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Determination of piribedil and its basic metabolites in plasma by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the determination of piribedil and its *p*-hydroxylated, catechol and N-oxide metabolites in plasma is described. After addition of an internal standard (buspirone), the plasma samples were subjected to a three-step extraction procedure. The final extracts were evaporated to dryness under nitrogen, and the residues were reconstituted in 100 μl of mobile phase (0.01 *M* phosphate buffer–acetonitrile, 50:50, v/v) and chromatographed by acetonitrile gradient elution on a C_{18} reversed-phase column coupled to an ultraviolet detector set at 240 nm. The method was selective for piribedil and its metabolites, and sufficiently sensitive and precise for studies aimed at elucidating the role of the metabolites in the parent drug's pharmacological effects.

INTRODUCTION

Piribedil is an alkoxybenzyl-4-(2-pyrimidinyl)piperazine derivative with vasodilatory activity [1]. Several effects have been documented on cerebral functions involving dopamine (cognitive functions, behavioural disorders, motor regulation, auditive and visual perception). Piribedil has proved active in patients with Parkinson's desease, particularly in the control of tremors [2].

The pharmacokinetics and metabolism of piribedil have been studied in humans and animals [3,4]. In alle species the drug is efficiently extracted by the liver resulting in extremely low bioavailability, and its systemic clearance is essentially due to hepatic metabolism. At present three major routes of biotransformation have been observed *in vivo* or *in vitro* in humans and animals: demethylation of the methylenedioxyphenyl bridge to produce a catechol, 1-(3,4-dihydroxybenzyl-4-(2-pyrimidinyl)piperazine (S 584, M_1), *p*-hydroxylation of the pyrimidine ring to give 1-(3,4-methylendioxybenzyl)-4-(5-hydroxy-2-pyrimidinyl)piperazine (*p*-hydroxypiribedil, M_2) and N-oxidation to yield piribedil N-oxide (M_3). In addition to these pathways, cleavage of the arylpiperazine side-chain to give 1-(2-pyrimidinyl)piperazine (PmP, M_4), a metabolite common to other psychotropic drugs, appears to be a minor metabolic route *in vivo* in animal studies [5,6]. The pharmacokinetics of these metabolites (see Fig. 1 for molucular structures)

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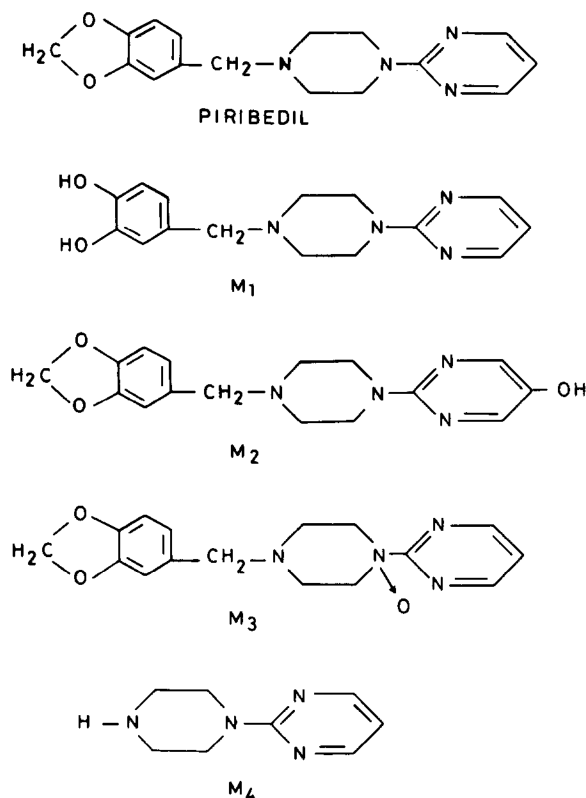

Fig. 1. Molecular structures of piribedil (PD) and its catechol (M_1), *p*-hydroxylated (M_2) and N-oxide (M_3) metabolites and 1-(2-pyrimidinyl)piperazine (M_4).

and their role in the parent drug's pharmacological effects in humans and animals is, however, still poorly understood.

Early methods for the analysis of piribedil and/or its basic metabolites were based on radioactively labelled parent drug combined with thin-layer chromatography. Most of our current knowledge of the kinetics and metabolism of piribedil in humans and animals stems from these studies [3,4]. Gas chromatography (GC) using a nitrogen-sensitive detector [4] or combined with mass spectrometry (GC–MS) [7,8] and high-performance liquid chromatography (HPLC) [6] have also been occasionally used to detect piribedil and/or some of its metabolites in animal studies. At present, however, no chromatographic methods are available for the simultaneous determination of piribedil and its basic metabolites in biological specimens.

As part of a study to assess the role of each metabolite in the parent compound's pharmacological effects we have developed an extraction procedure and gradient elution system for the simultaneous quantitation of piribedil, its hydroxylated metabolites M_1 and M_2, and piribedil N-oxide in rat plasma.

EXPERIMENTAL

Materials

Piribedil monomethane sulphonate, S 584 hydrochloride (metabolite M_1), *p*-hydroxypiribedil and piribedil N-oxide were kindly supplied by Les Laboratoires Servier (Gidy, France). Buspirone hydrochloride and PmP hydrochloride were kindly supplied by Mead Johnson Labs., (Evansville, IN, U.S.A.). Acetonitrile (Omnia Res, Milan, Italy) was of HPLC grade. All other reagents and solvents used were of analytical grade: monobasic potassium phosphate and chloroform (Farmitalia Carlo Erba, Milan, Italy) and 2-propanol and ethyl acetate (Merck, Darmstadt, F.R.G.).

Apparatus and chromatographic conditions

The analyses were conducted on an HPLC gradient system (Hewlett-Packard 1084B, Boblingen, F.R.G.) controlled by a computer terminal (HP 79850B LC model) and used in conjunction with a programmable variable-wavelength UV detector. Separation was on a reversed-phase column (μBondapak C_{18}, 30 cm × 3.9 mm I.D., particle size 10 μm) (Waters Assoc., Milford, MA, U.S.A.) protected by a Newguard pre-column with a C_{18} insert (15 mm × 3.2 mm I.D., particle size 7 μm) (Brownlee Labs., Santa Clara, CA, U.S.A.), at room temperature. Solvent A was 0.01 *M* potassium dihydrogenphosphate (pH 2.85), and solvent B was 0.01 *M* potassium dihydrogenphosphate (pH 2.85)–acetonitrile (50:50, v/v). The solvents were degassed by vacuum and ultrasound. The flow-rate was set at 1.2 ml/min and the detector wavelength at 240 nm. For elution during routine analysis a linear gradient from 10 to 90% solvent B was applied over 20 min. The column was reequilibrated within 5 min.

Sample preparation

To 0.5–1 ml of heparin-treated plasma, 0.05 ml of an aqueous solution of buspirone (1 or 10 μg/ml) and 0.5 g of sodium chloride was added. Then 5 *M* K_2CO_3 and 0.5 *M* phosphate buffer (pH 8) were added to a final volume of 2 ml, and the samples were mechanically shaken (15 min) with 11 ml of ethyl acetate–chloroform–2-propanol (85:15:5). The layers were separated by centrifugation at 600 *g* for 10 min, and the organic extract (10 ml) was transferred to new test-tubes containing 1.5 ml of 0.1 *M* hydrochloric acid, which were then shaken for 15 min. After centrifugation (600 *g* for 10 min), the organic phase was discarded and the acidic aqueous phase was made alkaline (see above) and re-extracted with 10 ml of chloroform–2-propanol (95:5), which then was evaporated to dryness at 35–45°C under a stream of nitrogen. The residues were dissolved in 0.1 ml of the mobile phase, and 10–100 μl were injected into the HPLC system.

Internal standard calibration graph

Stock solutions of piribedil and its metabolites were prepared in methanol at a

concentration of 1 mg/ml. They were stable for at least one month if stored at 0–4°C. Standard solutions were prepared from stock solutions by dilution with methanol.

Drug-free plasma containing known amounts of piribedil and its metabolites were analysed concurrently with each set of unknown samples. At least five different concentrations across the working range were measured in duplicate. Because of the wide range of piribedil and metabolite plasma concentrations encountered in the kinetic studies in various animal species and experimental conditions it was necessary to use different calibration graphs, in the ranges 0.005–5 μg/ml for piribedil, 0.01–0.25 μg/ml for metabolites M_1 and M_2 and 0.0–1 μg/ml for piribedil N-oxide. Calibration curves were calculated by the least-squares method. Peak-height ratios between the four compounds and the internal standard were used to generate the linear least-squares regression lines. The calibration graphs were all linear and not significantly different from zero ($p<0.05$) for all the intercepts on the ordinate. Concentrations of piribedil and its metabolites in the unknown samples were obtained by interpolation from these calibration curves using peak-height ratios obtained from unknown samples. Plasma blanks were used to monitor for interference.

The extraction on yields of piribedil and its metabolites were determined by comparing the peak-height ratios measured in extracts of plasma spiked with piribedil and its metabolites with the peak-height ratios for unextracted samples supplemented with the same amounts of piribedil and its metabolites. The internal standards were added to the sample just before injection into the chromatograph.

In vivo studies

Male CD-COBS rats (Charles River, Italy) weighing *ca.* 200 g were dosed intraperitoneally with piribedil monomethane sulphonate (60 mg/kg). To collect an adequate volume of blood for analysis of the parent drug and its metabolites, rats were decapitated and exsanguinated at predetermined times (5–240 min). Blood samples were collected in heparinized tubes and centrifuged, and the plasma fraction was stored frozen until assayed. Over the sampling interval the area under the plasma concentration–time curve (AUC) of piribedil and its metabolites was determined by the trapezoidal rule.

RESULTS AND DISCUSSION

Chromatography

In order to achieve simultaneous quantitation of piribedil and its basic metabolites, both isocratic elution and gradient elution were tried. The first experiments were carried out with isocratic elution, which is generally considered to be a more stable and reliable procedure than gradient elution. Various chromatographic systems involving different reversed-phase columns and mobile phases were in-

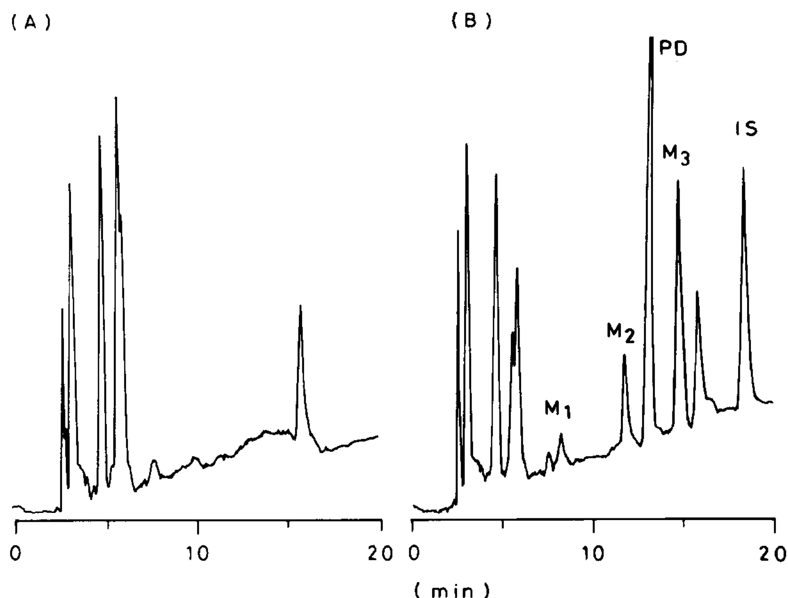

Fig. 2. Chromatograms of (A) an extract of a drug-free plasma sample and (B) plasma of piribedil-treated (60 mg/kg, intraperitoneally) rats containing (4 h after dosing) 0.01 μg/ml M_1, 0.02 μg/ml M_2, 0.07 μg/ml M_3 and 0.29 μg/ml piribedil. I.S. = internal standard (buspirone).

vestigated. Adequate separation of piribedil, *p*-hydroxypiribedil, piribedil N-oxide and endogenous constituents was easily achieved with a C_{18} column eluted isocratically with 0.01 *M* phosphate buffer–acetonitrile (60:40, pH 2.85), but the metabolites M_1 and PmP could not be resolved from the solvent front. Attempts to improve the resolution by manipulation of the pH, buffer ionic strength or organic modifier of the mobile phase were unsuccessful. Thus subsequent experiments were performed with the acetonitrile gradient described in Experimental. Under these conditions piribedil and its metabolites were adequately resolved and, with the exception of PmP, all the compounds eluted in a region of the chromatogram of normal plasma that was essentially devoid of endogenous components.

Examples of chromatograms of an unspiked plasma sample and a plasma sample of piribedil-treated (60 mg/kg, intraperitoneally) rats are shown in Fig. 2. Approximate retention times were 18 min for piribedil, 8, 12 and 22 min, respectively, for metabolites M_1, M_2 and M_3, and 25 min for the internal standard (buspirone). Even under these conditions, however, PmP (retention time *ca.* 4 min) could not be resolved from an interfering peak occasionally present in the chromatogram of normal rat plasma. This, and the fact that its extraction characteristics differed from those of the other metabolites (see below), precluded its simultaneous determination in rat plasma.

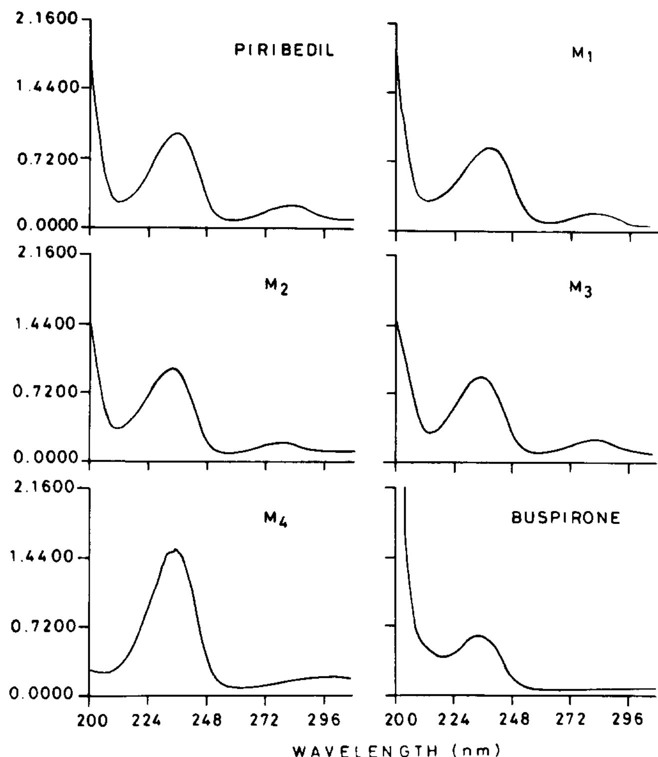

Fig. 3. UV spectra of piribedil, its metabolites and buspirone in the analytical mobile phase.

Fig. 3 shows the UV spectra of piribedil, its metabolites and the internal standard (buspirone). The wavelength of 240 nm was selected for quantitation because it elicited a good response for piribedil, its hydroxylated and N-oxide derivatives, and buspirone. The detection limit at a signal-to-noise ratio of 3 was *ca.* 2.5 ng/ml for piribedil and 5 ng/ml for its hydroxylated and N-oxide metabolites.

Extraction procedure

Ideally, a pre-chromatographic extraction procedure should give quantitative recoveries but, in practice, this is hard to accomplish, particularly when several compounds with different chemicophysical properties are to be analysed simultaneously. This was the case with piribedil, a highly liposoluble drug [3,6], and its (more polar) metabolites whose recovery was markedly affected by the pH and the nature of the solvent. Whereas piribedil and its *p*-hydroxylated and N-oxide derivatives were relatively insensitive to changes in pH in the range 7.5–10, the catechol metabolite (M_1) could be efficiently extracted only at pH 7.5-8 and PmP at pH above 9.0, *i.e.* above its pK_a value [6]. Piribedil was almost completely

extracted (recovery 80–90%) by most of the solvents tested (hexane, benzene, ethyl acetate, chloroform), but its metabolites, particularly M_1, could not be efficiently extracted even with the most polar solvent tested. A chloroform–2-propanol mixture (95:5) gave acceptable extractions for both the parent compound (*ca.* 90%) and its metabolites (70–80%), but interfering endogenous components were often present in the chromatograms. The first-step extraction procedure with an ethyl acetate–chloroform–2-propanol mixture and back-extraction into 0.1 *M* hydrochloric acid was thus used and this reduced the problem. Although this precluded the extraction of PmP, a phosphate buffer of pH 8 was chosen to alkalinize the plasma sample since higher pH values gave lower recoveries of M_1, which may play an important role in the pharmacological effects of the parent drug [9–12].

Under these conditions overall mean recovery was 70 ± 7% for piribedil and 60 ± 8, 64 ± 7 and 66 ± 7% for M_1, M_2 and M_3, respectively, with no significant dependence on concentration over the concentration range investigated; hence regression analysis over these concentration ranges revealed high linearity ($r > 0.99$) for piribedil and its metabolites. The lack of quantitative recovery was primarily the result of multiple-step extraction of the analytes from plasma.

Reproducibility and sensitivity

Intra-assay precision studies were carried out on plasma spiked with different amounts of piribedil and its three metabolites, processed as described above. Intra-assay precision was checked by replicate analysis of plasma samples on the same day. These studies indicated that intra-assay precision and accuracy of piribedil quantitation were relatively good at all the concentrations, the coefficient of variation (C.V) at 5 ng/ml being 8.3% and less than 10% at all higher concentrations (Table I). For the metabolites, the C.V. were higher than 15% at 5 ng/ml (data not shown) but lower (with the exception of M_1) than 10% at higher concentrations.

The inter-assay precision and accuracy were evaluated by preparing quality control samples at the start of the validation study. With each day's analysis, these plasma samples were assayed with standard samples over a period of two weeks, and the calculated concentrations were compared. The results are summarized in Table I for plasma samples spiked with 10 and 100 ng/ml of each compound. The mean day-to-day C.V. at 10 ng/ml was 7.4% for the parent drug and 14.5, 12.2 and 9% for metabolites M_1, M_2 and M_3, respectively. At 100 ng/ml it was less than 12% for all compounds.

Application

Currently, the method is being succesfully utilized in pharmacokinetics studies in animals. The plasma concentration–time profiles of piribedil and its *p*-hydroxylated, catechol and N-oxide metabolites in male rats given pharmacologically effective intraperitoneal doses of piribedil monomethane sulphonate

TABLE I

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH PIRIBEDIL AND METABOLITES M_1, M_2 AND M_3

Each value is the mean ± S.D. of five determinations; N.A. = not applicable; C.V. is shown in parentheses.

Amount added (ng/ml)	Amount found (ng/ml)			
	Piribedil	M_1	M_2	M_3
Within-day				
5	4.8 ± 0.4 (8.3)	N.A.	N.A.	N.A.
10	9.9 ± 0.6 (6.0)	12.5 ± 1.5 (12.0)	10.4 ± 1.0 (9.6)	10.8 ± 0.7 (6.5)
50	50.1 ± 0.7 (1.4)	51.8 ± 5.0 (9.6)	49.4 ± 5.9 (5.9)	49.6 ± 2.5 (5.0)
100	101.4 ± 3.2 (3.2)	100.2 ± 9.4 (9.4)	96.4 ± 7.4 (7.4)	102.6 ± 2.5 (2.4)
500[a]	501.0 ± 14.3 (2.9)	505.0 ± 30.2 (6.0)	508.0 ± 27.7 (5.5)	499.0 ± 16.5 (3.3)
Day-to-day				
10	10.8 ± 0.8 (7.4)	13.1 ± 1.9 (14.5)	11.5 ± 1.4 (12.2)	11.0 ± 1.0 (9.1)
100[a]	101.2 ± 5.3 (5.2)	99.6 ± 11.1 (11.1)	99.0 ± 8.9 (9.0)	102.1 ± 8.3 (8.1)

[a] The mean C.V. for piribedil at all higher concentrations was less than 5% (data not shown).

(60 mg/kg) are shown in Fig. 4. Piribedil rapidly appeared in rat plasma, maximum mean peak concentrations being reached within *ca.* 5 min. Its disappearance from plasma was slower than previously found after injection of lower doses in the rat [6,7,13], suggesting that its kinetics may be dose-dependent. The measured

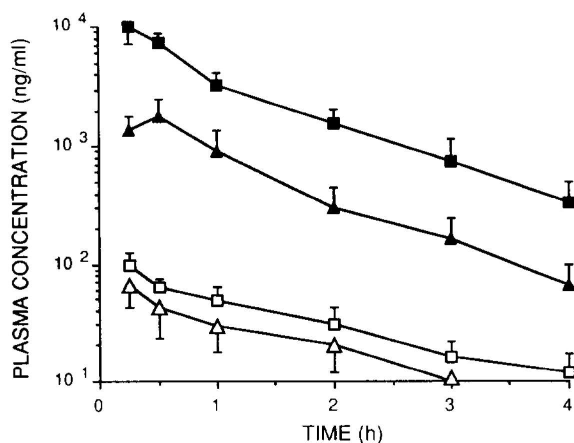

Fig. 4. Plasma concentration–time course of piribedil (■) and its metabolites M_1 (△), M_2 (□) and M_3 (▲) in rats injected intraperitoneally with piribedil monomethane sulphonate (60 mg/kg). Results are expressed as ng/ml (mean ± S.D.).

plasma concentrations of the three metabolites were always lower than those of piribedil, in terms of maximum concentrations and experimental area under the curve (expressed in nmol/ml · h). It cannot be excluded, however, that they may achieve higher concentrations than the parent drug at appropriate sites of drug action. Further investigation of these findings, which will include a dose-proportionality study and an investigation of the extent to which the metabolites enter the central nervous system compared with the parent drug, is now in progress.

CONCLUSION

An analytical procedure for simultaneous determination of the parent compound and its metabolites is a prerequisite for drug kinetics and concentration–response studies. Although some work has been done on the separation and quantitation of piribedil and its basic metabolites by GC [3,4], GC–MS [7,8] and HPLC [6], the major advantage of the procedure described here is that it allows simultaneous determination of the parent compound and its *p*-hydroxylated, catechol and N-oxide metabolites using an internal standard of similar molecular structure.

Interest in piribedil metabolites has been increasing in recent years in the light of the possible involvement of certain of them in the parent drug's pharmacological effects [9–12]. The method proposed is selective and sensitive enough for quantitating piribedil and metabolites in plasma after administration of pharmacologically effective doses of piribedil to animals. Moreover, it can be applied to determine brain concentrations of these compounds, after appropriate homogenization of the tissue (3% trichloroacetic acid, data not shown). Although this method, like other assays that have previously described [3–8], is probably not applicable for determination of the extremely low parent drug plasma concentrations at therapeutic dose [3,4], it does appear adequate to achieve our initial aim, which was to investigate the role of piribedil metabolites in the pharmacological effects of the parent compound.

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