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DETERMINATION OF PLASMA PROTEIN BINDING OF PROPAFENONE IN RATS, DOGS AND HUMANS BY HIGHLY SENSITIVE GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A highly sensitive method for the determination of propafenone in plasma has been established using gas chromatography—mass spectrometry with the deuterium-labelled compound as an internal standard. Plasma levels as low as 1 ng/ml were measured using 0.5-ml plasma samples. Plasma protein binding of the drug in rats, dogs and humans in vitro and in vivo was determined by the proposed method. About 90% of the drug was bound to the plasma protein in all species in vitro (20—500 ng/ml) and 69—88% in rats, 90—95% in dogs and 77—89% in humans after oral administration of the drug at doses of 50 mg/kg, 5 mg/kg and 200 mg per person, respectively.

INTRODUCTION

Propafenone hydrochloride is an antiarrhythmic drug with a structure common to β-blocking agents. It has been reported that the drug is effective in suppressing supraventricular and ventricular arrhythmias [1, 2].

Many methods have been reported for the determination of β-blocking agents in biological fluids using fluorimetry, high-performance liquid chromatography (HPLC), gas chromatography with electron-capture detection (GC—ECD), gas chromatography—mass spectrometry (GC—MS) and radioimmunoassay methods [3, 4].

For the determination of propafenone in plasma, HPLC [5, 6] and GC—ECD methods [7] have been reported. These methods had sufficient sensitivity (5—10 ng/ml) for the determination of the drug in plasma after administration of therapeutic dosages to humans. However, the values represent the sum of the

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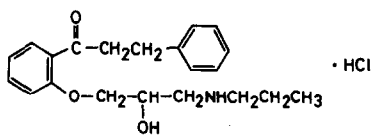
drug unbound and bound to the plasma protein. To analyse the pharmacokinetics of the drug in more detail, it is necessary to determine the binding of the drug to the plasma protein. In our preliminary experiments, the drug was bound strongly to the plasma protein, so that the unbound drug concentration in plasma was considered to be very low.

Therefore, in this study, a more sensitive method has been established using GC—MS, and the protein binding in vitro and in vivo were determined in rats, dogs and humans.

EXPERIMENTAL

Chemicals

Propafenone hydrochloride (Fig. 1) was supplied by Helopharm W (Berlin, F.R.G.). Deuterium-labelled propafenone hydrochloride (Fig. 1) was prepared by the previously reported method [8], except that epichlorohydrin labelled with five deuterium atoms (d_5-epichlorohydrin) (MSD Isotopes, Montreal, Canada) was substituted for unlabelled epichlorohydrin, and the reaction of the d_5-epichlorohydrin was conducted at 100°C for 40 min in dimethylformamide (DMF)—dioxane (1:1). The chemical and isotopic purity of the d_5-propafenone hydrochloride, which was checked by thin-layer chromatography and mass and nuclear magnetic resonance spectrometry, was > 99%. Other chemicals and reagents were obtained commercially and were of analytical-reagent grade.

(a) 

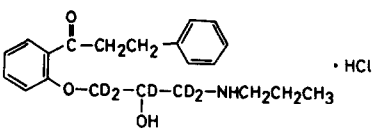
(b) 

Fig. 1. Structures of (a) propafenone and (b) d_5-propafenone hydrochloride.

Gas chromatography—mass spectrometry

A JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) and a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) were used. Separations were carried out on a glass column (1.8 m × 1.8 mm I.D.) packed with 3% OV-22 on Chromosorb W (80—100 mesh). The column temperature was maintained isothermally at 260°C. The injector, separator and ion-source temperatures were 300°C, 250°C and 200°C, respectively. The flow-rate of the carrier gas (helium) was 30 ml/min. The ionization potential and emission current were 70 eV and 300 μA, respectively. The multiplier voltage supply was set at 1.6—2.2 kV.

Determination of unchanged drug in plasma

To each plasma sample (0.1—0.5 ml), d_5-propafenone hydrochloride (30—100 ng) was added as an internal standard (I.S.). After addition of 0.5 ml

of 2 *M* sodium hydroxide solution, the mixture (pH > 12) was extracted with benzene (2 × 2 ml). The benzene layer was evaporated to dryness under reduced pressure and pentafluoropropionic anhydride (PFPA) (Pierce, Rockford, IL, U.S.A.) (50 μl) and ethyl acetate (50 μl) were added to the residue. The mixture was kept at room temperature for 10 min and then evaporated to dryness under a stream of nitrogen. Aliquots (2—5 μl) of the solution were injected into the column of the GC—MS system. Fragment ions at *m/z* 408 and 413 were used to monitor the pentafluoropropionic (PFP) derivatives of propafenone and the I.S., respectively. The peak height ratio was used to calculate the amount of propafenone in each sample by referring to a calibration graph.

Extraction recoveries

Control plasma (0.5 ml) containing propafenone hydrochloride (15 or 150 ng) was carried through the above procedure without addition of I.S. The I.S., dissolved in benzene (15 or 150 ng), was added to the benzene solution, extracted and the benzene solution was evaporated to dryness under reduced pressure. The subsequent procedure was carried out as described above. Recoveries were calculated by comparing the peak height ratios with those obtained when the drug and I.S., dissolved in benzene, were processed without the extraction procedure.

Determination of plasma protein binding

Plasma protein precipitation. The plasma protein was precipitated by ultracentrifugation (Beckman L 8-70) at 170 000 *g* for 18 h at 4°C. Protein in the supernatant was determined by the Lowry method [9] and the ratio of the precipitated protein was calculated.

In vitro study of plasma protein binding. The drug solution (0.8—20 μg/ml) was prepared in 0.01 *M* phosphate buffer (pH 7.4) and 0.05 ml of this solution was added to 1.95 ml of rat, dog or human plasma to prepare plasma samples (20—500 ng/ml). The plasma samples were incubated at 37°C for 1 h, then centrifuged at 170 000 *g* for 18 h at 4°C. The drug concentration in the supernatant was determined by the GC—MS method as described above. The drug bound to plasma protein (*B*) was calculated according to the equation

$$B\,(\%) = \frac{A - C}{A} \cdot 100$$

where *A* and *C* are the total drug concentration in plasma and the drug concentration in the supernatant after centrifugation, respectively.

In vivo study of plasma protein binding. Male Sprague—Dawley rats (170—200 g) and male beagle dogs (11—14 kg) were used. They were maintained with free access to food and water and the drug was administered orally in aqueous solution (50 mg/kg in rats and 5 mg/kg in dogs) after overnight fasting. Blood samples were obtained from the inferior cava in rats and the antecubital vein in dogs with a heparinized syringe. Plasma samples were stored at −20°C until required for assay.

We were requested to determine the plasma concentration in healthy male volunteers (52—82 kg, age 30—41 years) who had been administered

propafenone hydrochloride in gelatin capsules (200 mg) by clinicians [10]. Samples from these subjects were used for human studies.

The total drug concentration in plasma and the unbound concentration in plasma were determined by the method used for the in vitro study.

RESULTS AND DISCUSSION

Because of the low plasma concentration of β-blocking agents, many sensitive methods have been developed [3, 4]. Among them, the GC method currently in general use is based on the method used for the determination of propranolol [11], the first used β-blocker in therapy. Propranolol [11],

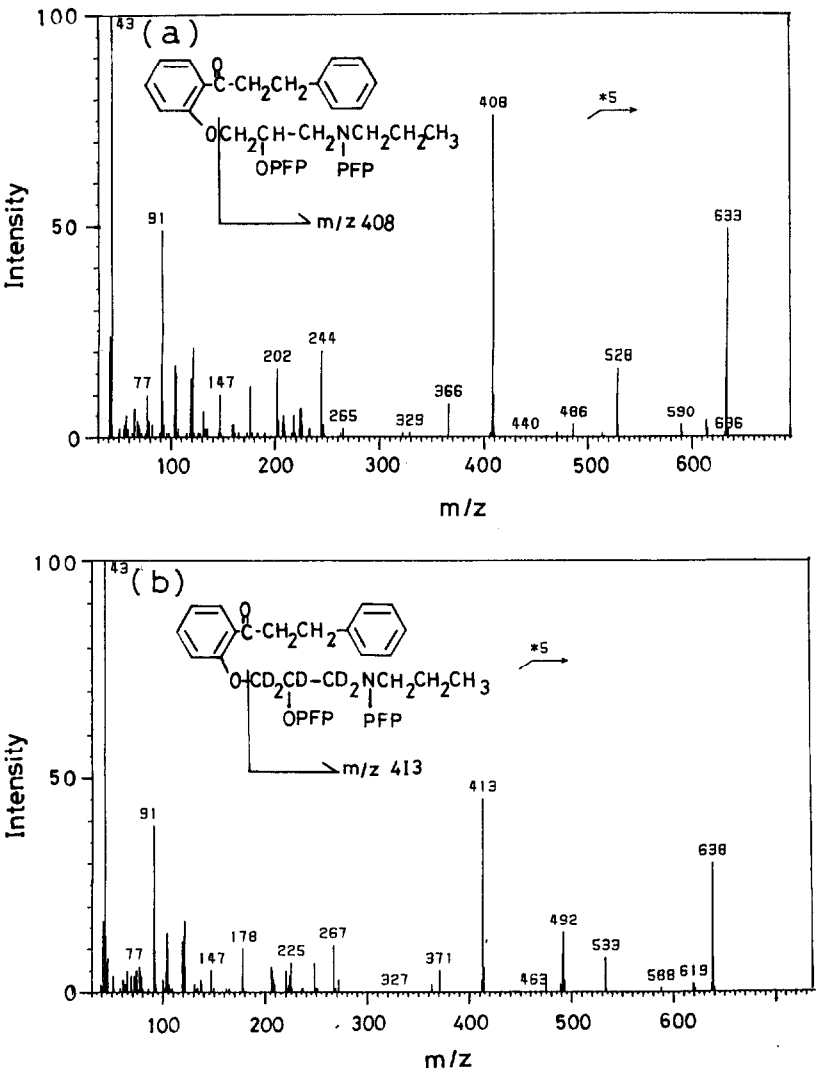


Fig. 2. Mass spectra of PFP derivatives of (a) propafenone and (b) d_5-propafenone.

alprenolol [11], metoprolol [12] and oxprenolol [11—13] were determined as the trifluoroacetyl (TFA) derivatives and timolol [14] and atenolol [15] as the heptafluorobutyric (HFB) derivatives. In the GC—ECD method used for the determination of propafenone [7], the drug was determined as the TFA derivative. The reaction of propafenone with TFAA was completed at 45°C after 45 min [7]. In the present method, we used PFPA instead of TFAA, because propafenone was completely derivatized with PFPA within 10 min at room temperature.

As shown in Fig. 2, the mass spectrum of the PFP derivative showed the molecular ion at *m/z* 633, indicating that the two PFP groups were introduced into the hydroxy and amino moieties in the structure. Another intense peak (base peak) was observed at *m/z* 408, which was used to monitor the drug. This peak shows the fragment ion caused by the cleavage of the side-chain, as shown in Fig. 2.

These derivatizations and fragmentations were similar to those of β-blocking agents with a common structure. For propranolol, two TFA groups were introduced into the hydroxy and amino moieties of the structure, showing the molecular ion at *m/z* 451, and the base peak at *m/z* 308 showed the fragment ion caused by the cleavage of the side-chain [16].

In this study, the deuterium-labelled compound was synthesized and used as an internal standard. Because the mass spectrum of the PFP derivative of propafenone showed an intense fragment ion at *m/s* 408, the deuterium atoms were introduced into this fragment (Fig. 2). The labelled propafenone was

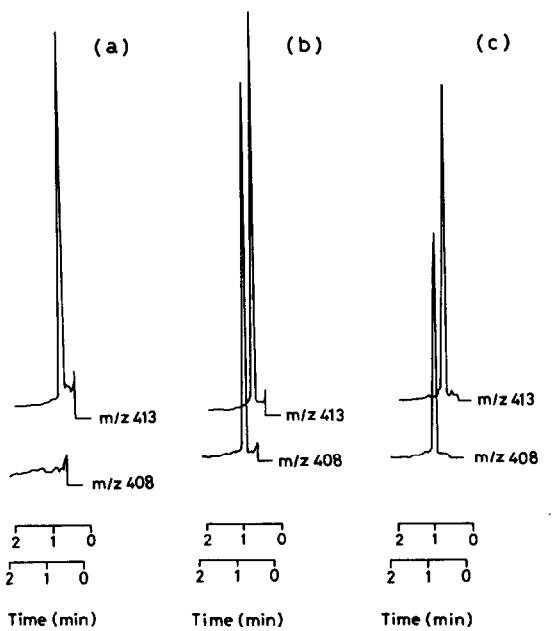


Fig. 3. Chromatograms obtained from human plasma samples. (a) Control plasma to which I.S. (100 ng/ml) was added; (b) control plasma to which propafenone · HCl (100 ng/ml) and the I.S. (100 ng/ml) were added; (c) plasma obtained 1 h after oral administration of propafenone · HCl (200 mg) to a human.

prepared by the procedure reported previously [8], except that d_5-epichlorohydrin was used instead of unlabelled epichlorohydrin. In the reported synthetic method, a large amount of epichlorohydrin was used both as a reagent and as a solvent. However, the amount of the labelled compound used was limited. Therefore, we modified the conditions of the reaction with d_5-epichlorohydrin. The reaction gave a high yield after 1 h at 100°C in DMF—dioxane (1:1).

The chromatograms of propafenone and I.S. extracted from plasma are shown in Fig. 3. The drug-free control plasma gave no interfering peaks.

A calibration graph obtained by the above procedure showed a linear response in the range 1—1000 ng/ml. The extraction recoveries for 30 and 300 ng/ml were 78.4 ± 0.8% (n = 6, mean ± standard error) and 84.4 ± 1.2% (n = 6), respectively. In this study, benzene was used as the extraction solvent, because endogenous substances that interfere in the assay were removed using this solvent, although the extraction recovery was lower than those obtained with other polar solvents.

The limit of determination, which was 1 ng/ml using 0.5-ml plasma samples, was five to ten times higher than those of the previous HPLC and GC—MS methods [4—6].

Plasma protein binding of the drug in vitro and in vivo were determined in rats, dogs and humans. Plasma protein precipitation was conducted by ultracentrifugation. To confirm the precipitation of plasma protein under the ultracentrifugation conditions used, the protein in the supernatant was determined by the Lowry method [9]. The protein was completely precipitated by centrifugation at 170 000 g for 18 h at 4°C (99.5 ± 0.03%; n = 4).

TABLE I

PLASMA PROTEIN BINDING OF PROPAFENONE IN VITRO

Propafenone · HCl concentration (ng/ml)	Propafenone · HCl bound* (%)		
	Rat	Dog	Human
20	—	92.4 ± 2.0	—
100	91.4 ± 0.6	91.4 ± 1.7	92.2 ± 0.7
500	90.5 ± 3.0	90.9 ± 0.9	91.1 ± 0.8

*Results are means ± standard errors (rat and dog, n = 4; human, n = 5).

The concentration of the drug in protein-free plasma was determined by the proposed method. The in vitro results are shown in Table I. The drug bound to plasma protein was about 90% in all species over the concentration range examined. In this study, the control plasma containing the drug was incubated at physiological temperature (37°C). However, we had to carry out the centrifugation at a lower temperature in order to prevent an increase in temperature caused by ultracentrifugation for a long period (18 h). Therefore, the protein binding determined in this study might be different from that under physiological conditions.

The in vivo results are shown in Table II. The drug was administered orally

TABLE II

TOTAL AND FREE PLASMA CONCENTRATIONS AND PROPAFENONE BOUND TO PLASMA PROTEIN AFTER ORAL ADMINISTRATION OF PROPAFENONE TO RATS, DOGS AND HUMANS

The drug was administered orally to rats (50 mg/kg, n = 4), dogs (5 mg/kg, n = 3) and humans (200 mg, n = 3). The results are means ± standard errors.

Time (h)	Total plasma concentration of propafenone · HCl (ng/ml)			Free plasma concentration of propafenone · HCl (ng/ml)			Propafenone · HCl bound (%)		
	Rat	Dog	Human	Rat	Dog	Human	Rat	Dog	Human
0.5	662 ± 88	520 ± 214	51 ± 13	200 ± 34	23 ± 8	9 ± 1	68.9 ± 4.9	95.3 ± 1.2	80.6 ± 4.7
1	391 ± 54	283 ± 99	199 ± 37	69 ± 7	14 ± 5	31 ± 5	80.6 ± 4.2	95.2 ± 0.7	84.6 ± 0.3
2	304 ± 126	80 ± 22	123 ± 40	34 ± 10	4 ± 1	15 ± 8	87.5 ± 1.7	94.3 ± 1.4	88.7 ± 2.7
4	71 ± 6	15 ± 4	56 ± 12	10 ± 1	0.7 ± 0.2	11 ± 2	85.9 ± 1.6	94.7 ± 1.2	80.8 ± 0.6
6	20 ± 15	3 ± 3	29 ± 10	3 ± 2	0.3 ± 0.3	4 ± 2	86.0 (n=2)	90.1 (n=1)	76.5 (n=2)

to rats (50 mg/kg), dogs (5 mg/kg) and humans (200 mg). Although these doses differed among the species, the total drug concentration levels were similar (Fig. 2). The drug bound to the plasma protein was 69—88% in rats, 94—95% in dogs and 76—89% in humans. The values in rats and in humans were lower than those in vitro, whereas in dogs there was no difference between the in vitro and in vivo values.

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