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Sensitive assay for pimozide in human plasma using high-performance liquid chromatography with fluorescence detection: application to pharmacokinetic studies

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Abstract

A method is described for the measurement of pimozide in human plasma using HPLC with fluorescence detection. The method is specific and sensitive in the range of concentrations seen in human plasma after conventional dosing (1–15 ng/ml) with a limit of quantification of 1 ng/ml. The calibration curves are linear for concentrations between 1 and 50 ng/ml. Within-day and inter-day coefficients of variation are less than 7.4% and 15.5%, respectively, at three concentrations of pimozide (2, 10 and 20 ng/ml). Intra-day and inter-day bias are less than 18.5% and 12.5%, respectively. A pharmacokinetic study conducted in a healthy volunteer administered 6 mg of pimozide orally demonstrates the utility of this method.

Keywords: Pimozide

1. Introduction

Pimozide (1-[1-[4,4-Bis(*p*-fluorophenyl)butyl]-4-piperidyl]-2-benzimidazolinone) is a highly potent, long-acting and specific neuroleptic drug belonging to the diphenylpiperidine group [1]. Currently pimozide is the only alternative to haloperidol approved by the United States Food and Drug Administration (FDA) for the treatment of Tourettes disorder. It has been used extensively in Europe for the treatment of schizophrenia [2]. The use of pimozide is limited by cardiotoxic and neurologic side effects that force physicians to dose patients very carefully. No quantitative relationship between plasma concentrations of pimozide and efficacy or adverse reactions has been described. An important step in any effort to delineate such relationships is the confident determination of concentrations of pimozide in human plasma.

An HPLC assay method for pimozide was described by Miyao et al. [3], but its utility depends on the availability of a synthetic internal standard. A radioimmunoassay was described by Michiels et al. [4] reporting a high selectivity of pimozide over its metabolites [5], but possible cross-selectivity with other neuroleptic drugs. This cross-selectivity reduces the ability of radioimmunoassay to specifically determine pimozide plasma concentrations in pa-

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tients on multi-drug regimens. Pimozide is a highly potent and extensively metabolized neuroleptic that is routinely administered orally to psychiatric patients in a daily doses of 2 to 20 mg. Plasma concentrations resulting from this regimen are in the range of 1 to 15 ng/ml [6]. In order to measure pimozide in human plasma at clinically relevant concentrations, we set out to develop a widely useable selective and sensitive assay using a HPLC system with fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

Pure reference standards of pimozide were obtained from Sigma (St. Louis, MO, USA). The internal standard dextromethorphan was obtained from Sigma as dextromethorphan hydrobromide. *n*-Hexane and isoamyl alcohol, both obtained from Fisher Chemical (Fair Lawn, NJ, USA), and acetonitrile, obtained from J.T. Baker (Phillipsburg, NJ, USA), were analytical grade and used without further purification. All other inorganic and organic reagents were analytical grade. Aqueous solutions were prepared with deionized water purified by a Nanopure Barnstead water purification system. The mobile phase was filtered using a 0.45 μm pore size nylon 66 filter and degassed by a continuous helium flow.

2.2. Stock solutions

Standard solutions of pimozide were prepared by dissolving 21 mg of pimozide in 200 ml ethanol in a volumetric flask followed by ultra-sonication for 5 min. Sequential dilutions to 100, 10, 1 μg/ml and 100, 50, 20, 10, 5, 2 and 1 ng/ml were made in ethanol, using 15 ml point-bottomed glass tubes with PTFE-lined screw-caps that were stored at 4°C. The internal standard was prepared by dissolving 20 mg of dextromethorphan in 40 ml of ethanol and then diluting to 1 μg/ml in ethanol, using 50 ml polypropylene tubes, and stored at 4°C. Sodium phosphate buffer (0.5 *M*) was prepared by dissolving 60 g NaH_2PO_4 in 1 l of nanopure water.

2.3. Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Model 600 dual-piston multi-solvent delivery system (Milford, MA, USA), a Perkin-Elmer Model LS 5B luminescence spectrofluorimeter (Norwalk, CT, USA) and a Waters Model 717 auto-sampler. Detector output was followed with Millennium Session Manager (version 2.10) software (Waters), using a Waters system interface module. The separation system consisted of a (15 cm×4.6 mm I.D.) stainless-steel column (Vydac) packed with 5 μm particle size (90 Å pore size) RP-C_{18} (Alltech Separations Group, Hesperia, CA, USA), Waters Nova-Pack C_{18} guard column (4 μm, 60 Å) and a mobile phase composed of 35% acetonitrile in 50 m*M* of NaH_2PO_4 buffer (adjusted to pH 3 using 1% phosphoric acid). The operating temperature was 20°C and the flow-rate was 1.0 ml/min. The column eluate was monitored by fluorescence with the detector excitation wavelength set at 285 nm and the emission wavelength set at 320 nm. The excitation and emission slit widths were 15 and 20 nm, respectively.

2.4. Extraction procedure

To 1.0 ml of plasma containing known concentrations of added pimozide in 15 ml point-bottomed glass tubes with PTFE-lined screw-caps, 50 μl of the internal standard solution (containing 1 μg/ml dextromethorphan in ethanol) and 1.0 ml of 1 *M* sodium hydroxide were added. After vortexing for 15 s, 5 ml of *n*-hexane–isoamyl alcohol (49:1) were added and the solution was then vortex-mixed for a further 15 s before being placed in a mechanical shaker for 20 min. After centrifugation at 2700 *g* for 10 min at 4°C, the organic layer was separated from the aqueous layer by transferring it to clean 10 ml conical-bottomed glass tubes and it was subsequently evaporated to dryness under reduced pressure. The samples were reconstituted with 200 μl of an ethanol–acetonitrile–water (10:45:45, v/v) solvent mixture, vortex-mixed for 15 s and placed in a mechanical shaker for 20 min. The volume injected into the HPLC system for the determination of pimozide concentration was 100 μl. Plasma cali-

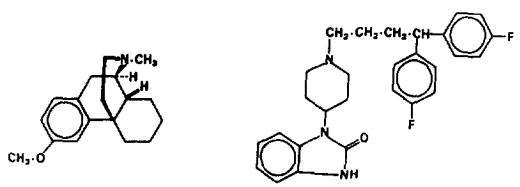

Fig. 1. Chemical structures of pimozide and the internal standard (dextromethorphan).

bration curves were prepared from 1.0 ml pimozide stock solutions containing 50, 20, 10, 5, 2 and 1 ng of pimozide by evaporating them to dryness under reduced pressure at room temperature in 15 ml point-bottomed glass tubes before adding 1.0 ml of drug-free plasma. All plasma calibration curves and direct injections were carried out in duplicate.

2.5. Pharmacokinetic study

A healthy male volunteer received a single 6 mg oral dose of pimozide, after giving written informed consent. Blood samples were collected at 0, 2, 4, 6, 8, 12, 16, 20, 24, 36, 48, 60, 72 and 96 h after receiving the pimozide dose. The plasma was separated for determination of pimozide concentrations by centrifugation at 1500 *g* for 20 min at 4°C and subsequent transfer of the plasma to clean 5.0 ml cryogenic polypropylene vials. The vials were stored at −20°C pending analysis. During the pharmacokinetic study the volunteer was monitored by electrocardiography (EKG) and telemetry in the Georgetown University Clinical Research Center in order to detect possible unwanted cardiotoxicity in the form of brady-arrhythmia, QT- prolongation or ventricular tachycardia. Pharmacokinetic parameters were determined using WINONLIN software version 1.0 (Apex, NC, USA).

3. Results

Despite the differences in their chemical structures (Fig. 1), pimozide and the internal standard (dextromethorphan) gave well resolved peaks (Fig. 2). The drug-free plasma used was consistently free of contaminating peaks at the retention times of the test compounds. Several commonly co-administered drugs in psychiatric therapy were tested in this system, in order to rule out any possible interference. The drugs tested were the neuroleptic haloperidol, the antidepressant fluoxetine and propranolol, omeprazole, levallorphan, dapsone, S-mephenytoin, phenytoin, quinidine, dextrorphan and the macrolide antibiotic clarithromycin. Among these, only dextrophan, a metabolite of dextromethorphan, was detectable with a retention time of 2 min that did not

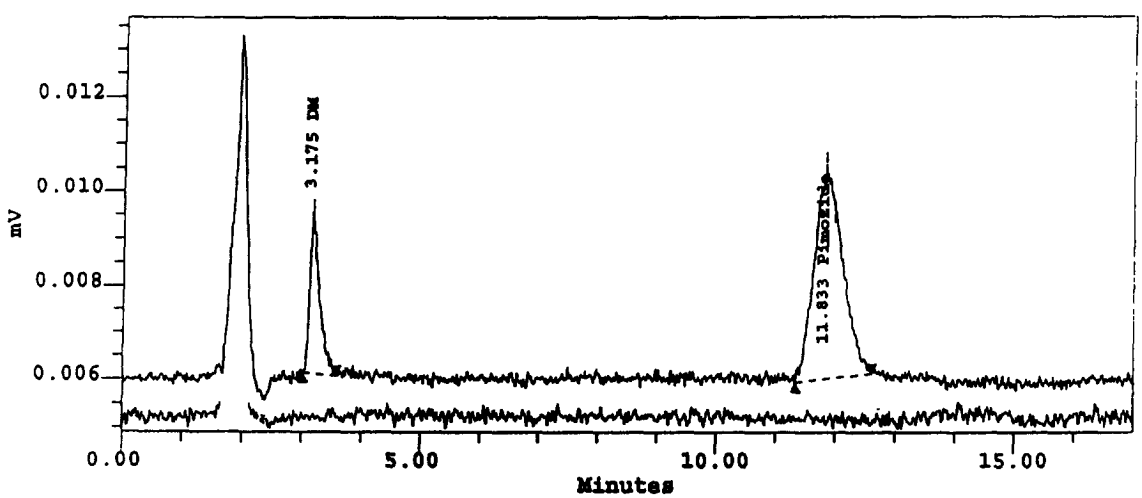

Fig. 2. Representative chromatogram with the pimozide peak at $t=11.83$ min and the internal standard (dextromethorphan) at $t=3.18$ min. The lower tracing represents a chromatogram of a blank plasma.

Table 1
Human plasma extraction ratios for pimozide

Pimozide (ng/ml)	Extraction ratio (%)
1	92.7±6.5
2	92.7±9.3
5	78.1±4.3
10	89.5±3.1
20	78.4±6.5
50	72.5±2.6

interfere with the internal standard that had a retention time of 3.2 min (Fig. 2).

The retention time of pimozide showed a high dependence on the relative amount of acetonitrile in the mobile phase, resulting in a narrow range of acetonitrile concentrations that allow resolution of dextromethorphan from the injection peak and efficient elution of pimozide. A concentration of 35% acetonitrile in phosphate buffer (pH 3.0) in the mobile phase and an ethanol–acetonitrile–water (10:45:45, v/v) mixture in the reconstituting solvent appeared to optimize the chromatography and was used for all subsequent experiments. Under these chromatographic conditions, the retention time of pimozide was 11.83 min (Fig. 2)

Plasma components did not fluoresce significantly near the pimozide or dextromethorphan peaks and thus the detection had relatively low background interference. The ratio of area under the curve of pimozide peaks to dextromethorphan was linear between 1 and 50 ng/ml pimozide. The correlation coefficient (r) of the generated standard curves was consistently higher than 0.95. The method had a limit of quantification of 1 ng/ml, with an inter-day and intra-day coefficient of variation of less than 21.9% and 18.7%, respectively, at that concentration.

The extraction ratio of the method was determined by comparing areas under curve of pimozide after extraction from plasma and in directly injected samples containing the same amounts. As can be seen in Table 1 the extraction ratio was always equal or greater than 72.5% in the sample range between 1 to 50 ng/ml pimozide (overall mean extraction ratio=83%). Similarly, the extraction ratio of the internal standard ranged between 81.2% to 96.3% (average, 88.9%) in the concentration of 1, 2, 5, 10, 20 and 50 ng/ml dextromethorphan. This is consistent with the linearity of the extracted standard curves described.

The day-to-day variance and bias determined with a 2, 10 and 20 ng/ml standard solution (n=3 for each day) were consistently less than 15.5% and 12.4%, respectively (Table 2).

The within-day variance and bias were also determined with a 2, 10 and 20 ng/ml standard solutions (n=3) as reflected in Table 2. While the variance remained below 7.4%, bias ranged between 8.8% to 18.4%.

3.1. Pharmacokinetic study

The plasma concentrations determined in the healthy volunteer after a single 6 mg oral dose of pimozide are represented in Table 3 and the con-

Table 3
Pimozide plasma concentrations (mean of duplicates measurements) after a single 6 mg oral dose in a healthy volunteer

Time (h)	Pimozide (ng/ml)	Time (h)	Pimozide (ng/ml)
0	0	15.9	2.4
2.1	5.7	19.9	2.6
3.9	5.3	23.9	2.3
6.1	3.7	36.1	2.0
8.2	3.7	48.2	1.1
11.9	3.5	57.9	0.0

Table 2
Within-day and between-day variation and bias as determined with a 2, 10 and 20 ng/ml pimozide concentration in plasma

Pimozide	Inter-day		Intra-day	
(ng/ml)	Variance (%)	Bias (%)	Variance (%)	Bias (%)
2	15.4	12.4	7.3	18.4
10	9.1	9	5.1	17.5
20	2.2	0.67	2.2	8.8

Values for each concentration are means of 3 measurements.

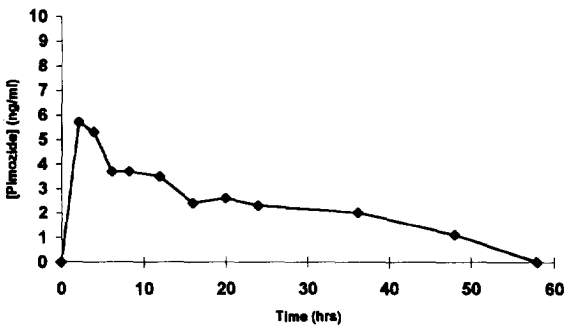

Fig. 3. Pimozide plasma concentration–time profile in a healthy volunteer after a single 6 mg oral dose of pimozide.

centration–time plasma profile is represented in Fig. 3. With the method described here, it was possible to detect plasma concentrations of pimozide up to 48.2 h of sampling.

The pharmacokinetic parameters of pimozide, derived from the plasma concentration–time profile, calculated according to a non-compartmental model after an extravascular dose, are represented in Table 4.

4. Discussion

This paper describes a specific and sensitive method to quantitate pimozide in human plasma using HPLC with fluorescence detection and describes its possible application to pharmacokinetic studies. The method has the ability to measure concentrations of pimozide as low as 1 ng/ml and this allows the method to be used to describe pimozide concentrations in human plasma at clinically relevant concentrations. The specificity for pimozide is a second important attribute of this method. The documented inability of the components of plasma and a wide range of commonly prescribed drugs to fluoresce at the appropriate wavelength or to interfere at the retention times of the dextromethorphan internal standard or pimozide is also important.

Table 4
Derived pharmacokinetic parameters of pimozide after administration of a single 6 mg oral dose of pimozide to a healthy volunteer, using non-compartmental modeling with extra-vascular dosage

T_{max} (h)	2.1
C_{max} (ng/ml)	5.7
T_{lag} (h)	0
Half-life (h)	22.7
MRT_{∞} (h)	38.3
AUC_{∞} (h ng ml^{-1})	183
Volume of distribution/F (l)	1071
Total clearance/F (ml/h)	32.7

Miyao et al. [3] described a method with different internal standard, chromatographic conditions and fluorescence wavelength settings, from those described here. The method we describe employs optimized fluorescent emission and excitation wavelengths and chromatographic condition that allow consistent separation of drug and internal standard from contaminants. We have employed an internal standard in this work that is cheap and readily available: dextromethorphan, whereas the method of Miyao et al., relies on a laboratory synthesis of methyl-pimozide.

The pimozide plasma concentrations determined in this pharmacokinetic study ranged between 1 and 8 ng/ml after a single 6 mg oral dose of pimozide. McCreadie et al. [6] reported pimozide concentrations between 2 and 20 ng/ml in patients over a six day period after a single 24 mg dose or multiple 6 mg daily doses of pimozide for four days, using a radioimmunoassay method described by Michiels et al. [4]. This radioimmunoassay reported a high selectivity of pimozide over its metabolites [5], but possible cross-selectivity with other neuroleptic drugs. This cross-selectivity reduces the ability of the radioimmunoassay to specifically determine pimozide concentrations in patients on multi-drug regimens.

The pharmacokinetic parameters derived from the pharmacokinetic study reported in this paper were modeled according to a non-compartmental model of an extravascular dose. The half-life we describe of 22.7 h in a healthy volunteer is significantly shorter than the only other pharmacokinetic data that have been reported: a mean half-life of 53 h described by McCreadie et al. [6] in nine chronic schizophrenics. Of note, these authors reported 9-fold and 12-fold interindividual differences in peak concentrations and AUC respectively. This wide variability in plasma pharmacokinetics could be due to coincident measurement of metabolites or other drugs by the radioimmunoassay that might also influence the measured plasma pharmacokinetic parameters. It is

also possible that schizophrenic patients, for reasons related to previous drug therapy or to the disease itself, may possess altered metabolic machinery in the form of a different cytochrome P450 isoform pattern of metabolism. Genetic metabolic polymorphism may also contribute to the wide range of pharmacokinetic parameters observed, as speculated by Logan et al. [7]. The method presented here should make it possible to conduct detailed studies of the pharmacokinetics and pharmacodynamics of pimozide that will allow better understanding of the properties of the drug in a variety of patient populations.

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