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Stereoselective determination of fenfluramine enantiomers in rat liver microsomal incubates

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Abstract

An enantioselective assay for *l*- and *d*-fenfluramine in rat liver microsomal incubates was developed. The method involves extraction of fenfluramine from the microsomal incubates, and formation of fenfluramine diastereomeric derivatives with the chiral reagent *S*-(−)-*N*-trifluoroacetyl prolyl chloride. Separation and quantitation of the diastereomeric fenfluramine derivatives are carried out by a capillary gas chromatographic system with flame ionization detection. The assay is linear from 1 to 50 μg/ml for each enantiomer. The analytical method affords average recoveries of 92.28 and 96.44% for *l*- and *d*-fenfluramine, respectively. The limits of detection and quantitation for the method are 0.1 and 1.0 μg/ml for the *l*- and *d*-fenfluramine isomers, respectively. The reproducibility of the assay was <10% (RSD). The method allowed study of the depletion of *l*- and *d*-fenfluramine in rat liver microsomal incubates. The stereoselectivity of fenfluramine phase I metabolism was observed.

Keywords: Enantiomer separation; Fenfluramine

1. Introduction

Many synthetic drugs that contain chiral centers are marketed as racemates. It is generally accepted that it is drug metabolism that introduces the greatest degree of stereoselectivity into drug disposition. It has been demonstrated that pharmacodynamic and pharmacokinetic differences involving stereoselective drug disposition is an important aspect in clinical evaluation of some chiral drugs. These drugs are administrated routinely in their racemic form, that is a 1:1 mixture of two enantiomers [1]. Separation of enantiomers used to be one of the most difficult issues in separation science. Therefore, chiral chromatography has been a field of considerable interest and has developed very rapidly during the past decade [1,2]. Also, chiral chromatography provides a powerful and stereoselective technology for simultaneous separation and determination of enantiomers of chiral drugs. Some enantiomers of chiral drugs have been separated and determined using chiral chromatography, such as NSAIDs [3], chiral amine drugs [4], ofloxacin [5], and mephenytoin [6].

Fenfluramine, *N*-ethyl-α-methyl-3-(trifluoromethyl)phenethylamine hydrochloride, is widely used as an appetite suppressant in the treatment of obesity. The chemical structure of fenfluramine contains a stereogenic center, but the compound is marketed and used clinically as a racemate, an

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equimolar mixture of *l*- and *d*-fenfluramine [7]. Only a few methods for quantifying fenfluramine enantiomers in plasma and urine by high-performance liquid chromatography or gas chromatography have been reported [8,9], including separation of *l*- and *d*-fenfluramine based on Pirkle-type stationary phase of HPLC [10]. In this article we describe a reliable enantioselective analytical method that uses a GC-flame ionization detector (FID) for assay of *l*- and *d*-fenfluramine in rat liver microsomal incubates. We also report an application of this method to study stereoselective phase I metabolism of fenfluramine enantiomers in vitro.

2. Experiments

2.1. Materials

Racemic fenfluramine hydrochloride (99.5%) and phenobarbital (99.5%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and nicotinamide (99%), Tris (99.8%), *ld*-isocitrate trisodium (98%), isocitrate dehydrogenase (40 units/mg protein), NADP (98%), NADPH (98%), and *N*-propylamphetamine hydrochloride (99.5%) from Sigma (St. Louis, MO, USA). *S*-(−)-*N*-Trifluoroacetyl prolyl chloride (*S*-(−)-TFPC, 97% ee), 0.1 *M* solution in dichloromethane, was purchased from Aldrich (WI, USA). Chloroform (99.8%), ethyl acetate (99.5%), triethylamine (99%), HCl (36.5–38%), $MgCl_2$ (99%), $NaHCO_3$ (99%), and NaOH (98%) were analytical or chromatographic grade and obtained from Jinsan (Shanghai, China). The rat liver microsomes were prepared as described by Gibson et al. [11].

2.2. Chromatography

The modular gas chromatography equipment was a GC-15A with a flame ionization detector (FID) and a C-R 4A data system (Shimadzu, Japan). The analytical column was a HP-1 fused-silica capillary column (15 m×0.25 mm I.D., 0.25 μm film thickness; Hewlett-Packard, USA). The oven temperature was held at 100°C for 4 min and then programmed linearly to 280°C for 1 min at a rate of 8°C/min. The injector and detector were maintained at 280°C. Nitrogen was used as the carrier gas at a head pressure of 1 kg/cm^2 and as make-up gas at a flow-rate of 15 ml min^{-1}.

2.3. Assay procedure

The pH of 1.0 ml of incubation media (2.0 mg/ml microsome protein and cofactors) was adjusted to 12–13 by adding 150 μl 10 *M* NaOH. *N*-Propylamphetamine (100 μl of 1 mg/ml) was added as an internal standard. Fenfluramine was extracted with 2.0 ml chloroform by rotatory shaking for 1 min. The chloroform layer was separated and dried with anhydrous sodium sulfate. Then 10 μl trimethylamine and 40 μl *S*-(−)-TFPC was added and gently rocked for 15 min at room temperature. The chloroform layer was washed with 2 ml water. After phase separation, the chloroform was evaporated to dryness under a gentle stream of air at 55°C. The residue was reconstituted in 50 μl ethyl acetate, and an aliquot of 1 μl was analyzed by GC-FID.

2.4. Calibration curves

The calibration curves for *l*- and *d*-fenfluramine were constructed by analyzing a series of blank microsomal incubates spiked with racemic fenfluramine in the concentration range 1.0 to 50.0 μg/ml enantiomers (free base). Extraction and derivatization of the samples were carried out as described above. Peak-height ratios (*l*-fenfluramine vs. *l*-propylamphetamine and *d*-fenfluramine vs. *d*-propylamphetamine) were measured and plotted against the concentration of *l*- and *d*-fenfluramine.

2.5. Recovery studies

A series of blank microsomal incubates, spiked with various amounts of fenfluramine, were processed as described under *Assay procedure*. The peak-height ratios of enantiomer and internal standard were compared with those obtained when equal amounts of fenfluramine in pure solvent were derivatized with *S*-(−)-TFPC without prior extraction.

2.6. Reproducibility studies

The drug-free incubation media, spiked with different concentrations (1.0 and 50.0 μg/ml) of *l*- or *d*-fenfluramine, were used for reproducibility studies. We determined intra-assay variability by analyzing samples in quadruplicate, and determined inter-assay variability by analyzing samples in quadruplicate on four separate days, according to the procedure described under *Assay procedure*. The relative standard deviations were calculated.

2.7. Incubation of fenfluramine with rat liver microsomes

The time-dependent study was performed with a 10.0-ml incubation mixture containing 40.0 μg *l*- or *d*-fenfluramine per ml of incubation mixture with 2 mg of protein equivalent of liver microsomes from phenobarbital (PB)-treated rats and other cofactors. The incubation mixture (1 ml nicotinamide, 8.5 ml Tris–HCl buffer, pH 7.4, 1 ml 0.15 mol/l $MgCl_2$, 30 mg *dl*-isocitrate trisodium, and 5.5 mg isocitrate dehydrogenase) was bubbled in oxygen for 1 min before use. After preincubation in air for 5 min at 37°C, 100 μl solution of regeneration system (100 mg/ml NADP and 30 mg/ml NADPH in 1% $NaHCO_3$) was added to the incubation mixture. During the incubation, a 1.0-ml aliquot was taken at 3, 5, 10, and 30 min into a test tube containing 150 μl 10 *M* NaOH followed by adding 100 μl of 1 mg/ml internal standard. The samples were then analyzed as described under *Assay procedure*.

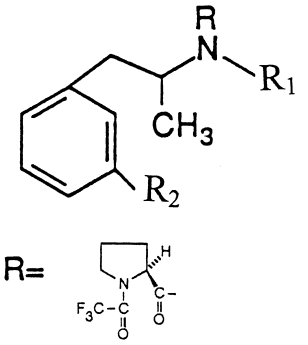
3. Results and discussion

Chiral chromatography has been widely applied to studies of chiral pharmacology [1,12–14]. Gas chromatography for separation and quantitation of drug enantiomers in biological fluids has been reported [12,15,16]. The methods used for chiral gas chromatography fall into two main categories. One is a direct method without the need of chiral derivatization by using a chiral stationary phase (CSP) and the other is an indirect method with the need to form diastereomers by reaction with chiral derivatizing reagents (CDR) and separation on an achiral stationary phase [2,8]. The direct method is simple and effective, but has high product costs and has a limitation for certain chiral drugs for each CSP. Achiral derivatization is required to improve enantiomer GC properties when the enantiomers of chiral drug are molecules with polar groups. CSPs demonstrate relative thermal instability and insufficient separation power in some cases [12]. The indirect method (CDR) is capable of resolving chiral drug enantiomers on an achiral GC stationary phase after derivatization of enantiomers with the optically pure chiral reagent. So, the CDR method is economic and flexible. A drawback of CDR is that it is contaminated by its antipode and the rates of each enantiomer derivatization reaction may be different. For the average laboratory, an achiral column could be used to analyze enantiomers of chiral pharmacological compounds and achiral compounds or chiral pharmacological compounds to be analyzed as racemates. Therefore, the CDR method is a useful means for separating drug optical isomers and complementary with the CSP method.

We developed the stereoselective GC analytical method for assay of *l*- and *d*-fenfluramine in rat liver microsomal incubates using *S*-(−)-*N*-trifluoroacetyl prolyl chloride as chiral resolving agent. It was reported that *l*-(−)-proline does not racemize during acylation or peptide synthesis since oxazolone formation is not possible. Furthermore, the rigid conformation of the prolyl–peptide bond is thought to enhance differences in the physical properties of its diastereomeric derivatives and to cause consequent enhancement of chromatographic separation.

Fenfluramine enantiomers were reacted with *S*-(−)-*N*-trifluoroacetyl prolyl chloride and converted into amide derivatives (Fig. 1). Good separation of the enantiomer pair of fenfluramine was achieved by means of an achiral fused-silica capillary GC column (Fig. 2). The analytical column efficiency was $>10^5$/column calculated on theoretical plate numbers, and the resolution between *l*- and *d*-fenfluramine was about 1.4.

The assay based on the peak-height ratios of the analyte and internal standard (y) versus concentration of the analyte (x) was linear. The linearity of the calibration curves for *l*- and *d*-fenfluramine was in the range 1.0 to 50.0 μg/ml. The regression equations of the calibration curves are $y = 0.4461x -$



Fenfluramine $R_1 = C_2H_5$ $R_2 = CF_3$

N-n-Propylamphetamine $R_1 = n\text{-}C_3H_7$ $R_2 = H$

Fig. 1. Structures of fenfluramine, internal standard and their *S*-(−)-*N*-(trifluoroacetyl)prolyl amide derivatives.

0.1735 for *l*-fenfluramine and $y = 0.4010x - 0.1367$ for *d*-fenfluramine. The correlation coefficients were 0.9998 and 0.9994 for the *l*- and *d*-enantiomer, respectively. The limit of detection (LOD) of the assay was measured as 0.1 μg/ml and the limit of quantitation (LOQ) was 1.0 μg/ml (RSD <15%, $n = 4$) for each enantiomer.

The recoveries of each enantiomer of fenfluramine

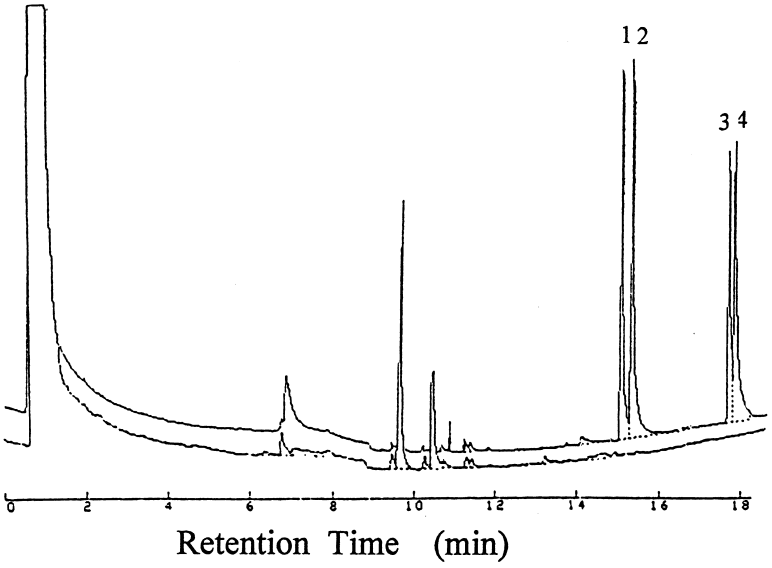


Fig. 2. Chromatogram of fenfluramine and propylamphetamine after pre-column chiral derivatization. Top: blank rat liver microsomal incubate spiked with fenfluramine and internal standard. (**1**) *l*-Propylamphetamine, (**2**) *d*-propylamphetamine, (**3**) *l*-fenfluramine, (**4**) *d*-fenfluramine. Bottom: blank rat liver microsomal incubate.

Table 1
Assay recoveries of enantiomers of fenfluramine from rat liver microsomal incubates ($\bar{x}\pm s$, $n=5$)

Target conc. (μg/ml)	Measured conc. (μg/ml)		Recovery (%)	
	l-Fenfluramine	*d*-Fenfluramine	*l*-Fenfluramine	*d*-Fenfluramine
1.0	0.910±0.03	0.976±0.07	91.0±2.7	97.6±6.8
50.0	46.8±2.9	47.7±1.6	93.5±5.8	95.3±3.2

by derivatization with *S*-(−)-TFPC are summarized in Table 1. The average recoveries of this analytical method were 92.28 and 96.44% for *l*- and *d*-fenfluramine, respectively.

The within-day and between-day coefficients of variation for the assay of enantiomers in microsomal incubates (Table 2) were less than 10% and 15% (RSD), respectively. We concluded that the reproducibility and precision of the enantioselective GC method for *l*- and *d*-fenfluramine in microsomal incubates were satisfactory.

The stereoselective GC method developed in the present article has been applied to the quantitation of fenfluramine enantiomers in rat liver microsomal incubates. Table 3 shows that the rate of *l*-fenfluramine metabolism is faster than that of the *d*-antipode. The *l*/*d*-fenfluramine ratio is about 0.66 when the time of metabolic reaction was from 3 to 30 min. A lower concentration was reported [13] for *l*-fenfluramine than for its antipode in plasma, red blood cells, and brain. The results of our present study agree with the previous report. It is clear that fenfluramine undergoes stereoselective metabolism in vitro and in vivo in the rat.

Table 2
Reproducibility and precision of the assay for *l*- and *d*-fenfluramine in the microsomal incubates ($\bar{x}\pm s$, $n=4$)

Target conc. (μg/ml)	Enantiomer conc. measured (μg/ml)	
	l-Fenfluramine	*d*-Fenfluramine
Within-day		
1.0	1.02±0.10	0.992±0.06
50.0	48.4±4.1	49.1±2.5
Between-day		
1.0	0.988±0.10	0.976±0.07
50.0	50.2±5.1	48.4±3.8

Table 3
Metabolic depletion of *l*- and *d*-fenfluramine ($\bar{x}\pm s$, $n=3$)

Incubation time (min)	Amount remained (μg)		l/d Ratio
	l-Fenfluramine	*d*-Fenfluramine	
3	25.0±3.3	37.3±5.8	0.67
5	23.2±1.6	36.0±5.9	0.64
10	22.5±5.2	33.8±7.9	0.66
30	20.6±4.4	30.6±7.2	0.67

In conclusion, a specific stereoselective analytical method was developed for the assay of *l*- and *d*-fenfluramine in rat liver microsomal incubates. The method was applied to the study of *l*- and *d*-fenfluramine depletion in vitro. The results show that fenfluramine undergoes enantioselective metabolism in rat liver microsomes.

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