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DETERMINATION OF 1,2-BENZISOXAZOLE-3-ACETAMIDOXIME HYDROCHLORIDE (PF-257) IN PLASMA USING ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method has been developed that permits the accurate and specific determination of a new psychotropic agent, PF-257, in plasma. PF-257 is extracted with ethyl acetate from alkaline plasma and, after a clean-up procedure, derivatized with heptafluorobutyric anhydride to form 3-[(5-*n*-heptafluoropropyl-1,2,4-oxadiazol-3-yl)methyl]-1,2-benzisoxazole (HOMB). The HOMB is assayed on a gas chromatograph equipped with an electron-capture detector.

Accurate determinations of PF-257 are possible in the concentration range from 1–40 ng/ml with a relative standard deviation of 6.8 %. The minimum detectable concentration in plasma is 0.1 ng/ml. Plasma levels of PF-257 in rats receiving intravenous or oral dosing (10 mg/kg) were determined.

INTRODUCTION

1,2-Benzisoxazole-3-acetamidoxime hydrochloride (PF-257 (ref. 1), Fig. 1) is a new psychotropic compound that possesses a novel pharmacological profile in experimental animals[2,3]. In order to study the pharmacokinetics of PF-257, a sensitive and specific assay method for the unchanged drug in plasma is necessary.

The gas chromatographic method described in this paper takes advantage of the unique reaction of the amidoxime of PF-257 with heptafluorobutyric anhydride (HFBA) to yield the 5-*n*-heptafluoropropyl-1,2,4-oxadiazole derivative with good electron-capture properties.

This specific derivatization permits the selective and sensitive assay of unchanged PF-257 in plasma, as was illustrated by measuring plasma levels in rats receiving an intravenous or oral 10 mg/kg dose of PF-257.

EXPERIMENTAL

Chemicals and reagents

PF-257 (m.p. 161–168°) and PF-257 base (m.p. 162–162.5°) were synthesized in

this laboratory[1]. HFBA was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Ethyl acetate (analytical-reagent grade) was distilled before use. All other chemicals used were of analytical-reagent grade.

Glass equipment

All centrifuge tubes, pipettes and flasks were silanized with 5% dimethyldichlorosilane in pyridine overnight, then washed with methanol and dried at 100°.

Gas chromatography

A JEOL, Model JGC-20KE, gas chromatograph equipped with a 10-mCi nickel-63 electron-capture detector was used. A 200 cm × 2 mm I.D. glass column was packed with 2% OV-225 on Gas-Chrom Q (80–100 mesh). The column temperature was 144° and the injector and detector temperatures were 200°.

Preparation of the PF-257 derivative[4] 3-[(5-n-heptafluoropropyl-1,2,4-oxadiazol-3-yl)-methyl]-1,2-benzisoxazole (HOMB, Fig. 1)

One gram of PF-257 base was dissolved in 2 ml of HFBA and heated for 5 min at 80°. The reaction mixture was added to 30 ml of benzene, and shaken with 100 ml of 5% of aqueous ammonia solution and with 100 ml of 0.01 *N* hydrochloric acid. The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo*, and the residue was recrystallized from *n*-hexane to give 540 mg (28%) of HOMB (colourless needles, m.p. 45–47°). Calculated for $C_{13}H_6N_3O_2F_7$: C, 42.29; H, 1.64; N, 11.38; F, 36.02%. Found: C, 42.57; H, 1.74; N, 11.34; F, 36.24%.

Examination of derivatization conditions

Reaction conditions were studied using the following derivatization procedure. PF-257 base and γ-benzene hexachloride (γ-BHC) as an internal standard were dissolved in 0.1 ml of solvent in a glass-stoppered 10-ml centrifuge tube. Thirty microlitres of HFBA were added and the reaction mixtures were heated at various temperature for various periods. One millilitre of water and 0.2 ml of *n*-hexane were added, and the tube

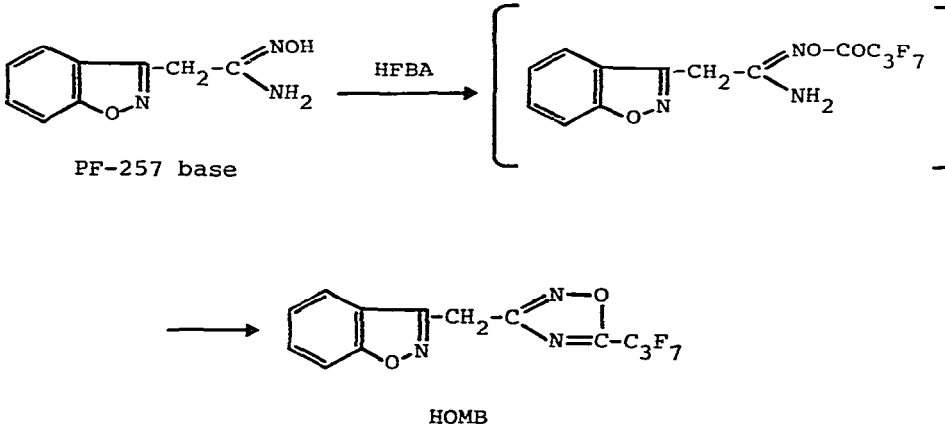

Fig. 1. Reaction of PF-257 with HFBA.

was shaken for 1 min. One millilitre of 5% aqueous ammonia solution was then added and the tube was shaken for 5 min to remove the excess of reagents[5]. After centrifugation, 5 μl of the organic phase were injected into the gas chromatograph.

The effects of solvents, catalysts and heptafluorobutyric acid on derivative formation were studied. Peak-height ratios of HOMB to the internal standard were calculated, and the percentage yields of HOMB were obtained from a calibration graph prepared from known amounts of synthetic HOMB and internal standard.

Assay procedure of PF-257 in plasma

To 1 ml of plasma sample were added 4 ml of 0.1 *M* carbonate buffer (pH 10) and 20 ml of ethyl acetate in a glass-stoppered 40-ml centrifuge tube. The tube was shaken for 15 min and centrifuged for 10 min, then 15 ml of the ethyl acetate layer were transferred into another glass-stoppered 50-ml centrifuge tube. The extraction was repeated with a further 15 ml of ethyl acetate.

The combined ethyl acetate extracts (30 ml) were shaken with 5 ml of 0.1 *N* hydrochloric acid for 10 min. After centrifugation, 4 ml of the aqueous layer were transferred into a glass-stoppered 15-ml centrifuge tube containing 0.5 ml of 0.5 *N* sodium hydroxide solution and 2 ml of 0.1 *M* carbonate buffer (pH 10). The tube was shaken with 3 ml of ethyl acetate for 15 min and centrifuged for 10 min. Two millilitres of the ethyl acetate layer were transferred into another glass-stoppered 10-ml centrifuge tube and the extraction was repeated twice more by adding 2 ml of ethyl acetate. The combined ethyl acetate extracts (6 ml) were evaporated to dryness at 50° under a gentle stream of air.

The residue was dissolved in 0.1 ml of ethyl acetate containing 5.5 ng of γ-BHC as an internal standard and 30 μl of HFBA. The reaction mixtures were allowed to stand at room temperature for 30 min, then 1 ml of water and 0.2 ml of *n*-hexane were added and the tube was shaken for 1 min. One millilitre of 5% aqueous ammonia solution was then added and the tube was shaken for 5 min. After centrifugation, 1–10 μl of organic phase were injected into the gas chromatograph.

Calibration graph

One millilitre each of control plasma samples containing 1–40 ng/ml of PF-257 was treated according to the assay procedure. Peak-height ratios of PF-257 to internal standard were measured and plotted against the amount of PF-257.

Animal experiments

Male Wistar rats weighing about 200 g were fasted for 16 h and anaesthetized with a 200 mg/kg intraperitoneal dose of sodium hexobarbital. The right carotid artery was canulated with a T-shaped polyethylene tube (1 mm O.D.) to form a loop for blood circulation. One end of the tube was clipped for drawing blood samples.

After canulation, PF-257 in a saline solution was administered intraveneously or orally at a dose of 10 mg/kg and about 0.3 ml of blood was drawn 5, 10, 15, 20, 30 and 60 min after intravenous dosing and 5, 10, 20, 30, 60 and 90 min after oral dosing. Blood samples were centrifuged on a Beckman/Spinco Model 152 Microfuge and plasma samples were frozen until analysis.

Aliquots of plasma samples containing 1–40 ng of PF-257 were diluted with the control plasma to a volume of 1 ml and analysed according to the assay procedure.

TABLE I

EFFECT OF SOLVENT ON REACTION OF PF-257 WITH HFBA

Solvent	*Yield (%) (Mean ± S.D.)*
n-Hexane	22.8 ± 6.0
Benzene	24.4 ± 10.5
Benzene + triethylamine (50 μl)	12.9 ± 5.3
Ethyl acetate	65.3 ± 2.2
Ethyl acetate + triethylamine (50 μl)	33.8 ± 2.8
Ethyl acetate + pyridine (50 μl)	56.1 ± 3.3
Ethyl acetate + heptafluorobutyric acid (30 μl)	38.1 ± 1.6
HFBA	32.6 ± 15.6

RESULTS AND DISCUSSION

Reaction conditions

Amidoximes can be acylated readily at room temperature by acid chlorides or anhydrides, and O-acylated amidoximes are easily dehydrated on heating to form the corresponding 3,5-disubstituted 1,2,4-oxadiazole[6]. With PF-257 and HFBA, O-heptafluorobutylated PF-257 was not obtained but a direct cyclization product, HOMB, was obtained (Fig. 1). This unique reaction product of amidoxime was found to have good electron-capture properties, leading to the selective and quantitative determination of unchanged PF-257 in plasma.

For the gas chromatographic determination, conditions for the acylation of PF-257 were examined. *n*-Hexane, benzene, ethyl acetate and the reagent HFBA itself were used as solvents, and pyridine and triethylamine as catalysts. The yields of HOMB varied with the solvent used, as shown in Table I. In ethyl acetate, the reaction proceeded with a reproducible yield of 65.3 ± 2.2% ($n = 10$). It is likely that the heptafluorobutyric acid formed during the reaction would inhibit the reaction, but addition of a catalyst (*e.g.*, pyridine or triethylamine) to neutralize the acid[5] did not increase the yield.

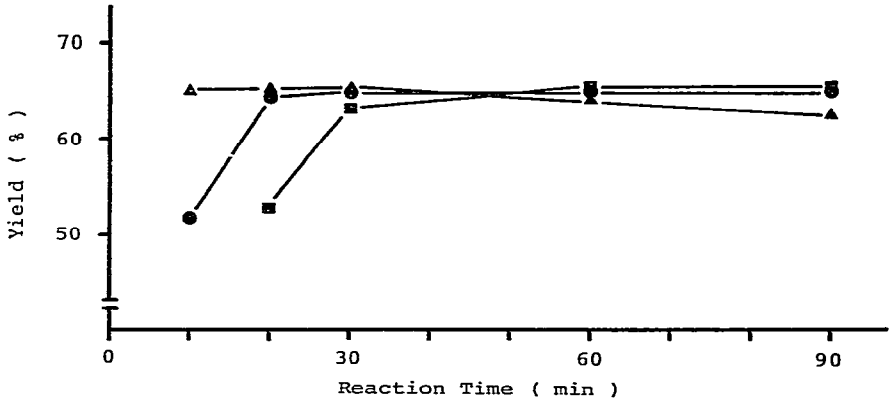

Fig. 2. Time course and yield of the reaction of PF-257 with HFBA in ethyl acetate. Reaction temperature: ▲, 40°; ●, room temperature; ■, 0°.

The time course and the yield of the reaction of PF-257 with HFBA in ethyl acetate are shown in Fig. 2. The reaction at 40° proceeded very rapidly but some decomposition seemed to occur after 30 min. At room temperature the yield was constant on reacting for 20–90 min, and at 0° the rate of reaction was slow. The formation of HOMB was not affected by the amount of HFBA. Therefore, the reaction conditions as described in the assay procedure were adopted.

When the reaction was performed in the unsilanized glass tube, the yield of HOMB was not constant and some adsorption on glass surface was observed after several hours.

Gas chromatographic properties of HOMB

HOMB had excellent gas chromatographic properties and gave symmetrical peaks when OV-17, XE-60, OV-225 and SP-400 were used as stationary phases. HOMB and the plasma blanks were perfectly separated on OV-225 (Fig. 3).

Combined gas chromatography-mass spectometry of HOMB was carried out with a Hitachi RMU-6LG mass spectrometer, and the results are shown in Fig. 4. The spectrum obtained was identical with that of HOMB obtained by the direct inlet method. Thermal decomposition of HOMB did not occur under the gas chromatographic conditions adopted.

HOMB was too volatile under a stream of air or nitrogen and *in vacuo* to concentrate the reaction media after derivatization; on the contrary, any losses of PF-257 were not observed in the evaporation process. Therefore, concentration of the reaction mixture was performed prior to derivatization, and the excess of HFBA after derivatization was removed according to the procedure reported by Walle and Ehrsson[5] and as described under *Examination of derivatization conditions.*

HOMB elicits a very high response on an electron-capture detector. The mini-

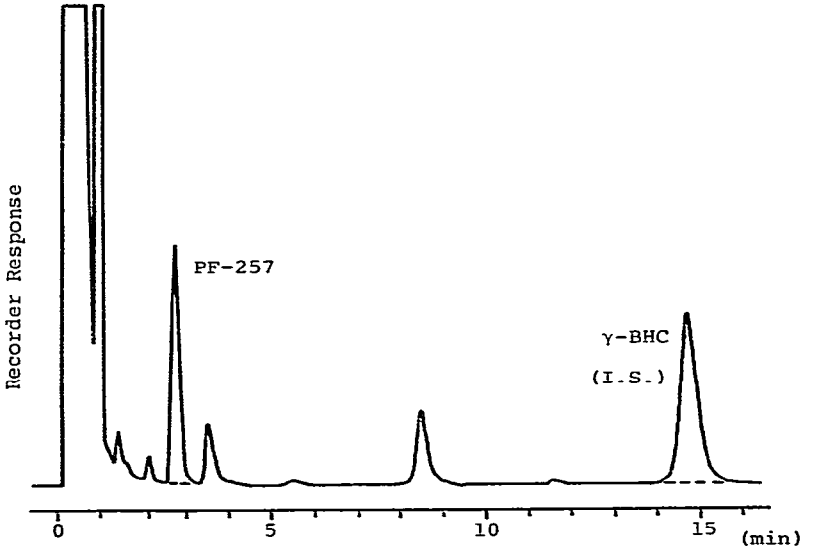

Fig. 3. Typical chromatogram of PF-257 in plasma (29.5 ng/ml). Broken lines represent the background from control plasma. Small peaks not assigned are due to plasma and reagent.

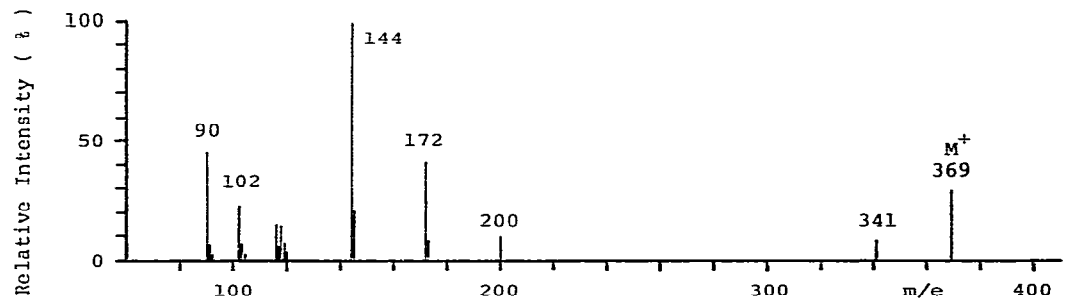

Fig. 4. Gaschromatographic–mass spectrometric spectrum of HOMB. Ionization voltage, 70 eV; chamber temperature, 200°; column, 2% OV-225, 1 m, 130°; carrier gas, helium, flow-rate 40 ml/min.

mum detectable amount[8], defined as the amount that gives a signal three times the background noise level, was $1.4 \cdot 10^{-16}$ mole/sec, corresponding to 1 pg under the gas chromatographic conditions used.

Internal standard

PF-257 derivatives such as 5-fluoro- or 5-chloro-1,2-benzisoxazole-3-acetamidoxime were examined. However, all of the peaks of their HFBA derivaties overlapped with those of the plasma and reagent blanks on gas chromatograms. Therefore, γ-BHC was used as an internal standard, as shown in Fig. 3.

Calibration graph

The caliblation graph obtained with 1–40 ng of PF-257 in 1 ml of plasma is shown in Fig. 5. The graph was a straight line over this 40-fold concentration range

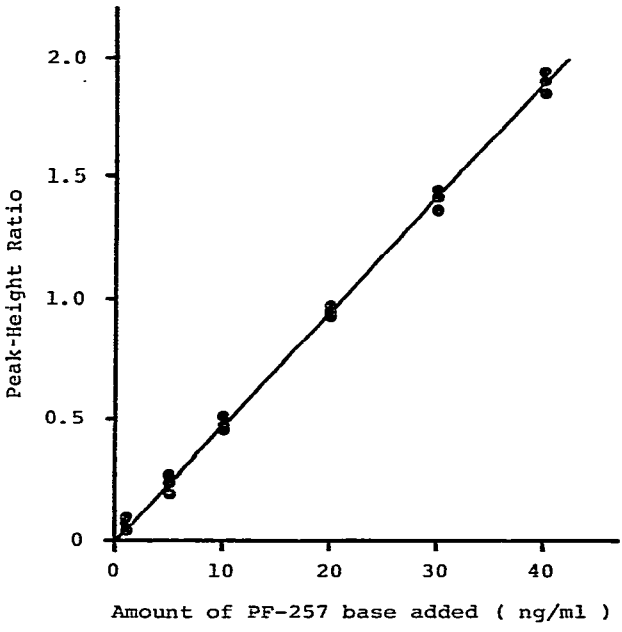

Fig. 5. Calibration graph for PF-257 in plasma.

and passed through the origin. The precision of the method was 6.8% (relative standard deviation) and the recovery from plasma was 77.5 ± 5.2%. The minimum detectable concentration was 0.1 ng/ml in plasma.

When the concentration of PF-257 in plasma exceeded 40 ng/ml, electron-capture detector did not respond linearly to the concentration. For accurate determinations, plasma samples containing over 40 ng/ml of PF-257 should therefore be diluted with the control plasma as described under *Animal experiments*.

Determination of PF-257 in rat plasma

Plasma levels of PF-257 following intravenous or oral administration at a dose of 10 mg/kg are shown in Fig. 6. Unchanged drug levels in orally treated rats were maximal 5 min after dosing, followed by a two-stage decrease with a second elimination half-life of *ca.* 20 min (elimination phase; 20–40 min post-dosing), suggesting very rapid absorption and elimination of PF-257.

Plasma levels in intravenously treated rats showed a similar two-stage decrease with *ca.*3–2-fold higher levels up to 60 min post-dosing.

The gas chromatographic method described would be sensitive and specific enough for the determination of unchanged drug in plasma following a low dose of PF-257 (possibly less than 1 mg/kg) and would therefore permit pharmacokinetic studies of PF-257 in man and experimental animals.

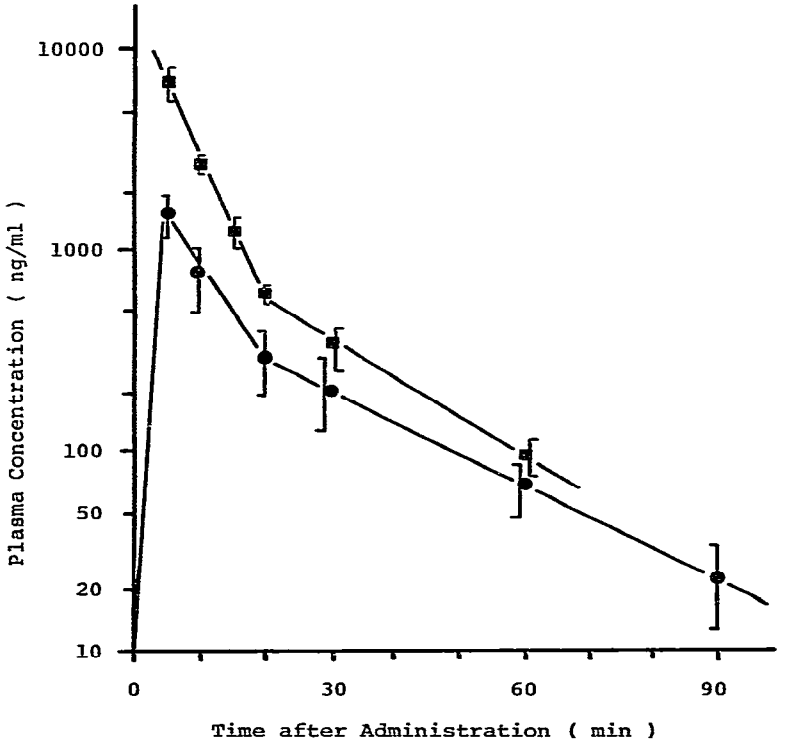

Fig. 6. Plasma levels of PF-257 in rats following oral (●, 4 animals) and intravenous (■, 3 animals) administrations at a dose of 10 mg/kg of PF-257. Values are means ± S.E.

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