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Brief Communications:

Pharmacokinetics of *d*- and *l*-norfenfluramine following their administration as individual enantiomers in rats

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Summary

The effect of fenfluramine and norfenfluramine enantiomers in rodent seizure models, and their correlation with the pharmacokinetics of *d*- and *l*-fenfluramine in rats have been recently reported. To complement these findings, we investigated the pharmacokinetics of *d*- and *l*-norfenfluramine in rat plasma and brain samples. Sprague Dawley rats were injected intraperitoneally (i.p.) with 20mg/kg and 1 mg/kg *l*-norfenfluramine. A 1 mg/kg dose of *d*-norfenfluramine was used because higher doses caused severe toxicity. The concentration of each enantiomer in plasma and brain was determined at different time points by Liquid Chromatograph/Mass Spectrometry. Pharmacokinetic parameters were compared between norfenfluramine enantiomers, and with those reported previously for fenfluramine enantiomers after a 20 mg/kg i.p. dose. All enantiomers were absorbed rapidly and eliminated with half-lives ranging from 0.9 h (*l*-fenfluramine) to 6.1 h (*l*-norfenfluramine, 20 mg/kg) in plasma, and from 3.6 h (*d*-fenfluramine) to 8.0 h (*l*-fenfluramine) in brain. Brain-to-plasma concentration ratios ranged from 15.4 (*d*-fenfluramine) to 27.6 (*d*-norfenfluramine), indicating extensive brain penetration. The fraction of *d*- and *l*-fenfluramine metabolized to norfenfluramine was estimated to be close to unity. This work is part of ongoing investigations to determine the potential value of developing enantiomerically-pure *l*-fenfluramine or *l*-norfenfluramine as follow-up compounds to the marketed racemic-fenfluramine.

Key words: fenfluramine, norfenfluramine, pharmacokinetics, enantiomers, chiral switch.

INTRODUCTION

Fenfluramine, initially marketed as an anorexic agent, is currently approved in the U.S. and Europe for the treatment of seizures associated with Dravet syndrome and Lennox-Gastaut syndrome in patients aged 2 years and older.[1,2] Although fenfluramine is generally described even in recent articles as a single molecular entity,[3-8] the marketed product is a racemic mixture of *d*- and *l*-enantiomers with different pharmacological properties.[9,10] In addition, *d*- and *l*-fenfluramine are biotransformed in the body to the corresponding de-ethylated active metabolites *d*- and *l*-norfenfluramine, which also differ in their pharmacological profile.[9,10] While both fenfluramine and norfenfluramine enantiomers possess anticonvulsant activity in animal models of seizures and epilepsy,[10,11] *d*-fenfluramine and *d*-norfenfluramine are thought to contribute to appetite suppressant activity, whereas *d*-norfenfluramine is considered to be primarily responsible for the cardiovascular toxicity which led to the demise of fenfluramine and *d*-fenfluramine as anorexic agents in 1997 as well as the structurally-related benfluorex (a prodrug of *d*- and *l*-norfenfluramine) in 2009.[9,12] In spite of the above considerations, the vast majority of pharmacokinetic studies conducted with fenfluramine have used non-enantioselective assays, which fail to differentiate between the four active chemical entities present in the systemic circulation following administration of fenfluramine.[6]

In a recent article, we described the pharmacokinetic profiles of *d*- and *l*-fenfluramine and their corresponding de-ethylated primary active metabolites following administration of the individual enantiomers of fenfluramine in rats.[10] We also described their relationship with antiseizure activity in the maximal electroshock (MES) test in rats and mice, and with neurotoxicity findings in the same species.[10] All enantiomers showed comparable antiseizure activity, but *d*-norfenfluramine had greater toxicity than the other compounds tested. Based on these results and other evidence,[9] *l*-fenfluramine and *l*-norfenfluramine were considered to be attractive candidates for development as enantiomerically-pure medications due to their potentially improved tolerability and safety compared with the corresponding *d*-enantiomers. The pharmacokinetic profile of *d*-fenfluramine and *l*-fenfluramine in rats was described in our previous study.[10] In the present study, we complemented recently reported findings by evaluating the pharmacokinetics of *d*- and *l*-norfenfluramine in the plasma and brain of rats after their single-dose administration as individual enantiomers. Overall, the data provided by these studies permit a complete assessment of the comparative pharmacokinetic properties of each of the individual enantiomers that contribute to the effects of the marketed fenfluramine.

2 MATERIALS AND METHODS

2.1 Dosing and sampling schedule

Experiments were conducted in male Sprague Dawley rats (184–248 g, age 6–8 weeks, Envigo, Israel). Animals were injected intraperitoneally (i.p.) with a single dose of *l*-norfenfluramine HCl (1 or 20 mg/kg) or *d*-norfenfluramine HCl (1 mg/kg) dissolved in saline, similarly to our earlier study where a 20 mg/kg dose of each fenfluramine enantiomer was tested. All doses are expressed as free base. Since the median effective doses (ED_{50}) of *d*- and *l*-norfenfluramine in the MES test in rats after i.p. administration are about 5 and 10 mg/kg respectively[10], a 20 mg/kg dose corresponds to the upper limit of the dose-response curve. Subsequent experiments were conducted using a 1 mg/kg dose of both enantiomers because higher doses of *d*-norfenfluramine were found to cause severe toxicity (see Results).

Plasma and brain samples for pharmacokinetic measurements were obtained at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 36, and 48 h after dosing for both enantiomers and additionally at 60 and 72 h after the 20 mg/kg dose of the *l*-enantiomer, with two animals being sacrificed at each time point. Details of the experimental procedure have been described elsewhere.[10] The study protocol was approved by the Ethics Committee of the Hebrew University-Faculty of Medicine.

2.2 Assay of norfenfluramine enantiomers and pharmacokinetic analysis

Norfenfluramine enantiomers concentrations in plasma and brain samples were quantified by Liquid Chromatography/Mass Spectrometry (LC/MS) as previously described.[10] Pharmacokinetic parameters were calculated by non-compartmental analysis based on statistical moment theory using the Phoenix Winnonlin Tripos L.P. software (Pharsight Co., Mountain View, CA, USA). Actual values of clearance (CL) and volume of distribution at steady state (Vss) could not be determined because the bioavailability (F) after i.p. administration is unknown and, therefore, they were reported as CL/F and Vss/F respectively. Vs*s* represents the apparent volume of distribution when the rate at which drug enters and leaves the slowly equilibrating tissues are exactly matched.[13]

The fraction of *d*- and *l*-fenfluramine metabolized to systemically-available *d*- and *l*-norfenfluramine (fraction metabolized, f_m) was calculated from the molar dose-normalized ratio between the area under the plasma concentration versus time curve (AUC) of each norfenfluramine enantiomer after i.p. administration of the respective parent compound and the AUC after i.p. administration of the metabolite (*d*- or *l*-norfenfluramine). This calculation assumes equivalent bioavailability of parent compound and its metabolite and a linear relationship between concentration and dose.[13,14] The AUC value of each norfenfluramine enantiomer after administration of the parent compound was derived from our recently published study conducted under the same experimental conditions.[10]

3 RESULTS

3.1. Tolerability data

According to the study protocol, *d*- and *l*-norfenfluramine were to be administered at a dose of 20 mg/kg to facilitate comparison with results recently reported for *d*- and *l*-fenfluramine, which were investigated at that dose.[10] The 20 mg/kg dose of *l*-norfenfluramine caused no visible manifestations of toxicity in 31 of 33 animals tested. Of the remaining two animals, one had tremor and one died 15 min after dosing. Administration of 20 mg/kg *d*-norfenfluramine, however, caused severe toxicity consisting of tremor and convulsions in all animals and death in 5 of the 7 rats tested at this dose. In subsequent experiments, 10 and 5 mg/kg *d*-norfenfluramine were also found to cause tremor and convulsions, whereas a 1 mg/kg dose caused no visible manifestations of toxicity and was therefore selected for subsequent pharmacokinetic studies of both enantiomers.

3.2. Pharmacokinetic data

At all sampling times, between-rat variability in plasma and brain tissue concentrations of each enantiomer was relatively minor (supplementary figures 1s and 2s). Mean plasma and brain concentrations of *d*- and *l*-norfenfluramine after administration of the 1 mg/kg dose are illustrated in Figure 1, whereas pharmacokinetic parameters for each enantiomer are summarized in Table 1. For comparison purposes, Table 1 also includes previously reported pharmacokinetic parameters calculated after administration of the individual enantiomers of fenfluramine.[10]

Both *l*- and *d*-norfenfluramine were rapidly absorbed and distributed readily into the brain, with peak plasma concentrations obtained at 0.25 h after dosing and peak brain concentration at 1 h and 0.5 h, respectively. Pharmacokinetic analysis based on plasma and brain data after administration of the same dose (1 mg/kg) indicated that *l*-norfenfluramine had a similar clearance (CL/F) and a 30% lower volume of distribution (Vss/F) in plasma compared with *d*-norfenfluramine, and that CL/F and Vss/F values calculated from brain concentrations were comparable for both enantiomers. The pharmacokinetics of *l*-norfenfluramine appeared to deviate from linearity, because its CL/F calculated from both plasma and brain concentration after a 20 mg/kg dose was approximately one-half of that estimated after a 1 mg/kg dose. Brain-to-plasma exposure (AUC) ratios ranged from were 19.6 for *l*-norfenfluramine after a dose of 20 mg/kg to 27.6 for *d*-norfenfluramine, indicating extensive brain penetration of both enantiomers.

Comparison of data from pharmacokinetic studies with fenfluramine and norfenfluramine enantiomers in plasma and brain tissue showed that pharmacokinetic parameters were of the same order of magnitude for all compounds (Table 1). A relative exception was *l*-fenfluramine, which stood out for its highest CL/F values in both plasma and brain.

Of note, 20 mg/kg *l*-norfenfluramine was associated with the largest AUC values (and, consequently, the lowest CL/F values) among all enantiomers tested.

Based on the ratio between plasma AUC values after i.p. administration of norfenfluramine enantiomers and their previously reported AUC values as formed primary metabolites- of the respective individual fenfluramine enantiomers[10], the fraction of fenfluramine metabolized to norfenfluramine (f_m) was estimated at 1.15 for *d*-fenfluramine and 0.92 and 1.97 for *l*-fenfluramine 20 mg/kg and 1 mg/kg, respectively. An f_m value of 1.97 (i.e., >>1) further suggests that *l*-norfenfuramine pharmacokinetics deviate from linearity[13] after administration of 1 and 20 mg/kg doses. In any case, the observed f_m values indicate complete biotransformation of fenfluramine-to-norfenfluramine for both enantiomers.

4. DISCUSSION

To our knowledge, this is the first time that the pharmacokinetics of individually administered norfenfluramine enantiomers have been reported in any species. These results complement our recently reported characterization of the pharmacokinetics of the individual enantiomers of fenfluramine in rats, and their relationship with antiseizure effects in rodent seizure models.[10] Overall, the combined data from these studies provide a complete characterization of the pharmacokinetic profile of *d*- and *l*-fenfluramine and their active de-ethylated primary metabolites following i.p. administration in rats. The rapid achievement of peak concentrations of *d*- and *l*-norfenfluramine in both plasma and brain is consistent with the early onset of seizure protection in the MES test as well as early onset of minimal motor impairment after administration of the same enantiomers to rats.[10] The fact that the concentration of both enantiomers in brain tissue was overall at least 20-fold the concentration in plasma indicates extensive brain penetration, and is likely to reflect extensive binding to lipids and/or proteins in the cerebral parenchyma. Therefore, brain tissue concentrations should not be considered as a measure of the unbound concentration available to interact with receptor sites. Similar considerations also apply to fenfluramine enantiomers, which were also found in brain at concentrations >15-fold higher than in plasma.[10]

A limitation of our comparison of the pharmacokinetic parameters of fenfluramine and norfenfluramine enantiomers is that not all compounds could be assessed at the same dose, and therefore interpretation of the data can be complicated by the occurrence of non-linear (dose-dependent) kinetics.[13] Evidence of non-linear kinetics has been reported before for *l*-fenfluramine[15], and our results suggest that *l*-norfenfluramine kinetics may also deviate from linearity.

Overall, our findings are relevant for the design and interpretation of future studies on the antiseizure activity of fenfluramine and norfenfluramine enantiomers in rat models. In studies conducted to date, the individual enantiomers

of fenfluramine and norfenfluramine have been found to possess antiseizure activity in the MES test in rats and mice,[10] and in the zebrafish model of Dravet syndrome.[11] Among all enantiomers, *d*-norfenfluramine shows the greatest potency as an anticonvulsant in the MES test, but also the greatest potential to cause neurotoxicity.[10] The toxicity of *d*-norfenfluramine was confirmed in the present study, and resulted in the need to administer a dose as low as 1 mg/kg for pharmacokinetic assessment.

The pharmacology of fenfluramine and norfenfluramine enantiomers is complex,[7,9] and caution is required in extrapolating preclinical data to the clinical setting. Fenfluramine's cardiovascular adverse effects have been ascribed primarily to activation of 5-HT_{2B} receptors by norfenfluramine, and in particular by *d*-norfenfluramine which is more potent than *l*-norfenfluramine in activating this receptor.[9] The mechanisms responsible for the appetite suppressing effects of fenfluramine are diverse, with activation of the 5-HT_{2c} receptor by the metabolite norfenfluramine playing an important role.[9] Again, *d*-norfenfluramine is more potent than *l*-norfenfluramine in activating this receptor, and also displays a greater anorexic action than *l*-norfenfluramine in rodent models.[9] Accordingly, the observation that the *l*-enantiomers of both fenfluramine and norfenfluramine retain antiseizure activity makes them attractive candidates for development as follow-up compounds to the currently marketed racemic-fenfluramine for the treatment of seizure disorders.[9,10] Potential advantages of the *l*-enantiomers over racemic-fenfluramine include a reduced risk of cardiovascular effects, a lesser potential to cause anorexia and weight loss and, in the case of *l*-norfenfluramine, a longer half-life and a reduced risk of being involved in adverse drug-drug interactions.[9,10] The fact that two companies have recently filed patent applications for the use in epilepsy of the individual *l*-enantiomers of fenfluramine or norfenfluramine demonstrate the incentive and advantage in developing these enantiomers as follow-up compounds to racemic-fenfluramine.[9,16] Even though our data advocate against developing the *d*-enantiomers of fenfluramine and norfenfluramine for epilepsy treatment, it should be noted that the 5-HT_{2C} receptor is among the receptors putatively involved in the antiseizure action of fenfluramine, and that *d*-norfenfluramine has higher affinity and activity for this receptor compared with *l*-fenfluramine and *l*-norfenfluramine.[9,11] Moreover, evidence for antiseizure activity in the zebrafish model of Dravet syndrome is less conclusive for *l*-norfenfluramine than for the other enantiomers.[11] Ultimately, a decision to proceed with clinical development of *l*-fenfluramine or *l*-norfenfluramine requires further evaluation of their comparative activity and safety in animal models.

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CONFLICT OF INTEREST

NE, RH, CS and DB have nothing to disclose. RS is an employee of Xenon Therapeutics. EP received speaker's or consultancy fees from Eisai, Janssen, PMI Life Sciences, Sanofi group of companies, Shackelford Pharma, SKL Life Science, Takeda, UCB Pharma and Xenon Pharma, editorial fees from John Libbey and royalties from Wiley and Elsevier. MB received consultancy fees from Clexio Therapeutics, Guidepoint, Pharma2B, Selene Therapeutics, Shackelford Pharma and US WorldMeds.

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TABLE 1. Mean pharmacokinetic parameters of *l*- and *d*-norfenfluramine calculated from plasma (upper panel) and brain (lower panel) concentrations in rats (n=2 at each time point) after i.p. administration of single 1 and 20 mg/kg doses of *l*-norfenfluramine and a single 1 mg/kg dose of *d*-norfenfluramine. Results from previously

reported data[10] for the *l*- and *d*-enantiomers of fenfluramine and metabolically-formed norfenfluramine after administration of single 20 mg/kg i.p. doses of *l*- or *d*- fenfluramine are shown for comparison purposes.

Pharmacokinetic parameter (plasma)	***l*-Fenfluramine (20 mg/kg)**	***l*-Norfenfluramine (20 mg/kg)**	***l*-Norfenfluramine (1 mg/kg)**	***d*-Fenfluramine (20 mg/kg)**	***d*-Norfenfluramine (1 mg/kg)**
CL/F (L/h/kg)	7.0	1.2	2.6	1.5	2.1
Vss/F (L/kg)	8.9	10.6	11.3	7.4	15.1
$t_{1/2}$ (h)	0.9	6.1	3.0	3.5	4.95
AUC_{inf} (mg/L/h)	2.8	16.6	0.39	13.8	0.47
C_{max} (mg/L)	2.3	2.7	0.10	3.0	0.08
t_{max} (h)	0.25	0.25	0.25	0.25	0.25
f_m		0.92	1.97		1.15
Metabolically formed norfenfluramine					
$t_{1/2}$ (h)	6.2	-		8.0	-
AUC_{inf} (mg/L/h)	13.4	-		9.6	-
C_{max} (mg/L)	1.1	-		0.38	-
t_{max} (h)	3.0	-		12	-

Pharmacokinetic parameter (brain)	***l*-Fenfluramine (20 mg/kg)**	***l*-Norfenfluramine (20 mg/kg)**	***l*-Norfenfluramine (1 mg/kg)**	***d*-Fenfluramine (20 mg/kg)**	***d*-Norfenfluramine (1 mg/kg)**
CL/F (L/h/kg)	0.42	0.06	0.10	0.09	0.08
Vss/F (L/kg)	4.8	0.59	0.51	0.49	0.55
$t_{1/2}$ (h)	8.0	6.6	3.5	3.6	4.95
AUC_{inf} (µg/g/h)	47.7	325	9.87	212	13.1
C_{max} (µg/g)	27	35	1.7	42	1.7
t_{max} (h)	0.5	1	1	0.5	0.5
Brain-to-plasma (AUC_{inf}) ratio	16.7	19.6	25.6	15.4	27.6

Metabolically formed norfenfluramine				
$t_{1/2}$ (h)	7.1	-	7.9	-
AUC_{inf} (μg/g/h)	255	-	221	-
C_{max} (μg/g)	14	-	9.5	-
t_{max} (h)	3	-	4	-
Brain-to-plasma (AUC_{inf}) ratio	19.1	-	23.1	-

Abbreviations: AUC, area under the drug (or metabolite) concentration- time curve; CL, clearance; C_{max}, peak concentration; F, bioavailability (after i.p, administration); $t_{1/2}$, half-life; t_{max}, time of peak concentration; Vss, volume of distribution at steady state.

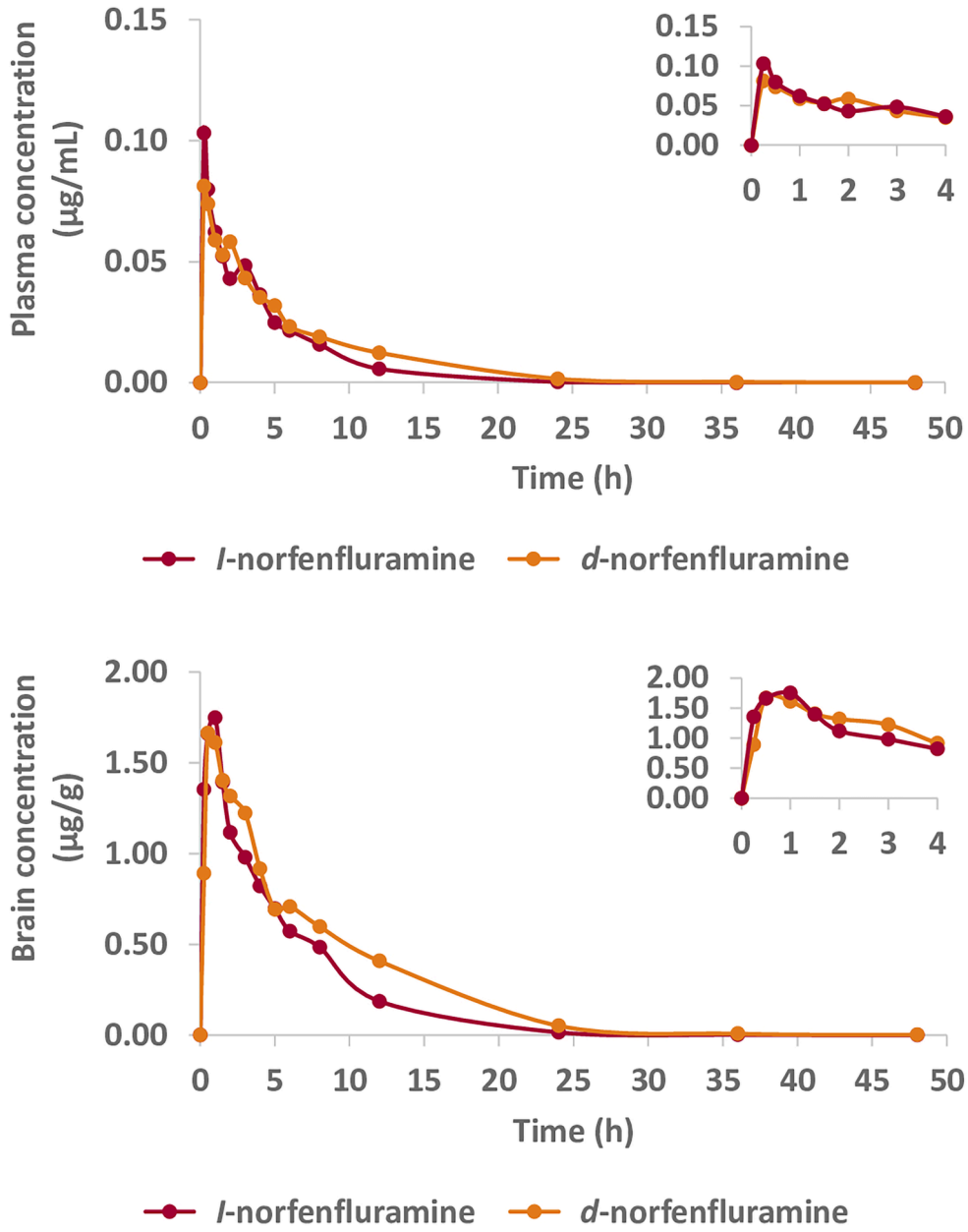
Legends to Figures

FIGURE 1. Plasma concentrations (upper panel) and brain tissue concentration (lower panel) of *d*- and *l*-norfenfluramine following of a single 1 mg/kg i.p. dose of each individual enantiomer in rats. The insert depicts the plasma and brain concentrations in the first 4 hours after dosing. Each point is the mean of measurements in 2 animals.

Supplementary Figures

FIGURE 1s. Plasma concentrations of *l*-norfenfluramine and *d*-norfenfluramine after single i.p. doses of each individual enantiomer in rats. The doses administered were 1 mg/kg (A) and 20 mg/kg (B) for *l*-norfenfluramine and 1 mg/kg for *d*-norfenfluramine (C). The concentrations shown are concentrations in individual animals and lines have been drawn to join all the highest (upper) and the lowest (lower) concentrations found at each time point in each pair of animals.

FIGURE 2s. Brain tissue concentrations of *l*- (upper panel) and *d*- norfenfluramine (lower panel) following a single i.p. dose of each individual enantiomer in rats. The doses administered were 1 mg/kg (A) and 20 mg/kg (B) for *l*-norfenfluramine and 1 mg/kg for *d*-norfenfluramine (C). The concentrations shown are concentrations in individual animals and lines have been drawn to join all the highest (upper) and the lowest (lower) concentrations found at each time point in each pair of animals.



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