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In vitro metabolism of the alkaloid piplartine by rat liver microsomes☆

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ABSTRACT

Because piplartine (PPT) has demonstrated biological activities, such as cytotoxic, anxiolytic, antidepressant, antifungal and antiplatelet activities, this molecule is a relevant drug candidate. The metabolic fate of drug candidates is an essential requirement in assessing their safety and efficacy. Based on this requirement, the biotransformation of PPT by cytochrome P450 enzymes (CYP) was investigated for the first time. To determine the *in vitro* enzymatic kinetic parameters, an HPLC method was developed and validated to quantify PPT. All samples were separated on a reversed-phase C18 column using a mobile phase of acetonitrile:water (40:60, v/v). The method exhibited a linear range of 2.4–157.7 μmol/L, with the following calibration curve: $y = 0.0934\ (\pm 0.0010)x + 0.0027$, $r = 0.9975$. The lower limit of quantitation was verified to be 2.4 μmol/L, with an RSD below 7%. The precision and accuracy were assessed for both within-day and between-day determinations; neither relative standard (RSD%) deviations nor relative errors (RER) exceeded a value of 15%. The mean absolute recovery was 85%, with an RSD value below 6%. The enzymatic kinetic parameters revealed a sigmoidal profile, with $V_{max} = 4.7 \pm 0.3$ μmol/mg mL^{-1}/min, $h = 2.5 \pm 0.4$, $S_{50} = 44.7 \pm 0.3$ μmol/L and $CL_{max} = 0.054$ μL/min/mg protein, indicating cooperativity behavior. Employing a mammalian model, PPT metabolism yielded two unreported monohydroxylated products (*m/z* 334). The identification and structural elucidation of the metabolites were performed by comparing their mass spectra with those spectra of the parent drug. For the first time, the *in vitro* metabolism studies employing microsomes were demonstrated to be a suitable tool for data regarding enzymatic kinetics and for the metabolites formed in the PPT mammalian metabolism.

1. Introduction

Throughout human history, plant extracts have been employed in traditional medicines to treat many different ailments. These extracts offer a rich source of structurally diverse compounds with a wide range of biological activities [1,2]. The genus *Piper* belongs to the Piperaceae family, which consists of 14 genera and over 1950 species that are widely distributed throughout tropical and subtropical regions. Several biologically active compounds have been isolated from this family, and amide alkaloids have been found to be the most characteristic ones. Piplartine, 5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H)-pyridinone (Fig. 1), is the major amide isolated from *Piper tuberculatum* [3,4]. This

☆ Electronic supplementary information available: Representative chromatograms of UHPLC-DAD analyses. (Fig. S1); High resolution ESI-MS (Q-ToF) (positive mode) spectrum of protonated piplartine $[M+H]^+ = m/z$ 318 (Fig. S2); Representative total ion chromatogram (TIC) of protonated piplartine oxidized metabolites $[M(OH)+H]^+ = m/z$ 334 of UHPLC analyses (Fig. S3).

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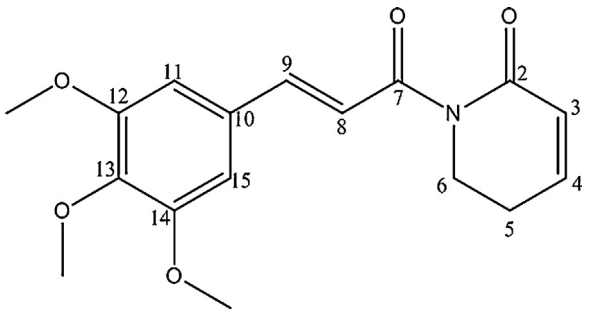

Fig. 1. Chemical structure of the piplartine.

secondary metabolite has demonstrated several biological activities, such as antifungal, anti-platelet aggregation, insecticidal, antiparasitic, anxiolytic, and antidepressant activities [3,5,6], and among these activities, cytotoxic properties have been highlighted. PPT presents the ability to kill cancer cells of several histotypes, including hematological, colon, lung, breast, central nervous system, pancreatic, nasopharyngeal, osseous, bladder, renal and prostate [7,8].

When considering PPT as a promising drug candidate, its metabolism requires clarification. Drug-metabolism studies are key points in early drug development [1]; therefore, *in vitro* systems have been extensively used for this purpose due to ethical, economic, and scientific reasons. Liver microsomes are one of the most commonly employed *in vitro* systems because, in addition to the characteristics mentioned above, liver microsomes present good reproducibility and their preparation is straightforward [9,10].

P450 cytochromes (CYP) are the most important enzymes in drug metabolism. Most P450 reactions follow simple Michaelis–Menten kinetics, which is based on a single active site for the interaction between the substrate and the enzyme. This model easily provides the kinetic constants (K_m and V_{max}) and promotes understanding concerning possible metabolic-based drug interactions [11]. In contrast, non-Michaelis–Menten kinetics may also be observed for some P450 reactions. In these cases, the kinetics apparently results from an allosteric effect that commonly yields a sigmoidal velocity saturation curve [12–15].

In view of the potential of PPT as a primary compound against cancer, the main aim of this paper was to provide the first data regarding PPT metabolism in a mammalian model. For this purpose, a bioanalytical method for PPT analysis in rat liver microsomes was comprehensively developed and validated. Moreover, PPT metabolism was characterized by determining its enzymatic kinetic parameters (V_{max}, K_m) and its clearance. Additionally, metabolite formation was investigated using mass spectrometry.

2. Experimental

2.1. Chemicals and reagents

PPT was isolated from *P. tuberculatum* Jacq (Piperaceae) according to a previously published procedure [5]. Briefly, a dry powder of inflorescences from *P. tuberculatum* (500 g) was extracted by incubation with MeOH (2 l × 2 l) at room temperature for three days. The solutions were filtered and concentrated under vacuum conditions, yielding a dark syrup (47 g). Part of this extract (2 g) was submitted to a silica chromatography column using a gradient of hexane–ethyl acetate at increasing polarities, which yielded 35 fractions. Fraction 20 (350 mg) was recrystallized with hot methanol; a white crystalline compound was obtained (150 mg), and the molecular structure and purity analysis were determined using ^{13}C and ^{1}H NMR spectral data, confirming this compound as piplartine.

Stock standard solutions of PPT were prepared at the concentration of 2000 μg mL^{-1} in HPLC grade methanol (Panreac, Barcelona, Spain). The analytical curve for PPT was obtained by dilutions in the same solvent in the range of 2.4–157.7 μM. Carbamazepine (CBZ) (Sigma–Aldrich, Steinhein, Germany), which was prepared in HPLC grade methanol, was employed as an internal standard (IS) at a concentration of 500 μg mL^{-1}. All solutions were stored at −20 °C and protected from light. PPT and CBZ have similar physico-chemical properties and are structural close-related: PPT:LogP 2.424 ± 0.366 and pKa −1.88 ± 0.20; CBZ:Log P 1.895 ± 0.597 and pKa −0.49 ± 0.20 and 13.94 ± 0.20. [16–18]. Sodium chloride and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Sodium hydroxide and potassium chloride were obtained from Nuclear (Sao Paulo, Brazil). Glycerol and Tris (hydroxymethyl) aminomethane were obtained from J.T. Baker (Phillipsburg, NJ, USA); ethylenediaminetetraacetic acid (EDTA) was obtained from Carlo Erba (Milan, Italy). $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. HPLC conditions

A Shimadzu (Kyoto, Japan) High Performance Liquid Chromatography System, which was composed of a LC-20AT solvent pump unit; a CTO-20A column oven; a DGU-20A$_5$ online degasser; a CBM-20A system controller and a SPD-M20A (190–800 nm) diode array detector, was used. Injections were automatically performed (20 μL) using a 50 μL loop SIL-10AF. Data were collected using the LC solution software SPD-M20A PDA *utility* (Shimadzu, Kyoto, Japan). The resolution of the PPT was performed at 32 °C on a Shim-pack VP-ODS column acquired from Shimadzu (250 mm × 4.6 mm, 4.6-μm particle size, Kyoto, Japan). A Shim-pack GVP-ODS Shimadzu C18 column (10 mm × 4.6 mm, 4.6-μm particle size) was used as a guard column. The mobile phase used was a mixture of acetonitrile:water (40:60, v/v) at a flow rate of 1 mL min^{-1}.

2.3. Animals and microsome preparation

Male Wistar rats weighting 180–220 g were obtained from the School of Pharmaceutical Sciences of Ribeirao Preto–University of Sao Paulo. The experiment was approved by the Ethical Committee from the University of Sao Paulo (#11.1.1047.53.4), according to the Guiding Principles for Research Involving Animals and Human Beings from the American Physiological Society. Animals were fed under normal conditions and acclimatized with a 12 h light/dark cycle. The animals were sacrificed by decapitation, and the livers were removed and placed in ice-cold 0.05 mol/L Tris–HCl buffer (pH 7.4), which contained 0.15 mol/L KCl. The livers were minced with scissors and washed three times with Tris–HCl buffer. Then, the slices were homogenized using a MA 181 potter equipment (Marconi, SP, Brazil). The homogenate was centrifuged using a HIMAC CF 15D2 centrifuge (Hitachi, Tokyo, Japan) at 10,000 × *g* for 15 min at 4 °C, and the resulting supernatant was ultracentrifuged using a XL-70 Beckman ultracentrifuge (Beckman, Carlsbad, CA, USA) at 100,000 × *g* for 60 min at 4 °C to obtain the microsomal pellet. The obtained pellet was resuspended in HEPES–HCl buffer (pH 7.4; 0.05 mol/L), which contained 20% glycerol and 0.001 mol/L EDTA, and stored at −170 °C until use. The protein concentration was determined by the Biuret method using a BCA kit (Labtest, MG, Brazil) [10,19].

2.4. Microsomal incubation conditions

Optimal conditions for microsomal incubation were determined in the linear range for the disappearance of PPT from the microsomal medium. Incubations were performed with the reconstituted