeutic levels have been reported to be in the range 50 (216 n*M*)–150 ng/ml (648 n*M*) [23,24]. On the other hand, D-Fen (15 mg twice daily), the therapeutic levels have been reported with an average of 20 ng/ml (86 n*M*) [25], while for Phen the reported value after 37.5 mg/day orally was up to 90 ng/ml (603 n*M*) [26]. Consequently, development of sensitive and precise method for their determination in plasma is required.

The methods developed for the independent determination of Phen and Fen in biological fluids, used either GC [12–16] or high-performance liquid chromatography (HPLC) [17–21]. In these methods, the minimum sample volume used for their quantitation in plasma or urine was 1 ml with minimum quantitation limits of 10 ng/ml (67 n*M*) and 2.5 ng/ml (11 n*M*) for Phen [21] and Fen [13], respectively. Cho et al. [16] reported a GC–MS method for the determination of Phen in plasma. The authors did not document the lower limit of detection for their method, but they used 2 ml of plasma for analysis and reported a value of 1.6 ng/ml (11 n*M*) after 240 min of Phen administration to rats. Clausing et al. [22] described an HPLC method using dansyl chloride (Dns-Cl) as a derivatizing reagent for determining D-Fen and fluoxetine (FLX) in plasma. Although the method was sensitive with a quantitation limit of 2.3 ng/ml (10 n*M*) using a small volume of plasma sample (100 μl), sample preparation needed a clean-up step in order to remove the excess Dns-Cl after 4 h incubation.

The reports concerning the chiral separation of D-Fen, L-Fen, in addition to their metabolites D-norfenfluramine (D-Norf) and L-norfenfluramine (L-Norf) in biological fluids are few. Most of the methods utilized GC coupled either with electron-capture detection (ECD) [27–30] or flame ionization detection (FID) [31]. Among these, the most sensitive method reported by Srinivas et al. [29] with detection limit of 2 ng/ml for Fen enantiomers. On the other hand, only one paper described a chiral HPLC method with UV detection for the determination of DL-Fen and DL-Norf in plasma and urine [32]. The separation of 3,5-dinitrophenylurea derivatives of Fen and Norf enantiomers was achieved on a Pirkle-type chiral column. In this method 1 ml plasma was used with a quantitation limit of 10 ng/ml (43 n*M*) per enantiomer and a detection limit of 1 ng/ml (4.3 n*M*).

Previously, we reported a HPLC method with fluorescence detection for the simultaneous determination of sympathomimetic amines after derivatization with Dns-Cl. The studied sympathomimetic amines were ephedrine (E), norephedrine (NE), 2-phenylethylamine (2-PEA), 4-bromo-2,5-dimethoxyphenylethylamine (2-CB), Phen and DL-Fen in human plasma, using FLX as the internal standard (I.S.). The method was then modified for the simultaneous determination of Phen and Fen in human and rat plasma [33]. Both methods showed high sensitivity with detection limits at signal-to-noise ratio of 3 ($S/N=3$) ranging from 16 (1 n*M*) to 255 (17 n*M*) fmol on column for the studied amines in plasma, whereas the detection limits for Phen and Fen were 54 (3 n*M*) and 48 (4 n*M*) fmol, respectively. In addition, we developed a HPLC method with UV detection for the simultaneous determination of the above sympathomimetic amines, except 2-CB, following derivatization with 4-(4,5-diphenyl-1*H*-imidazole-2-yl)benzoyl chloride (DIB-Cl) using cyclohexylamine as the I.S. The method was also modified for the simultaneous determination of Phen and Fen in human and rat plasma, using FLX as the I.S. [34]. The latter showed satisfactory sensitivity and was sufficient for the therapeutic drug monitoring of DL-Fen and Phen.

In this work, we could improve the detection limits of some of the amines described above including Phen and DL-Fen using HPLC with fluorescence detection following derivatization with DIB-Cl. Furthermore, a sensitive and reliable chiral HPLC method for the simultaneous determination of Phen,

DL-Fen and DL-Norf, in plasma is developed. The application of the method to the monitoring of Phen and the enantiomers of Fen in rat plasma samples after the administration of a single intraperitoneal (i.p.) dose of 1 mg/kg of Phen and racemic Fen is also described.

2. Experimental

2.1. Chemicals

DL-Fen·HCl, D-Fen·HCl and Phen·HCl were obtained from Sigma (St. Louis, MO, USA). DL-FLX·HCl was purchased from Tocris Cookson (Bristol, UK). E was obtained from Dainippon Pharmacy (Osaka, Japan), 2-PEA from Nacalai Tesque (Kyoto, Japan) and NE from Aldrich (Milwaukee WI, USA). DIB-Cl was synthesized in our laboratory [35] and 2-CB was synthesized according to DeRuiter et al. [36]. DL-Norf and D-Norf were enzymatically prepared by incubating DL-Fen and D-Fen, respectively, with rat liver microsomal preparation [34]. Ethyl acetate, acetonitrile and methanol of HPLC grade were obtained from Wako. Water was deionized and passed through a water purification system (Pure Line WL21P, Yamato Kagaku, Tokyo, Japan). Other reagents were of analytical grade. Solid-phase extraction (SPE) was carried out by Bond Elut cartridges (50 mg C_{18}, 1 ml; Varian, USA).

2.2. HPLC system and chromatographic conditions

The simultaneous separation of the DIB derivatives of sympathomimetic amines was performed using a gradient HPLC system (Shimadzu, Kyoto, Japan) consisting of two pumps (LC-6A) with a system controller (SCL-6A), a recorder (R-112) and a fluorescence detector (RF-550) which was set at an excitation wavelength of 325 nm and an emission wavelength of 430 nm. An injector Rheodyne 7125 (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). For the enantiomeric separation, besides the HPLC system mentioned above, a third pump and a signal cleaner (SC 77, SIC, Tokyo, Japan) were set as shown in Fig. 1 and the column was a Chiralcel OD-R (250×4.6 mm I.D.; Daicel, Osaka, Japan).

The mobile phase was a mixture of A (methanol–water–ethyl acetate, 69:30:1, v/v) and B (acetonitrile–water, 70:30, v/v) for the simultaneous achiral separation. The flow-rate was 1.0 ml/min. The time program for the gradient elution was set as follows: from 0 to 20 min, B was 40% and increased to 95% from 20 to 26 min, then B was maintained at 95% until 49 min, where B was programmed to return to the initial condition (40%).

For the enantiomeric separation, a gradient system was also used where the mobile phase was a mixture of A (acetonitrile–0.01 *M* citrate buffer, pH 2.7–0.05 *M* perchlorate buffer, pH 2.0, 50:25:25, v/v) and B (acetonitrile). The flow-rate was 1.0 ml/min. The solution pumped by the third pump was ammonia solution in water (4%). The flow-rate was 0.1 ml/min. The time program for the gradient elution is shown in Table 1.

2.3. Plasma samples

Human blood samples were drawn from healthy volunteers in our laboratory. Male Wistar rats (290–345 g) were used in the experiment. Blood samples were centrifuged at 2000 *g* for 10 min and obtained

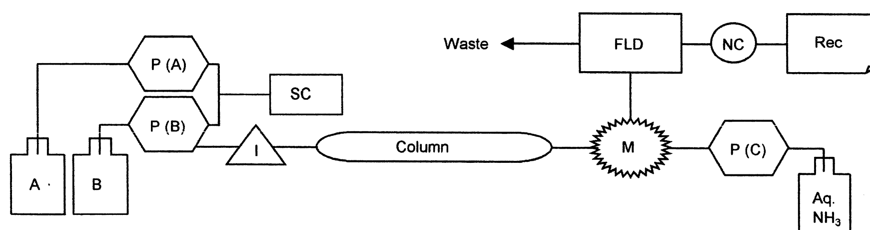

Fig. 1. The chiral HPLC system for the separation of DIB derivatives of DL-Fen, DL-Norf and Phen. P, Pump; SC, system controller; I, injector with 20-μl sample loop; M, mixing tee; FLD, fluorescence detector; NC, noise cleaner; Rec, recorder; column, Chiralcel OD-R; A, a mixture of acetonitrile–0.01 *M* citrate buffer, pH 2.7–0.05 *M* perchlorate buffer, pH 2.0 (50:25:25, v/v); B, acetonitrile.

Table 1
Chromatographic gradient program for the chiral-HPLC of DIB derivatives of DL-Fen, DL-Norf and Phen

Time (min)	A (%)	B (%)
Initial (0)	100	0
0–20	100	0
20–21	95	5
21–27	90	10
37	90	10
38	85	15
38–44	85	15
44 (initial condition)	100	0

plasma samples were kept frozen at −20°C prior to use. A detailed description of the extraction method of the amines from plasma can be found elsewhere [33]. In brief, to 100 μl of the plasma 10 μl of an aqueous solution of 5 μ*M* FLX (I.S.) was added and then were extracted under alkaline condition (borate buffer, 0.1 *M*, pH 10.6) with ethyl acetate. After the evaporation of the organic layer, the obtained residue was derivatized with DIB-Cl as described below.

2.4. Derivatization with DIB-Cl

For the optimization of the derivatization conditions with DIB-Cl, standard solutions of the amines in methanol were used. The residues of the evaporated standards or extracted plasma were derivatized as follows: 150 μl of 1.5 m*M* DIB-Cl in acetonitrile and 50 μl of 0.01 *M* carbonate buffer (pH 9.0) were added to the residue, vortex mixed and then incubated at 60°C (at room temperature for the enantiomeric separation) for 10 min. The reaction was stopped by adding 10 μl of 25% ammonia solution. Samples were then diluted with acetonitrile (1:1, v/v) and 20 μl of the resultant solution were injected onto the achiral column. For the chiral system, samples were applied for SPE as described below.

2.5. SPE for DIB derivative samples

SPE was performed using a 12-tube Vac Elut vacuum system (Varian). Cartridges were conditioned with 5×1 ml of acetonitrile and then 5×1 ml of deionized water. Samples were diluted with water (1:1, v/v) and loaded onto the cartridges and were allowed to dry for 1 min in vacuo. Cartridges were washed with 8×1 ml of acetonitrile–methanol–water (5:50:45, v/v) mixture and dried for 3 min in vacuo. The DIB derivatives were eluted with 2×75 μl acetonitrile, from which 20 μl were injected into the chiral HPLC system.

2.6. Method validation

Quantitation of the amines in plasma was performed via the internal standard method. The calibration curves were prepared over the ranges 10–5000 n*M* for E, 2-CB, and DL-Fen, 100 to 5000 n*M* for NE, 50 to 5000 n*M* for 2-PEA, and 5 to 2500 n*M* for Phen in human plasma for the achiral system. For the chiral system, calibration curves were prepared over the ranges 2–2500 and 2–1000 n*M* for Phen in human and rat plasma, respectively, and from 5 to 5000 and 5–2500 n*M* for D- and L-Fen, in human and rat plasma, respectively. The precision was calculated as the relative standard deviation (RSD) within a single run (intra-assay) and between different assays (inter-assay). The limit of detection (LOD) was calculated as the peak height at $S/N=3$ on column. The recovery as well as the accuracy was also evaluated.

3. Results and discussion

3.1. Optimization of derivatization conditions with DIB-Cl

The derivatization reaction with DIB-Cl was optimized using standard solutions of the studied sympathomimetic amines by the achiral separation system. In the studied concentration range of DIB-Cl (0.03–3 m*M*), the yields of DIB derivatives were almost constant from 0.15 to 3 m*M*, except for Phen and Fen where their derivatives continued to increase until 1 m*M* and hereafter kept constant. For carbonate buffer concentration, in the studied range (0.01–1 *M*), no difference in the derivatives yields was noticed. Fig. 2 shows the effect of pH on the DIB derivatives yields, where in the range from 9.0 to 10.5, the yields were constant for the DIB derivatives of E, NE, 2-PEA, 2-CB and Fen, while for DIB-Phen, the yield decreased at pH more than 10. The derivatization reaction was very fast where within 10

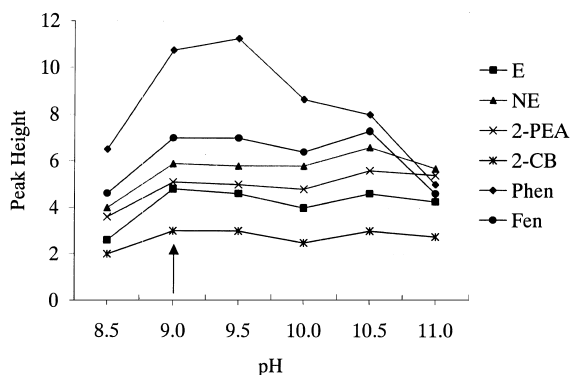

Fig. 2. Effect of pH on the derivatization of the sympathomimetic amines with DIB-Cl. Other conditions: 1.5 m*M* DIB-Cl, 0.01 *M* carbonate buffer, and incubation time for 10 min at 60°C. The arrow indicates the selected pH.

min, the reaction was almost complete and kept constant for 8 h. Furthermore at the three examined temperatures (room temperature, 45°C and 60°C) the yields of DIB derivatives were almost the same, with a slight increase at 45°C. From these results, the best derivatization conditions for DIB-Cl concentration and carbonate buffer concentration were 1.5 m*M* and 0.01 *M*, respectively, at pH 9.0 and finally for the reaction time and temperature for 10 min at 60°C. The same derivatization conditions were used for the chiral separation of Phen, DL-Fen and DL-Norf except for the temperature, where the reaction was occurred at room temperature. The reason we used 60°C temperature for the achiral system is to decrease the effect of the interfering peaks derived from DIB-Cl and eluted close to E and NE. The peak heights of these interfering peaks decreased as the temperature increased, which led us to choose 60°C. For the chiral separation, since different chromatographic conditions were used, the reaction at room temperature gave satisfactory results.

3.2. HPLC separation of DIB derivatives

For the achiral system, the resolution of DIB-NE peak from an interfering peak derived from DIB-Cl could not achieved without the addition of ethyl acetate to mixture A. Other solvent modifiers were also tried including dichloromethane, tetrahydrofuran and isopropanol. The best resolution and separation were obtained with the addition of 1% ethyl acetate. Fig. 3 shows typical chromatograms for normal human plasma (A) and spiked one with known concentrations of the studied compound (B). The retention times of the DIB derivatives of E, NE, 2-PEA, 2-CB, Phen, Fen and FLX were 16, 18, 27, 34, 35, 41 and 51 min, respectively. Sharp and defined peaks were obtained under the chromatographic conditions used. For the specificity of the current method, other sympathomimetic amines such as amphetamine and methamphetamine were examined to check their interfering with the studied compounds. Both amines did not interfere with any of the compounds.

The effects of different buffers and pH of the mobile phase were studied for the separation of the enantiomers using a Chiralcel column. The elution time for the DIB derivatives of the enantiomers increased with the increase in buffer pH and its concentration. Buffers such as acetate, Tris, citrate and perchlorate buffers were tried. In all cases, the D-enantiomers of Fen and Norf were always coeluted except with perchlorate buffer where a little resolution was obtained. The best separation and resolution (R_s=0.94) was obtained using a mixed buffer system of citrate buffer (0.01 *M*, pH 2.7) and perchlorate buffer (0.05 *M*, pH 2.0). Columns other than Chiralcel used in this study were not tried. Simultaneously, because of the low pH of the buffers, the fluorescence intensities of the DIB derivatives were very low. This permitted us to introduce a third pump eluting an alkaline solution to increase the fluorescence intensities. The retention times of L-Norf, D-Norf, D-Fen, Phen, and L-Fen were 16, 22, 24, 32 and 42 min, respectively. Racemic L- and D-FLX were eluted at retention times of 37 and 38 min, respectively.

SPE was necessary to remove excess DIB-Cl, which interfered dramatically with the enantiomers. Different SP cartridges packed with 100 mg of C_{18} (Bond Elut, Varian; Supelclean, Supelco; Daisopak, Daiso) and with 100 mg of C_{18}-OH (Bond Elut, Varian) in addition to 50 mg of C_{18} (Bond Elut, Varian) were examined. The best result was obtained with the C_{18} cartridge (50 mg) where less interfering peaks as well as clean chromatograms were attained.

As well, different washing mixtures were examined; the best recoveries with minimum interfer-

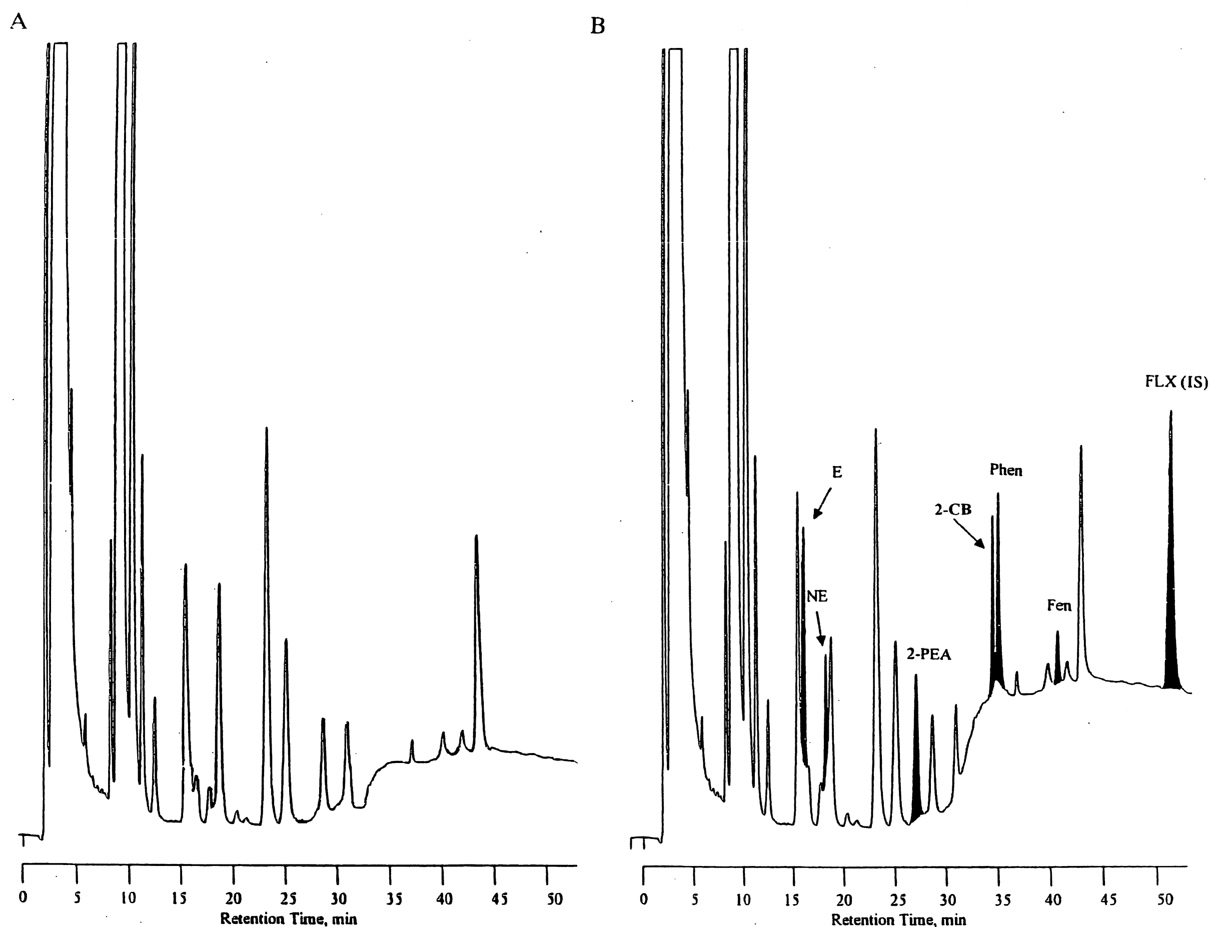

Fig. 3. Typical chromatograms of human plasma, (A) free and (B) spiked with known concentrations of the sympathomimetic amines by the achiral system. The peaks represent 250 nM for E, NE and 2-PEA, and 100 nM for 2-CB, Phen and DL-Fen.

ences were achieved with acetonitrile–methanol–water (5:50:45, v/v).

3.3. Method validation

3.3.1. Achiral HPLC system validation: calibration curves, precision, recovery and accuracy

Calibration curves for the quantitation of the sympathomimetic amines in human plasma were linear with correlation coefficients (r) ≥0.997 in the studied ranges. The regression equations and r-values for each compound are shown in Table 2. At S/N of 3, the LODs of the six amines on column ranged from 6 fmol (1.2 nM) for 2-CB to 476 fmol (100 nM) for NE. For Phen and Fen the LODs were 10 fmol (2 nM) and 18 fmol (3.75 nM), respectively (Table 2). The LODs for NE and 2-PEA are considered high (476 and 248 fmol on column, respectively) and this is because that NE is not well separated from an interfering peak obtained from DIB-Cl, which increases its detection limit. 2-PEA is an endogenous compound in plasma and a metabolite of phenylalanine. To determine its LOD in plasma, the sensitivity was decreased until the endogenous 2-PEA could not be detected; the spiked concentration of 2-PEA which did not interfere with endogenous amount was 50 nM (248 fmol on column). On the other hand, although E eluted on the shoulder of the reagent peak, its LOD was low and this is due to the high fluorescence intensity of its DIB derivative. To

Table 2
Studied ranges, regression equations and detection limits of the sympathomimetic amines using the achiral system

Compound	Range (nM)	Regression equation[a]	r value	Detection limit, fmol/inj. (nM), S/N=3
E	10–5000	y=0.0070 (0.0005)x+0.250 (0.051)	0.999	29 (6)
NE	100–5000	y=0.0033 (0.0008)x−0.237 (0.046)	0.999	476 (100)
2-PEA	50–5000	y=0.0042 (0.0012)x+0.361 (0.050)	0.998	248 (50)
2-CB	10–5000	y=0.0044 (0.0006)x+0.07 (0.01)	1.000	6 (1.2)
Phen	5–2500	y=0.0087 (0.0004)x+0.267 (0.04)	0.997	10 (2)
DL-Fen	10–5000	y=0.0016 (0.0004)x+0.045 (0.008)	0.999	18 (3.75)

[a] The regression equations represent the average of three calibration curves with the standard deviations shown in parentheses.

improve the separation of E and NE, changing the mobile phase or the time program could modify the resolution, but simultaneously it will affect the separation of the other compounds. Further, to eliminate the large interfering peaks of the derivatizing reagent, SPE can be applied, however, since in this study the main target was the simultaneous determination of Phen and Fen, the obtained separation was considered acceptable. The precision of the method was evaluated by four replicates for spiked human plasma with known concentrations of the compounds at two levels, 100 and 2500 nM for E, NE, 2PEA, 2-CB and Fen while for Phen at 50 and 1250 nM. The intra-day and inter-day RSDs ranged from 0.1% for 2-CB at 2500 nM to 13.3% for NE at 100 nM and 2% for 2-CB at 100 nM to 12.2% for NE at 100 nM, respectively. The RSD values for NE at its quantitation limit are high compared to those of other amines, which could be due to its elution on the shoulder of an interfering peak. As well, the inter-day RSD of Phen at its quantitation limit is also slightly high (12%), but could be acceptable for biological samples [37]. The recoveries from spiked human plasma for the compounds ranged from 85 to 113%, and accuracy expressed as the concentration found to that of the nominal concentration ranged from 95 to 113% (Table 3).

Table 3
Precision, recovery and accuracy of the sympathomimetic amines in spiked human plasma using the achiral system

Compound	Spiked (nM)	Found (nM)	Precision (RSD, %, n=4)		Recovery (n=3) (%±SD)	Accuracy[a] (%)
			Intra-day	Inter-day		
E	100	101	4.0	4.8	100±2.9	101
	2500	2494	9.5	9.0	113±6.1	100
NE	100	95	13.3	12.2	85±3.9	95
	2500	2455	0.9	6.6	89±4.3	98
2-PEA	100	113	4.9	9.8	111±0.5	113
	2500	2486	3.0	5.6	99±9.3	99
2-CB	100	106	0.5	2.0	106±7.9	106
	2500	2510	0.1	5.8	110±10.2	100
Phen	50	50	2.5	12.0	92±5.6	100
	1250	1255	3.4	5.5	110±8.2	100
DL-Fen	100	107	3.5	2.2	109±5.6	107
	2500	2515	0.7	5.6	102±7.4	101

[a] Found/nominal×100.

3.3.2. Chiral HPLC system validation: calibration curves, precision, recovery and accuracy

Calibration curves in spiked human and rat plasma were obtained by plotting the peak ratios of the peak heights of Phen and the enantiomers to that of the peak height of the first peak of the I.S., eluted at 37 min. We did not identify the two peaks related to FLX enantiomers because only the racemic mixture is available. The calibration curves in human plasma were linear in the studied ranges with an *r*-value of 0.999 for D-Fen, L-Fen and Phen. The LODs at $S/N=3$ were 27 fmol (2.0 n*M*), 19 fmol (1.4 n*M*), and 47 fmol (3.5 n*M*) for D-Fen, Phen and L-Fen, respectively. Furthermore, calibration curves were prepared in spiked rat plasma. The studied ranges were 5 to 2500 n*M* for D- and L-Fen, respectively, and 2 to 1000 n*M* for Phen. The regression equations and LODs are shown in Table 4 for human and rat plasma.

The precision of the chiral method was evaluated by analyzing four replicates of spiked human and rat plasma with known concentrations; 50 and 1250 n*M* for D- and L-Fen, and 25 and 500 n*M* for Phen. The intra-day RSDs ranged from 3.6 to 10.9% for human plasma and from 3.8 to 5.6% for rat plasma, while the inter-day RSDs ranged from 3.8 to 8.3% and from 4.9 to 10.1% for human and rat plasma, respectively. The accuracy ranged from 92 to 108% in human plasma and from 93 to 108% in rat plasma (Table 5). Recoveries were 69 and 76% for D- and L-Norf, respectively, 79 and 84% for D- and L-Fen, respectively, and 59% for Phen. Before applying to SPE, samples were diluted with water in 1:1 (v/v), because without this step, inconsistent recoveries for Phen were obtained, which could be improved after the addition of water.

Both the achiral and chiral methods provided simple and highly sensitive methods using small sample size for the simultaneous determination of Phen and Fen compared to other methods. Among the HPLC methods, Clausing et al. [22] and Zeng et al. [32] reported sensitive HPLC methods for the determination of D-Fen and Fen enantiomers, in plasma with quantitation limits of 2.3 for D-Fen [22] and 10 ng/ml for each enantiomer [32], but both methods needed long derivatization reaction time, i.e., 4 h followed by a clean-up step in the former and 1.5 h in the latter. Also, as a comparison with our previous Dns-Cl method [33], we could improve the LOD from 51 to 10 fmol on column for Phen and from 54 to 18 fmol on column for Fen by using DIB-Cl as the derivatizing reagent. Moreover, the LODs for the other amines were also improved. The previous LOD for E and 2-CB with Dns-Cl were 36 and 15 fmol, while in the present study are 29 and 6 fmol on column, respectively. On the other hand, the LOD for NE could not be improved because of the interfering peak derived from the derivatizing reagent, but it can be improved if NE peak was well separated from that interfering peak. Finally, for 2-PEA the LOD was almost the same in both studies.

Both achiral and chiral methods showed better sensitivity and practicality than other published GC and GC–MS methods for the separate determination of Fen or Phen [9,12–16,27–31]. Only Palmer et al. [9] reported the simultaneous determination of Phen and Fen by GC–MS, but the method lacked the sensitivity for their determination in plasma. Again

Table 4
Studied ranges, regression equations and detection limits of the DL-Fen and Phen spiked in human and rat plasma using the chiral system

Compound	Range (n*M*)	Regression equation[a]	*r* value	Detection limit, fmol/inj. (n*M*), $S/N=3$
Human plasma				
D-Fen	5–5000	$y=0.0004\ (0.00007)x-0.0003\ (0.00008)$	0.999	27 (2.0)
L-Fen	5–5000	$y=0.0003\ (0.00006)x+0.0064\ (0.0012)$	0.999	47 (3.5)
Phen	2–2500	$y=0.0019\ (0.00035)x-0.0246\ (0.0048)$	0.999	19 (1.4)
Rat plasma				
D-Fen	5–2500	$y=0.0004\ (0.00005)x+0.0003\ (0.00003)$	0.998	19 (1.4)
L-Fen	5–2500	$y=0.0006\ (0.00005)x+0.0021\ (0.0008)$	0.999	57 (4.3)
Phen	2–1000	$y=0.0031\ (0.00085)x-0.0056\ (0.0012)$	0.999	23 (1.7)

[a] The regression equations represent the average of three calibration curves with the standard deviations shown in parentheses.

Table 5
Precision, recovery and accuracy of the compounds in spiked human plasma using the chiral system

Compound	Spiked (n*M*)	Found (n*M*)	Precision (RSD, %, $n=4$)		Accuracy[a] (%)
			Intra-day	Inter-day	
Human plasma					
D-Fen	50	54	10.9	8.3	108
	1250	1352	9.0	6.8	108
L-Fen	50	46	7.5	6.1	92
	1250	1309	5.6	7.9	105
Phen	25	24.5	4.3	7.1	98
	500	510	3.6	3.8	102
Rat plasma					
D-Fen	50	52	5.6	10.1	104
	1250	1320	4.0	5.7	106
L-Fen	50	52	4.1	9.4	104
	1250	1279	3.8	6.2	102
Phen	25	27	5.4	8.4	108
	500	466	3.8	4.9	93

[a] Found/nominal×100.

among the sensitive GC methods used for the enantiomeric separation of Fen and Norf, Srinivas et al. [29] described a GC–ECD method for Fen and Norf enantiomers in plasma and obtained an LOD of 10 n*M* for Fen enantiomers. However, there are no papers reported simultaneous determination for Phen and the enantiomers of Fen and Norf either using HPLC or GC, which represent an advantageous point of the present study.

3.4. Determination of Phen and enantiomers of Fen and Norf in rat plasma

The chiral system was applied for the determination of Phen, D- and L-enantiomers of Fen and Norf in rat plasma after a simultaneous administration of single i.p. doses of 1 mg/kg of Phen and DL-Fen. Fig. 4 shows two chromatograms obtained from rat plasma, before (A) and after (B) 30 min of drug administration. Concerning Norf enantiomers, currently are not available in our laboratory, which caused us to prepare them enzymatically by incubating D-Fen or DL-Fen with rat microsomes [34]. For the quantitation of Norf enantiomers, D-Fen calibration curve was used for both D- and L-Norf due to their retention times, which were closer to D-Fen. Although such quantitation method is not accurate, it is adequate to show the changes in the Norf enantiomers plasma levels with time. Following the single doses of 1 mg/kg of Phen and Fen, it was possible to detect Phen, DL-Norf and DL-Fen to 10 h with average concentrations ($n=3$) of 159, 420, 497, 23 and 20 n*M* for Phen, D-Norf, L-Norf, D-Fen and L-Fen, respectively. Fig. 5 illustrates their plasma levels with time. The mean ratio between L- and D-Fen was 0.76 with a range of 0.59–0.89 ($n=3$). This suggests a more rapid metabolism of the L-enantiomer compared to the D-enantiomer, which is evidenced by the higher plasma levels of the L-enantiomer of Norf compared to the D-enantiomer. This result agrees with other reported data [28,38]. The plasma levels of Phen and combined concentrations of D- and L-Fen were 306 and 340 n*M*, respectively. Compared to our previous paper [33], the maximum plasma levels of Phen and Fen after the simultaneous administration of a single dose of 5 mg/kg of each Phen and Fen to rats were 1730 and 1744 n*M*, respectively, which correlate with the results obtained from this study.

As a preliminary study, some experiments were conducted where Phen and Fen were administered individually (three rats for each drug). In spite of small sample size of rats number ($n=3$ for each treatment) in all experiments, i.e., individual or combined administration, we noticed some difference in the pharmacokinetic parameters of Phen, Fen

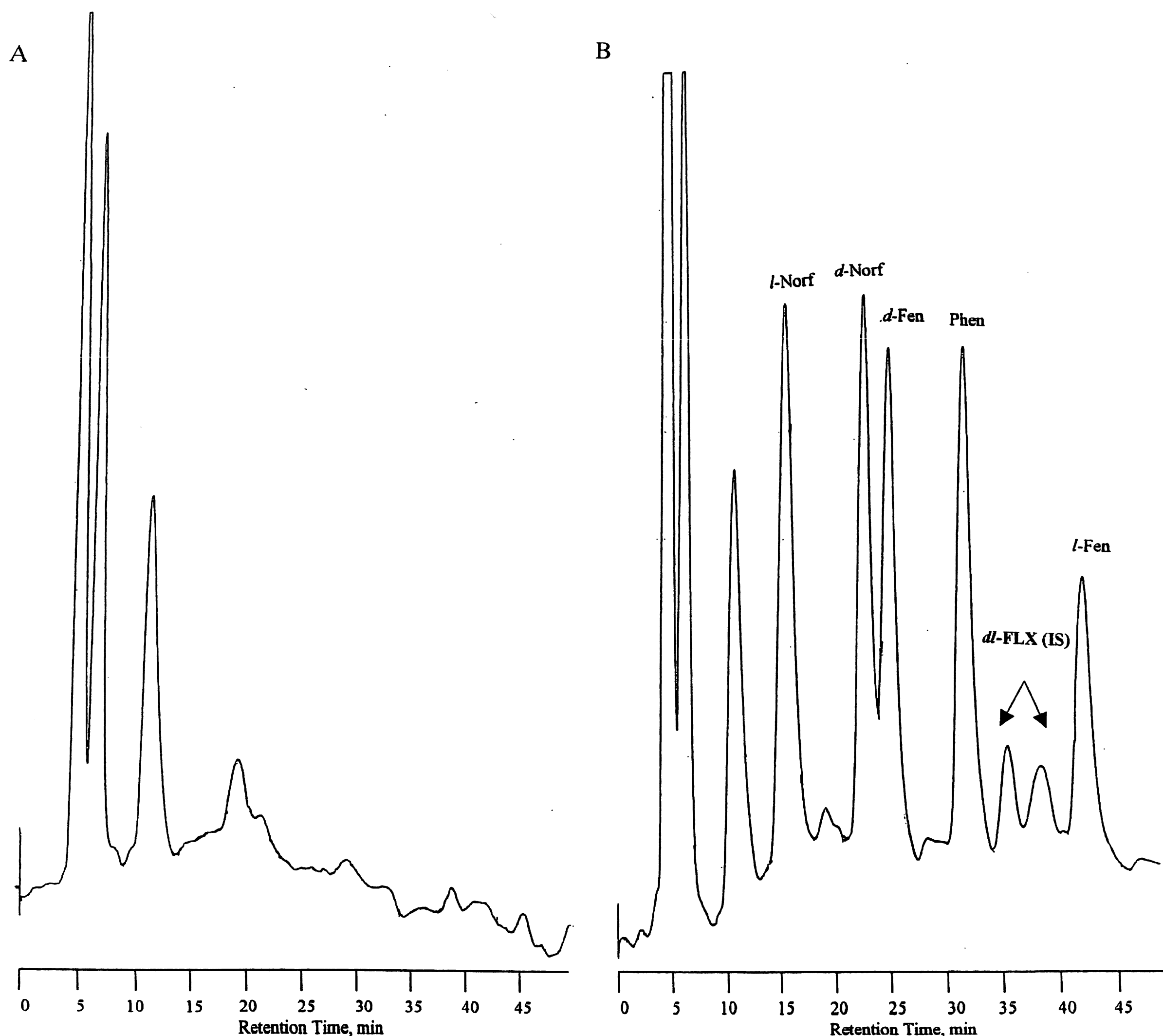

Fig. 4. Chromatograms of rat plasma samples, (A) before and (B) 30 min after the simultaneous administration of single i.p. dose of Phen and DL-Fen (1 mg/kg each). The peaks represent 253, 239, 177, 112 and 89 n*M* of L-Norf, D-Norf, D-Fen, Phen and L-Fen, respectively.

and Norf in the case of the simultaneous administration compared to those of the individual administration (data not shown). Such results indicated to us the necessity for further studies with larger number of rats so that a statistical analysis can be performed to examine the possibility of pharmacokinetic interaction between Phen and Fen when they were administered in combination and the results will be published elsewhere.

Furthermore, other two experiments were carried out; in one of these experiments, a rat was administered with 5 mg/kg of each DL-Fen and Phen, and in the other experiment one rat was administered with 5 mg/kg of D-Fen and Phen, in order to check for in vivo racemization. Low plasma levels of L-Fen in addition to the metabolite L-Norf were detected compared to the antipodes in the latter experiment. The maximum plasma levels were 196 and 137 n*M* for L-Fen and L-Norf, respectively, compared to 2900 and 1286 n*M* for D-Fen and D-Norf, respectively. On

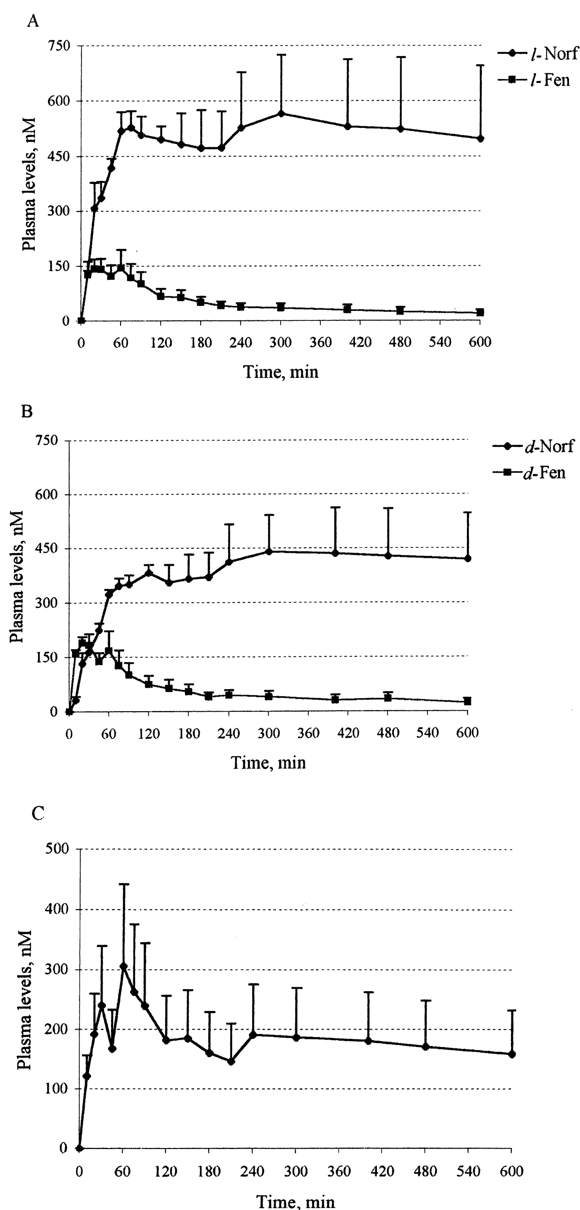

Fig. 5. Time-courses of plasma levels of (A) L-Norf and L-Fen, (B) D-Norf and D-Fen and (C) Phen after the simultaneous administration of single i.p. doses of 1 mg/kg Phen and DL-Fen to rats (n=3).

the other hand, the maximum plasma levels for the rat administered with DL-Fen were 1847 and 1957 nM for D- and L-Fen, respectively, and 1239 and 2971 nM for D- and L-Norf, respectively. When we checked for the purity of the D-Fen standard, about 1.6% of L-Fen was obtained as impurity, which suggested the existence of L-Fen in the rat plasma was due to the impurity rather than racemization.

4. Conclusion

In conclusion, a simple and sensitive HPLC method for the simultaneous determination of six sympathomimetic amines including E, NE, 2-PEA, 2-CB, Phen and DL-Fen in human plasma has been developed, which should be useful for forensic and toxicological studies. Moreover, a new sensitive chiral HPLC method for the simultaneous quantitation of Fen and Norf enantiomers in addition to Phen in human and rat plasma has been developed, using FLX as I.S. The chiral method has been applied for the determination of Phen and the enantiomers of Fen and Norf after the simultaneous administration of a single i.p. dose of DL-Fen and Phen to rats. The method showed to be highly sensitive with detection limits of 0.46, 0.81 and 0.21 ng/ml for D-Fen, L-Fen and Phen in human plasma and of 0.32, 0.99 and 0.25 ng/ml for D-Fen, L-Fen and Phen in rat plasma, respectively, which makes it the most sensitive method for their determination in plasma.

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