ORIGINAL ARTICLE

Studies on in vivo incorporation of fenfluramine and norfenfluramine into pigmented and nonpigmented hair by HPLC-fluorescence detection

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Abstract The incorporation profiles for fenfluramine (Fen) and its metabolite norfenfluramine (Norf) in black hair and white hair of Zucker rats and in white hair of Wistar rats after intraperitoneal (i.p.) administration of Fen or *N*-nitrosofenfluramine (*N*-Fen) were studied in great detail. The target compounds were determined by high-performance liquid chromatography with fluorescence detection using 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride as a derivatizing reagent. After repeated i.p. administrations of Fen (5 mg/kg) for 4 days, to Zucker rats, shaft and root samples of black and white hair were obtained 1 week after the first administration. It was surprising that Fen and Norf levels in root samples of white hair were much higher than those in shaft or root samples of black hair, strongly suggesting that unknown mechanisms other than the action of melanin, takes place in the white hair root. Time course profiles for Fen and Norf after administration of a single i.p. dose of Fen or *N*-Fen were constructed using Zucker and Wistar rats. The percent level of Fen or Norf in white hair was 15-50% of that in black hair at any interval within 600 min after a single administration of Fen using Zucker rats. Even with Wistar rats having white hair only, we could demonstrate the time courses for incorporation of Fen and Norf into white hair. Finally, time course profiles for Fen and Norf were also followed after a single i.p. administration of *N*-Fen; this experiment was characterized by the result that the levels of Norf were much higher than those of Fen for both black and white hair samples of Zucker rats at any interval tested.

Keywords Fenfluramine · Norfenfluramine · *N*-Nitrosofenfluramine · Hair analysis · Melanin · Zucker rats

Introduction

There are some studies showing that pigmentation is an important factor in determining drug incorporation into hair [1-5]; this is due to the presence of melanin that has relatively high contents of negatively charged carboxyl groups and semiquinones, facilitating its binding to compounds with cationic properties at physiological pH like amines by ionic interactions. For weak acids and neutral compounds, the binding is probably not as strong as the weak bases due to the absence of the ionic interactions that are required to strengthen other type of interactions like Van der Waals, hydrophobic and charge transfer interactions [1]. Hence, the incorporation of weak acids and neutral compounds into pigmented and nonpigmented hair is generally low.

Recently, fenfluramine (Fen) or *N*-nitrosofenfluramine (*N*-Fen) was found to be adulterated in Chinese diet products, which caused serious health damages. Fen was listed as one of the most important drugs of abuse to be screened in Japan [6]. Fen and its metabolite norfenfluramine (Norf) are weak bases, and are positively charged at physiological pH like other amphetamines (APs). Thus both compounds are expected to be similar to APs and other weak base compounds in their incorporation into hair [7-10]; the incorporation of Fen and Norf into pigmented hair is expected to be much higher than into nonpigmented hair. However, our previous report [10] for the determination of Fen and Norf in hair of patients suffering from hepatotoxicity following *N*-Fen ingestion showed that their in vivo incorporation of Fen and Norf into white hair is comparable to that into black hair. The objective of this work is to further study the in vivo incorporation of Fen and its metabolite Norf into white and black hair using rats after administration of either with Fen or *N*-Fen.

Materials and methods

Chemicals

Fen-HCl was obtained from Sigma Chemical (St. Louis, MO, USA). Norf-HCl and *N*-Fen were synthesized from Fen-HCl as previously described [12]. 4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) was synthesized in our laboratory [13] and can be obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile and methanol of high-perfomence liquid chromatography (HPLC) grade were purchased from Wako Pure Chemical (Osaka, Japan). Water was deionized and passed through an automatic water distillation apparatus (Aquarius GSR-500, Advantec, Tokyo, Japan). Other common chemicals used were of analytical grade.

Animal experiments

Male Wistar (280-345 g) and Zucker (210-320 g) rats were purchased from Otsubo Experimental Animals (Nagasaki, Japan) and housed at constant room temperature with an alternating 12-h light and dark cycle and with free access to food and water. Animals were housed individually in hanging wire cages to prevent contamination from bedding, urine or saliva of rats. In this work, 4 experiments were achieved. (1) The back hair of Zucker rats (n=3) was shaved followed by repeated intraperitoneal (i.p.) administrations of Fen (5 mg/kg) once a day for 4 days. The newly grown back hair 1 week after the first injection was collected from the pigmented (black) and nonpigmented (white) areas by shaving (shafts) and plucking (roots plus shafts). Hair samples were kept at 4^{o}C until analysis. (2) The back hair of Zucker rats (n=3) was shaved. They were anesthetized with ethyl carbamate (1.5 g/kg, i.p.). Before Fen administration, hair (black and white) and blood samples were obtained for blank tests,

then the rats were administered with a single i.p. dose of Fen (5 mg/kg). Fen and Norf concentrations in hair and blood were monitored at 15, 30, 45, 60, 90, 120, 180, 240, 360, 480 and 600 min. Hair samples were obtained by plucking (roots and shafts), and blood samples were collected in a tube containing EDTA and centrifuged at 1500*g* for 10 min. The obtained plasma and hair samples were stored at -20 and 4^{o}C, respectively, until analysis. (3) In this experiment, Wistar rats were used (*n*=3). Hair and blood samplings were performed in a similar way to that in the above experiment 2 using Zucker rats except that the sampling intervals were not longer than 180 min. (4) The last experiment was also similar to the above experiment 2 expect that Zucker rats were administered with a single dose of *N*-Fen (25 mg/kg, i.p.), followed by hair and plasma samplings.

Sample pretreatments

Hair samples were treated directly without washings. Pigmented and nonpigmented hair samples were treated separately as described previously [11]. Hair samples were weighed and appropriate volumes of methanol were added to prepare a final hair concentration of 1 mg/ml followed by sonication for 1 h. After sonication, the sample solvent was decanted and analyzed for Fen and Norf. For the analysis, 400 μl of the sample solution was pipetted into a vial and evaporated to dryness.

Plasma samples were extracted as described previously [12]. In brief, to 50 μl of plasma, 100 μl borate buffer (0.1 M, pH 10.6) and ethyl acetate were added. Samples were centrifuged at 1500*g* for 10 min, and the organic layer was separated and evaporated to dryness. The obtained residues of hair and plasma samples were then subjected to derivatization with DIB-Cl as follows. To each residue, 25 μl of carbonate buffer (0.02 M, pH 9) and 75 μl of 2 mM DIB-Cl suspension in acetonitrile

were added. Samples were allowed to stand at room temperature for 10 min for the derivatization reaction, which was then stopped with 5 μl aqueous ammonia (25%). From the reaction mixture, 20 μl were injected into HPLC.

Chromatographic conditions

For separation of DIB-derivatives of Norf and Fen, the same conditions described in our previous reports [11,12] were used. In brief, an isocratic HPLC system with fluorescence detection at excitation wavelength of 325 nm and at emission of 430 nm was used. The column was Daisopak SP-120-5-ODS-BP (250 mm × 4.6 mm i.d., 5 μm particle size; Daiso, Osaka, Japan), and the mobile phase consisted of a mixture of acetonitrile/water (65:35, v/v) with a flow rate set at 1.0 ml/min. The method proved to be valid and linear in the examined ranges of 50 pg to 144 ng/mg for Fen and 30 pg to 127 ng/mg for Norf with limits of detection of 1.9 and 1.1 pg on column for Fen and Norf, respectively [11].

Results and discussion

HPLC separation of the DIB-derivatives

Under the chromatographic conditions used in these experiments, the DIB-derivatives of Norf and Fen were well separated from the reagent peaks and the DIB-derivatives of hair endogenous compounds within 30 min with retention times of 16 and 23 min, respectively. Figure 1 illustrates chromatograms obtained from (a) white hair of a Wistar rat after 30 min, and (b) white hair and (c) black hair of a Zucker rat after 360 min of single i.p. administration of Fen (5 mg/kg). The peaks of Norf and Fen

represent the following concentrations: (a) 123 and 2010 pg/mg; (b) 609 and 923 pg/mg; (c) 4.1 and 6.5 ng/mg, respectively.

Fenfluramine (Fen) and norfenfluramine in hair after repeated administration of Fen

The Fen and Norf levels in the shafts of Zucker rats were briefly reported [11], but their levels in the roots have not been reported. The incorporation of Fen and Norf into pigmented and nonpigmented hair of Zucker rats 1 week after the first administration of Fen (5 mg/kg) is shown in Fig. 2 a (Fen) and b (Norf). Fen and Norf levels in the roots were calculated from the difference between their concentrations in hair samples obtained from plucking and shaving. Fen and Norf concentrations in the shafts of white hair represent 55 and 59%, respectively, of their concentrations in black hair [11]. While in the roots, Fen and Norf levels in the white hair roots were much higher than those of black hair (Fig. 2). The role of melanin in enhancing drug incorporation into the shafts is obvious, but the mechanism for the high concentration of Fen and Norf in the white hair roots is not clear and remains to be explored. In our preliminary study, we found that the incorporation of methamphetamine (MP) and its metabolite, amphetamine (AP) into the roots of white hair of Zucker rats was negligible as compared to that into black hair roots (data is not shown). These results are compatible with those in the report by Nakahara et al. [14] showing that MP levels in black hair roots were about 17 times higher than those in white hair roots. Many other studies showed that non-ionic compounds like *N*-acetylamphetamine [15] and negatively charged compounds like cannabinoids (tetrahydrocannabinols and their carboxyl metabolites) [16, 17] and many of steroids [18, 19] could be found in hair, suggesting that melanin is not the only drug binding site in hair. Other researchers [1, 5, 20] introduced other processes that may contribute to drug incorporation into hair

such as drug transport by hair cells. Borges et al. [5] studied the influx and efflux of AP and *N*-acetyamphetamine in keratinocytes, pigmented melanocytes and nonpigmented melanocytes as a model for incorporation and efflux of these drugs from hair cells; from their results, they concluded that drugs were incorporated into cells not only by a simple diffusion but also by a transport system that allowed a selective uptake of certain drugs and exclusion of others. While others suggested the role of hair components such as lipids as a critical factor in addition to melanin for drugs to be incorporated into hair [21].

Time courses for fenfluramine (Fen) and norfenfluramine in plasma and hair of Zucker rats after a single dose of Fen

The time courses for the levels of Fen and Norf in plasma and black and white hair of Zucker rats were followed for 10 h after a single dose of Fen (5 mg/kg) as shown in Fig. 3. Fen was rapidly incorporated into black and white hair roots and was detectable within 15 min after its administration. In plasma, Fen showed T_{max} at 25 ± 10 min (Table 1) and an elimination half life of 2.2 ± 0.2 h; while Fen in the black hair increased until 45 min, then decreased at 60 min and increased again, reaching a plateau from 120 to 480 min (Fig. 3 a). In the white hair, Fen showed maximum levels during the intervals from 45 to 120 min and then gradually decreased. The percent ratios of Fen concentrations 600 min to their respective C_{max} values in plasma, and black and white hair were calculated at resulted in the values 3.3 ± 0.3, 88 ± 13 and 37 ± 10%, respectively, indicating that Fen decline in hair does not follow its plasma pattern, and its decline in white hair is faster than the black hair. The latter is not surprising, because the accumulation of Fen in white hair was found active only in the root portion (Fig. 2 a), and Fen concentrations decrease according to the growth of the

shaft portion. Other pharmacokinetic parameters of Fen in plasma and hair are summarized in Table 1. To investigate the effect of pigmentation on the incorporation of Fen into black and white hair, the percent ratios of C_{max} and AUC_t for Fen in the white hair to those in black hair were calculated at 60 ± 19 and 49 ± 12%, respectively. Fen levels in black hair were higher than those in white hair, although there was no significant difference between them (P=0.17, one tail; P=0.34, two tail t-test) due to the small number of each experiment.

The time course profiles for Fen metabolite Norf in Zucker rats under the same conditions are shown in Fig. 3 b. Norf was also rapidly incorporated in the hair roots and was detectable within 15 min after Fen administration. As expected, the metabolite Norf in plasma showed delayed T_{max} at 160 ± 20 min (Table 1). The Norf levels in black hair continued to increase until 600 min. The obtained pharmacokinetic parameters for Norf are also summarized in Table 1. The percent ratios of Norf levels in white hair to those in black hair were 22 ± 9 and 22 ± 8%, for C_{max} and AUC_t, respectively.

Time courses for fenfluramine (Fen) and norfenfluramine in plasma and hair of Wistar rats after a single dose of Fen

The aim of this experiment was to investigate the incorporation of Fen and Norf into white hair of Wistar rats on the assumption that melanin is not a contributing factor for drug incorporation into hair, and also to compare Fen and Norf levels in white hair of Wistar rats with those in white hair of Zucker rats. The time course profiles of Fen and Norf in plasma and hair of Wistar rats after a single dose of Fen (5 mg/kg) are shown in Figs. 4 a and b, respectively. Fen and Norf were rapidly incorporated into hair roots and were detectable within 15 min of Fen administration. The time course

profiles for Fen and Norf in plasma and hair of Wistar rats were almost similar to those in plasma and white hair of Zucker rats. The percent ratios of Fen concentration at 180 min to its C_{max} in plasma and hair were calculated at 28 and 71%, respectively, indicating that Fen decline in hair does not follow its plasma pattern, which is similar to the results obtained with the Zucker rats. There is a poor correlation between Fen levels in plasma and hair with r=0.656. Though more data points are required, Fen obviously retained mainly in hair roots and showed plateau through the interval from 90 to 180 min, whereas in this interval its levels in plasma declined. Norf in plasma, on the other hand, showed almost similar profiles to those of hair with r-values of 0.899, though levels in plasma were much higher than those in hair.

The incorporation of Fen, the precursor drug, into hair is much higher than that of Norf for Wistar rats (Fig. 4). Best representation for this matter could be obtained by calculating the ratios of their AUC values in plasma and hair according to the equation $AUC_{0\text{-}180}$ (Fen)/$AUC_{0\text{-}180}$ (Norf). This resulted in 1.61 and 3.44 for plasma and hair, respectively. In black and white hair of Zucker rats, Fen incorporation was also higher than Norf with ratios of ($AUC_{0\text{-}600}$ was used) 1.04, 1.60 and 1.65 for plasma, black hair and white hair respectively. These results are consistent with those for other drugs describing that the precursor compounds levels in hair are much higher than those of their metabolites. These drugs include cocaine [22], nicotine [23] and codeine [3]. This phenomenon may be explicable by higher polarity (hydrophilicity) of the metabolites, which are more difficult to cross hair cell membranes and enter the hair forming cells than less polar precursor drugs [24].

Moreover, to examine the effect of species differences on the incorporation of these compounds into white hair of Wistar and Zucker rats were evaluated; melanin is completely lacking in Wistar rats, while it is available in Zucker rats. To make the

comparison, the intervals from 0 to 180 min were carefully evaluated for both species (Figs. 3 and 4). The plasma levels and time courses of Fen and Norf in Wistar rats were almost the same (P>0.8) as those in Zucker rats; these were supported by their $AUC_{0\text{-}180}$ summarized in Table 2. Although Norf levels in plasma was slightly lower for Zucker rats ($AUC_{0\text{-}180}$ =14.6 ± 3 μg·min/ml), but it was not significantly different from its levels in Wistar rats ($AUC_{0\text{-}180}$ =18.5 ± 3.3 μg·min/ml, P>0.4). Fen and Norf levels in white hair of Zucker rats were comparable to those in Wistar rats (P>0.5). The results showed that the presence of melanin in the skin of Zucker rats did not affect its incorporation into the white hair.

Time courses for fenfluramine and norfenfluramine in white and black hair samples of Zucker rats after a single dose of *N*-nitrosofenfluramine

In our previous report [11], we could demonstrate the presence of Fen and Norf as metabolites in human hair after ingestion of *N*-Fen. In this animal experiment, we administered *N*-Fen to Zucker rats (25 mg/kg, i.p.) and followed the time courses of Fen and Norf in black and white hair of the animals until 14 days (20160 min) as shown in Fig. 5. Contrary to the results of human hair [11], the concentrations of Norf were much higher than those of Fen for both black and white hair samples after *N*-Fen administration to rats.

Norf showed rapid incorporation into hair root of black and white hair within 15 min with a continuous increase. In black hair, Norf showed a plateau from 120 to 600 min, and then declined to almost constant concentrations observed from 48 h (2880 min) to 14 days (20160 min), which possibly reflects the amounts of Norf trapped in the hair shafts. While in the white hair, the increase of Norf was not so marked over

the examined period. Norf levels in the black hair were higher than those in white hair from 30 min to 24 h (1440 min).

On the other hand, the data of Fen (Fig. 5 b) did not show consistent patterns unlike the data of Norf; the Fen concentrations in black hair were higher than those in white hair at 9 points of period, while the former ones were lower than the latter at 5 points of period. The inconsistent profile and low concentrations of Fen led us to estimate the poor formation of Fen in rat body after *N*-Fen administration. The major metabolic product from *N*-Fen is probably Norf, which is incorporated into rat hair cells; only trace amounts of Fen may be produced from *N*-Fen in rat body.

In conclusion, although more investigations are required to clarify many of the ambiguous results obtained here, it seems correct that Fen and Norf behave in a different way regarding their incorporation into hair as compared to other AP derivatives; the extent of incorporation of Fen and Norf into nonpigmented hair is much greater than that of other APs [14]. Thus the incorporation of different compounds into pigmented and nonpigmented hair should not be generalized because of the presence of various determining factors; melanin in hair is really one of the important factors, but other endogenous constituents in hair should not be ruled out for enhancing the incorporation of weak base compounds into hair.

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Figure Captions

Fig. 1 Chromatograms obtained from (**a**) white hair of Wistar rat, (**b**) white hair of Zucker rat and (**c**) black hair of Zucker rat after 30 min for **a** and 360 min for **b** and **c**, with single i.p. administration of Fen (5 mg/kg). The detector sensitivity in a and b is 4 times higher than in c. Fen: fenfluramine; Norf: norfenfluramine

Fig. 2 Shaft and root levels of (**a**) Fen and (**b**) Norf in white and black hair of Zucker rats administered i.p. with repeated doses of Fen (5 mg/kg) for 4 days, once a day. Shaft and root sampling was obtained 1 week from the first administration (*n*=3)

Fig. 3 Time courses for levels of Fen (**a**) and Norf (**b**) in plasma, black hair and white hair of Zucker rats after administration of a single i.p. dose of Fen (5 mg/kg, *n*=3)

Fig. 4 Time courses for levels of (**a**) Fen and (**b**) Norf in plasma and white hair of Wistar rats after administration of a single i.p. dose of Fen (5 mg/kg, *n*=3)

Fig. 5 Time courses for Norf (**a**) and Fen (**b**) levels in black hair and white hair of Zucker rats after administration of a single i.p. dose of *N*-nitrosofenfluramine (25 mg/kg, *n*=3)

Table 1 Pharmacokinetics of fenfluramine (Fen) and norfenfluramine (Norf) in plasma, white and black hair of Zucker rats administered with Fen (5 mg/kg, *n*=3)

	C_{max} (ng/ml or g)	T_{max} (min)	AUC_t (μg·min/g)
Fen			
Plasma	339 ± 23	25 ± 10	47 ± 11
Black hair	2952 ± 1921	135 ± 57	1370 ± 979
White hair	1051 ± 437	85 ± 22	306 ± 138
Norf			
Plasma	111 ± 23	160 ± 20	45 ± 6
Black hair	2151 ± 1396	600 ± 0	836 ± 537
White hair	482 ± 200	320 ± 140	186 ± 63

Data were expressed as means ± standard deviation (SD)

Table 2 Comparison of plasma and white hair AUCs at 180 min (μg·min/ml or g) of Wistar rats (*n*=3) with those of Zucker rats (*n*=3)

		Plasma AUC_{180}	White hair AUC_{180}
Fen			
	Wistar	29.9 ± 6.4	127.2 ± 49.1
	Zucker	32.4 ± 8.1	128.3 ± 58.7
Norf			
	Wistar	18.5 ± 3.3	36.9 ± 6.4
	Zucker	14.6 ± 3.0	49.6 ± 17.4

Data were expressed as means ± SD

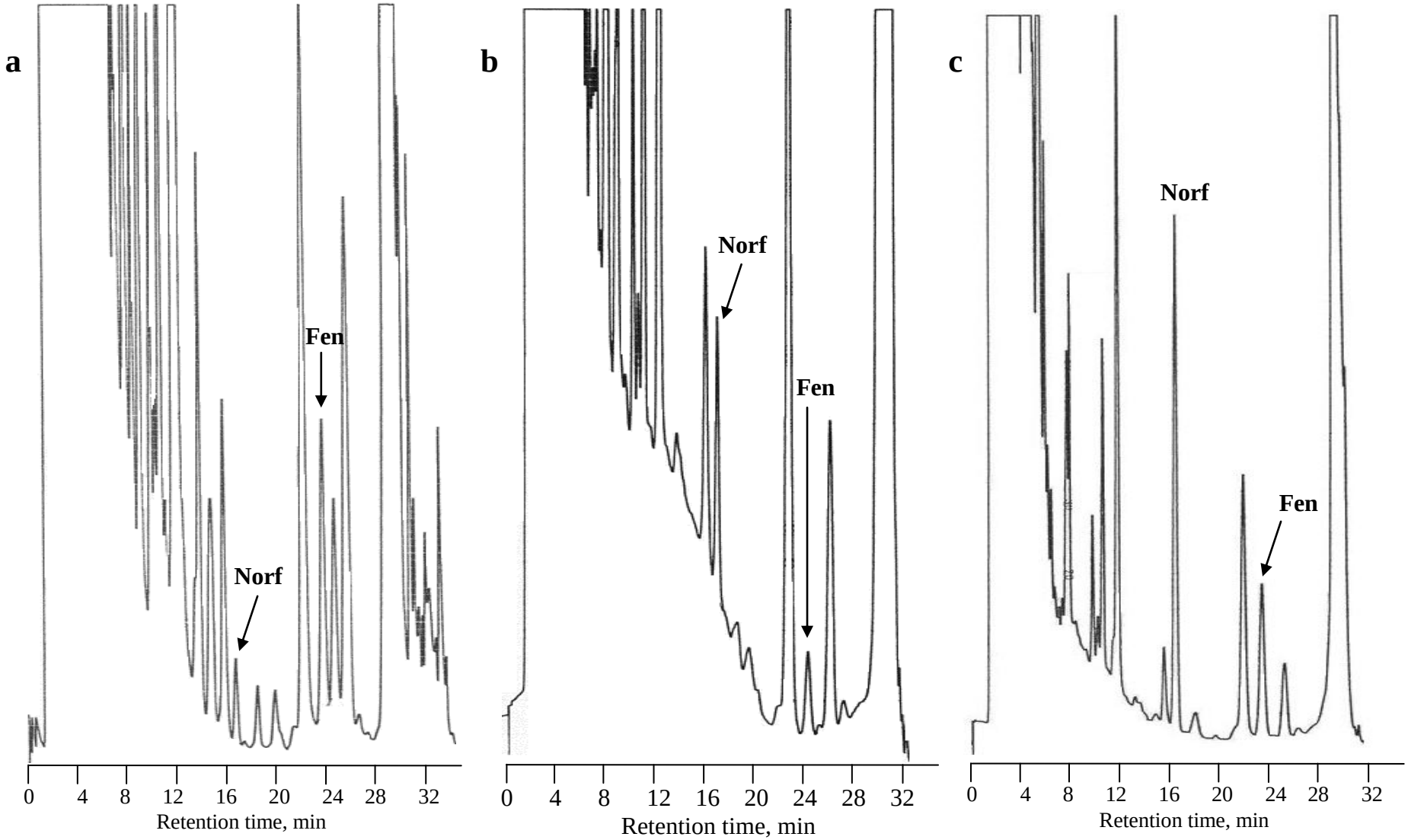
a
Norf
Fen
0 4 8 12 16 20 24 28 32
Retention time, min
b
Norf
Fen
0 4 8 12 16 20 24 28 32
Retention time, min
c
Norf
Fen
0 4 8 12 16 20 24 28 32
Retention time, min

Fig. 1.

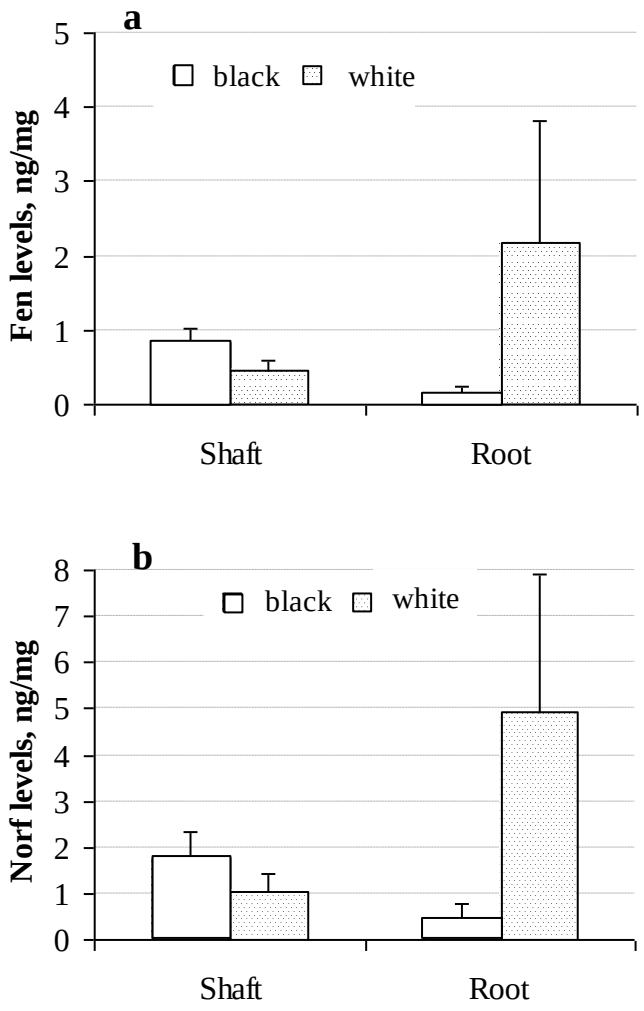
a
black
white
Fen levels, ng/mg
0
1
2
3
4
5
Shaft
Root
b
black
white
Norf levels, ng/mg
0
1
2
3
4
5
6
7
8
Shaft
Root

Fig. 2.

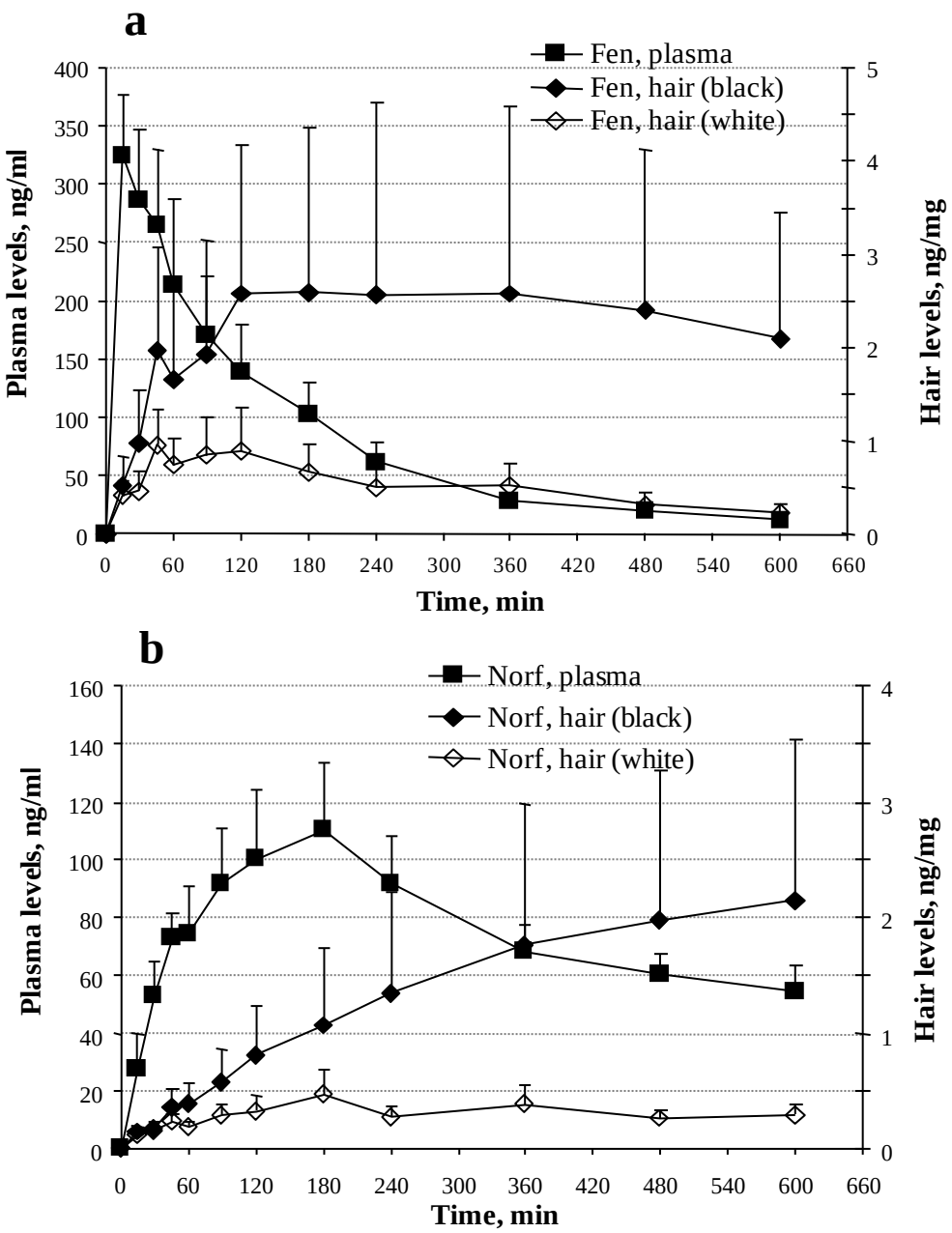
a
Fen, plasma
Fen, hair (black)
Fen, hair (white)
Plasma levels, ng/ml
Hair levels, ng/mg
Time, min
b
Norf, plasma
Norf, hair (black)
Norf, hair (white)
Plasma levels, ng/ml
Hair levels, ng/mg
Time, min

Fig. 3.

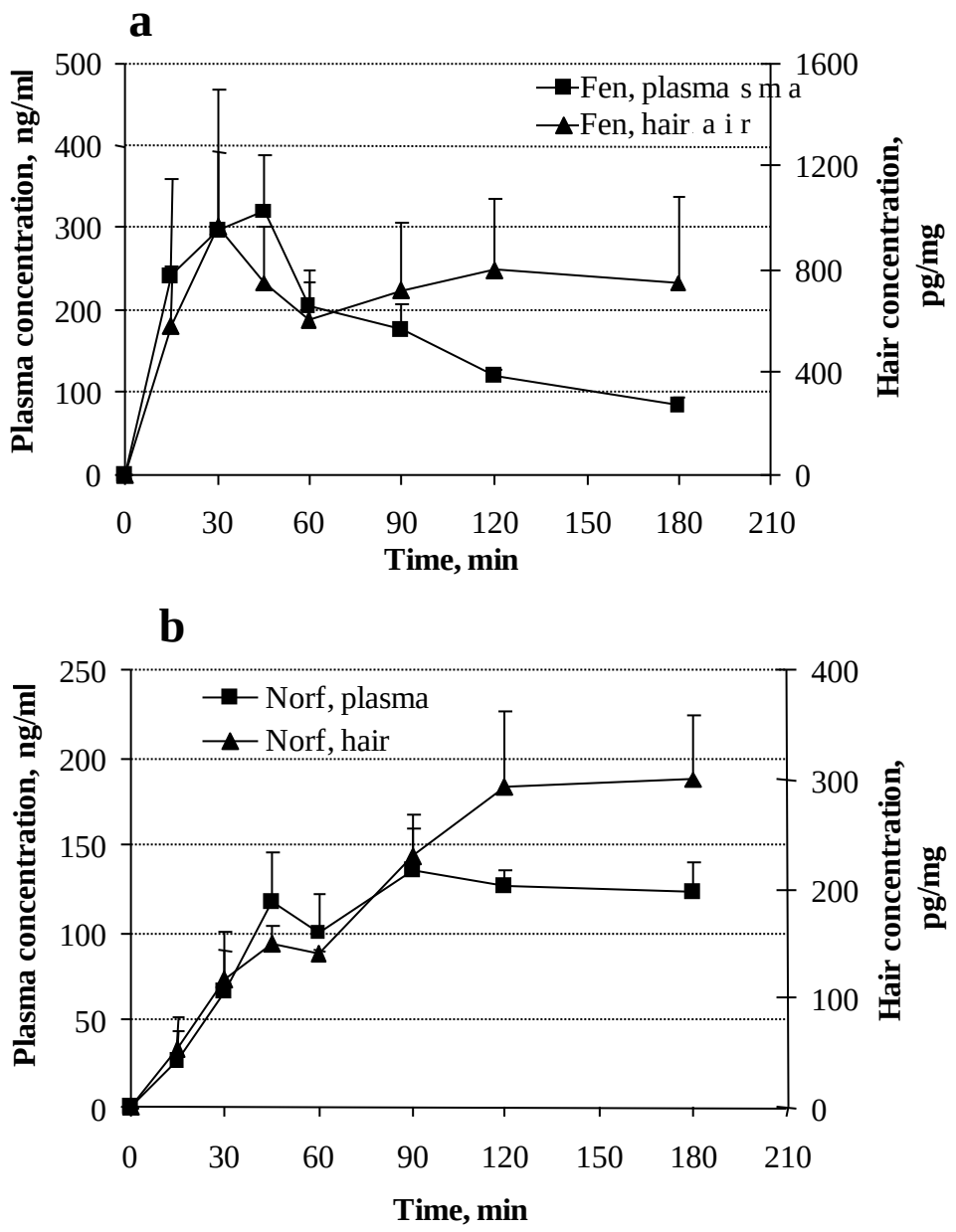
a
Plasma concentration, ng/ml
Hair concentration, pg/mg
Fen, plasma
Fen, hair
Time, min
b
Norf, plasma
Norf, hair
Time, min

Fig. 4.

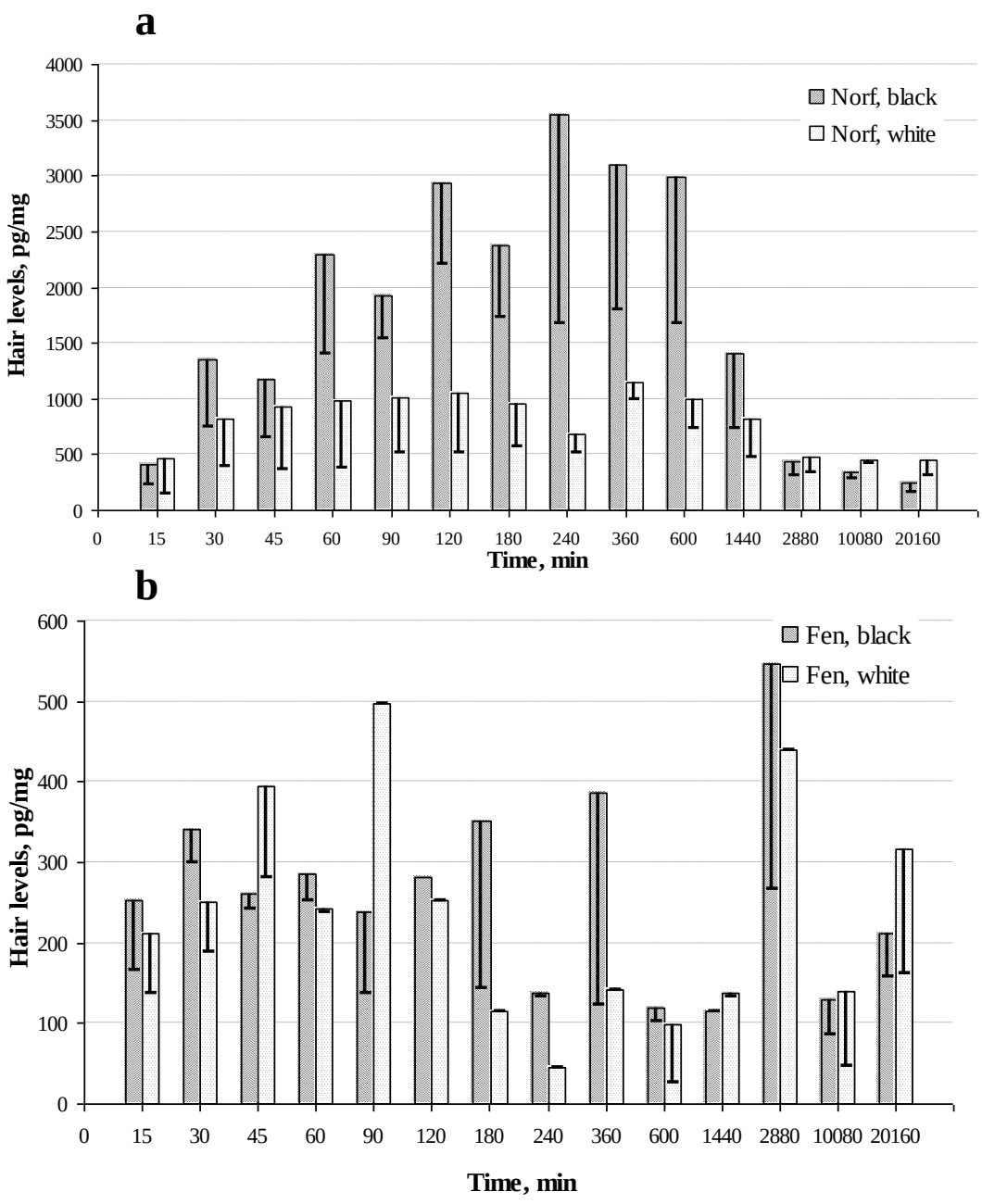
a
Norf, black
Norf, white
Hair levels, pg/mg
Time, min
b
Fen, black
Fen, white
Hair levels, pg/mg
Time, min

Fig. 5.