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Note

A sensitive assay method for pimozide in human plasma by high-performance liquid chromatography with fluorescence detection

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Pimozide (Orap®) is a highly potent, long-acting neuroleptic, belonging to the diphenylbutylamine series [1]. The drug is administered orally to psychiatric patients in daily doses of 2—8 mg [2]. Since plasma levels are expected to be very low, a highly sensitive assay method was required.

Recently immunological assay [3] and a high-performance liquid chromatographic (HPLC) method with UV detection [4] were reported. However, the latter method was not sensitive enough for use with human samples and the former involved complicated protein-conjugation steps for antibody preparation.

This paper describes a rapid, sensitive and selective HPLC—fluorescence method for the determination of pimozide in human plasma. The method was used to determine plasma concentrations in pimozide in patients given a single oral dose of 3 mg.

EXPERIMENTAL

Chemicals and reagents

Pimozide (I) and the internal standard (II) (Fig. 1) were synthesized at the Research Laboratories of Fujisawa Pharmaceutical Co. Ltd. The internal standard was obtained by the methylation of pimozide with sodium hydride and methyl iodide and purified by HPLC under analytical conditions.

All inorganic reagents were analytical grade. Aqueous solutions were prepared with deionized water purified by a Millipore Milli-Q® water purification system.

n-Hexane and isoamyl alcohol were analytical grade and used without further purification. Acetonitrile (chromatographic grade) was purchased from Katayama Chemical Industries Ltd., Osaka, Japan.

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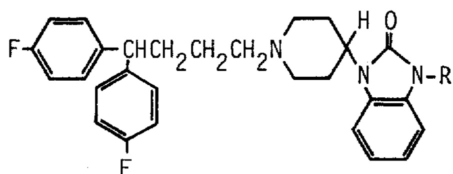

R=H : Pimozide

R=CH_3: Internal standard

Fig. 1. Chemical structure of pimozide and internal standard.

Apparatus

An HPLC system, including a Model 6000A pump and a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model FS-970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.), was used.

A Varian Model 5000 liquid chromatograph equipped with a Valco loop injector and a Model UV-5 fixed-wavelength detector (Varian, Walnut Creek, CA, U.S.A.) was used for UV detection.

Chromatographic conditions

A 15 cm × 4 mm I.D. stainless-steel column was packed with TSK-GEL LS-410 ODS SIL (particle size 5 μm, Toyo Soda Industries Co. Ltd., Tokyo, Japan) for the analytical column and a 1 cm × 4 mm I.D. stainless-steel column, packed with the same material, was used for the guard column.

Chromatography was performed in a reversed-phase mode using a mobile phase of 48% (v/v) acetonitrile in 20 m*M* KH_2PO_4 adjusted to pH 2.5 with 20 m*M* phosphoric acid. The operation temperature was ambient and the flow-rate was 1.0 ml/min.

The column eluate was monitored by fluorescence detection with excitation at 210 nm and emission above 320 nm or by UV at 280 nm (0.005 a.u.f.s.). The fluorescence detector range was 0.2 μA full scale and the time constant value was 6 sec.

Extraction procedure

To 1.0 ml of plasma in a round-bottomed glass tube, 0.1 ml of the internal standard solution (containing 20 ng of internal standard), 1.0 ml of 1 *N* sodium hydroxide and 5 ml of *n*-hexane—isoamyl alcohol (98:2, v/v) were added and the mixture was shaken reciprocally for 10 min. After centrifugation at 1900 *g* for 5 min, the organic layer was transferred to a conical-bottomed glass tube for re-extraction into 0.1 or 0.2 ml of aqueous acid. After the mixture was shaken and centrifuged, the organic layer was aspirated off and discarded. Almost all of the aqueous layer was injected into the high-performance liquid chromatograph.

All glass tubes were silanized before use.

Standard solution

Stock solutions of pimozide and the internal standard were prepared by dissolving 10 mg of material in 100 ml of 1% phosphoric acid. The standard

solutions for the calibration curve were made by diluting the stock solutions with 1% phosphoric acid. These solutions were stored at 4°C until analyzed.

Human study

Plasma samples were obtained from three male in-patients (age 28—37 years, body weight 61—82 kg) at 1, 4 and 24 h after oral dosing with a 3-mg tablet of pimozide. Blank plasma was obtained from healthy volunteers. The samples were stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Recovery

The effect of kind and volume of inorganic acid for back-extraction was investigated. The results are given in Table I. The recoveries at the 100 ng/ml level of pimozide and the internal standard were, respectively, 62.3% and 21.7% when 0.1 ml of hydrochloric acid was used. The recovery became poorer as the concentration of hydrochloric acid was increased. However, in the case of phosphoric and sulfuric acid, the recoveries were better than 80%. The recovery was also improved by increasing the volume of acid to 0.2 ml.

TABLE I

EXTRACTION RECOVERY OF PIMOZIDE AND INTERNAL STANDARD FROM SPIKED *n*-HEXANE—ISOAMYL ALCOHOL (98:2)

A 5-ml volume of *n*-hexane—isoamyl alcohol, containing 100 ng/ml pimozide and internal standard (I.S.), was extracted with inorganic acid. Aqueous layers were analyzed by HPLC.

Extraction solvent	Volume (μl)	Recovery (%)	
		Pimozide	I.S.
0.1 *M* HCl	100	62.3	21.7
	200	79.2	34.3
0.2 *M* HCl	100	47.2	10.4
	200	66.0	20.7
0.5 *M* HCl	100	44.2	10.7
	200	59.8	17.4
0.1 *M* H_3PO_4	100	81.8	59.9
	200	90.1	69.8
0.2 *M* H_2SO_4	100	84.1	67.3
	200	93.6	80.1

The poor recovery of the internal standard compared with pimozide, especially with hydrochloric acid, may be due to its high lipophilic character. When phosphoric acid or sulfuric acid was used as the solvent for back-extraction, however, the ratios of pimozide against the internal standard were almost constant.

From these results and on account of column life, phosphoric acid was used for back-extraction.

Detector study

First, a UV detection method was investigated. The maximum detection sensitivity was 215 nm; however, many of the background peaks could not be separated from the peak of pimozide. At 280 nm, the maximum absorbance wavelength of pimozide, no interference peak was found at retention times of pimozide or internal standard.

The lower limit of detection was 5 ng/ml for this method. After a single oral dose of 4 mg of radioactive pimozide to humans, plasma levels of pimozide were found to be lower than 2 ng/ml [5].

Consequently, a more sensitive assay method was required and a fluorescence detection method was examined. Since pimozide has no UV absorption

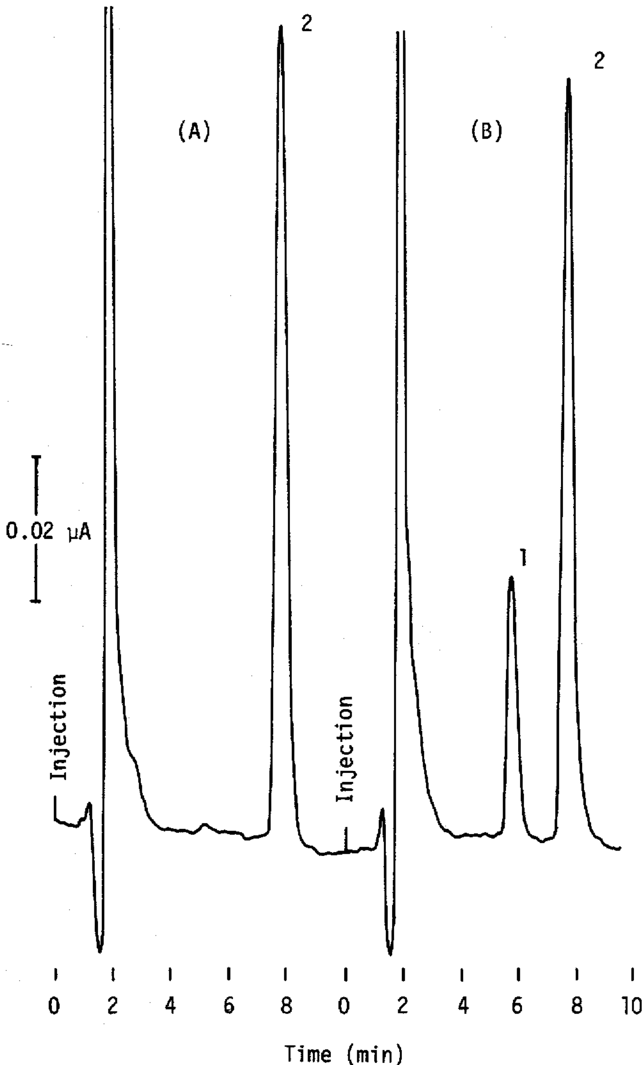

Fig. 2. Chromatograms of (A) blank plasma treated according to the method described under Experimental, and (B) blank plasma sample spiked with 5 ng/ml pimozide. Peaks: 1 = pimozide, 2 = internal standard.

above 300 nm, a variable-wavelength fluorescence detector, equipped with a deuterium lamp (FS-970), was used. The lower limit of detection by this method could be lowered to 0.3 ng/ml.

Chromatograms

Fig. 2 shows chromatograms of pimozide and the internal standard from spiked human plasma. As shown in Fig. 2, there is no background peak at the retention time of pimozide. The retention times of pimozide and internal standard were 6 min and 8 min, respectively.

Calibration curve

The calibration curve for human plasma level of pimozide was obtained as follows. Samples of blank plasma were spiked with pimozide at concentrations of 0.3, 0.5, 1, 2, and 5 ng/ml and the internal standard at a fixed concentration of 20 ng/ml.

The samples were taken through the extraction procedure described under Experimental and were injected into the liquid chromatograph. The ratio of peak height of pimozide to the internal standard was calculated for each chromatogram. A linear regression analysis of these data at the five concentrations of pimozide gave the slope, intercept and correlation coefficient for the human plasma calibration curve. The equation of the curve was $Y = 0.0687X + 0.0004$, and the correlation coefficient was 0.9998.

The lower limit of detection was 0.3 ng/ml at a signal-to-noise ratio of 3 and a sample volume of 1.0 ml.

Reproducibility

Reproducibility was obtained by adding known amounts of pimozide to the plasma and comparing the five samples with a single calibration curve. The results are given in Table II. The coefficients of variation were 4.4% and 2.8% at concentrations of 0.3 ng/ml and 2 ng/ml, respectively. Even at a concentration at the lower limit of detection, good accuracy and precision were obtained.

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF PIMOZIDE IN HUMAN PLASMA

$n = 5$.

Added (ng/ml)	Found (ng/ml) (mean ± S.D.)	C.V. (%)
0.3	0.32 ± 0.01	4.4
2.0	1.86 ± 0.05	2.8

Concentration of pimozide in human plasma

Chromatograms obtained from the plasma of subject A.K. are shown in Fig. 3. Generally, a psychotic patient requires several drugs (i.e. haloperidol, chlorpromazine, etc.) in combination. This subject (A.K.) was also given

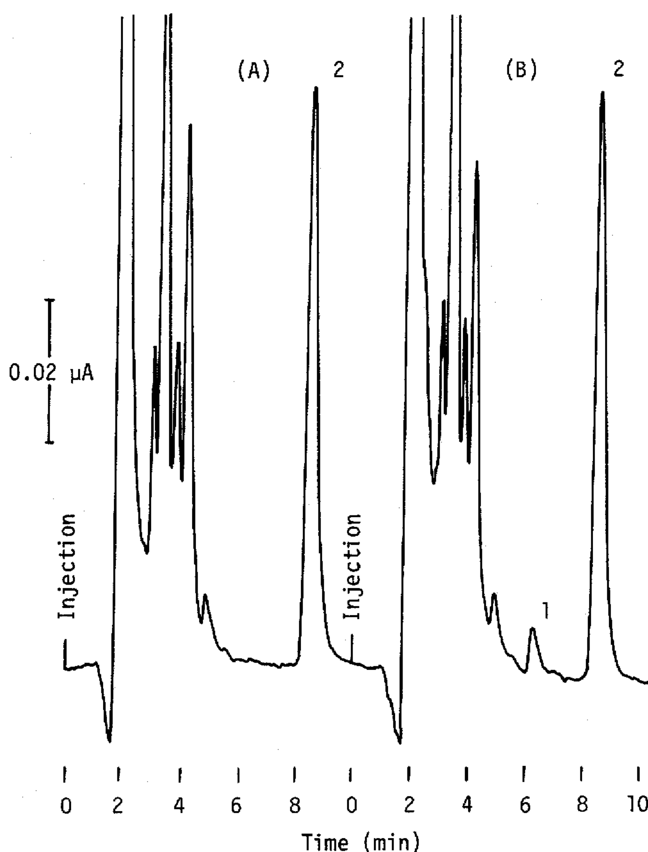

Fig. 3. Chromatograms of clinical plasma extracts: (A) before dosing; (B) 4 h after dosing (containing 0.9 ng/ml pimozide). Peaks: 1 = pimozide, 2 = internal standard. Plasma extracts were obtained from subject A.K. who was given a 3-mg tablet of pimozide in combination with haloperidol, sulpiride, levomepromazine, thiothixene and thioridazine.

TABLE III

PLASMA LEVELS OF PIMOZIDE AFTER A SINGLE ORAL DOSE OF A 3-mg TABLET

Time after dose (h)	Subject			Mean ± S.E.
	K.H.	O.K.	A.K.	
1	n.d.*	0.5	n.d.*	0.2 ± 0.2
4	0.9	3.3	0.9	1.7 ± 0.8
24	1.7	2.2	2.6	2.1 ± 0.2

*n.d. = not detected.

haloperidol, sulpiride, levomepromazine, thiothixene and thioridazine daily; however, no interfering peak was found in the chromatogram obtained from the plasma before dosing with pimozide (Fig. 3A). It can be considered that the differences in extraction recovery, sensitivity to fluorescence detection and plasma levels between other drugs and pimozide cause no interference.

These results, therefore, show that the present method is suitable for monitoring drug levels of pimozide in patients.

After a single oral dose of a 3-mg tablet of pimozide, plasma concentrations were below 3.3 ng/ml at all times of observation. Even at 24 h after dosing, a level of 1.7—2.6 ng/ml of pimozide was found.

McCreadie et al. [6] reported plasma levels of pimozide after a single 24-mg dose by the immunological assay method. They found 15 ng/ml of pimozide at 24 h after dosing. The present results agree with McCreadie's findings on the long-acting character of pimozide.

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