Analysis of amide compounds in different parts of *Piper ovatum* Vahl by high-performance liquid chromatographic

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ABSTRACT

Background: *Piper ovatum* (Piperaceae) has been used in traditional medicine for the treatment of inflammations and as an analgesic. Previous studies have showed important biological activities of the extracts and amides from *P. ovatum* leaves. **Objective:** In this study, a high-performance liquid chromatographic (HPLC) method was developed and validated for quantitative determination of the amides in different parts of *Piper ovatum*. **Materials and Methods:** The analysis was carried out on a Metasil ODS column (150 x 4.6 mm, 5μm) at room temperature. HPLC conditions were as follows: acetonitrile (A), and water (B), 1.0% acetic acid. The gradient elution used was 0–30 min, 0-60% A; 30–40 min, 60% A. Flow rate used was 1.0mL/min, and detection at 280nm. **Results:** The validation using piperlonguminine, as the standard, demonstrated that the method shows linearity (linear correlation coefficient = 0.998), precision (relative standard deviation <5%) and accuracy (mean recovery = 103.78%) in the concentration range 31.25 – 500μg/mL. The limit of detection and quantification were 1.21 and 4.03μg/mL, respectively. This method allowed the identification and quantification of piperlonguminine and piperovatine in the hydroethanolic extracts of *P. ovatum* obtained from the leaves, stems and roots. All the extracts showed the same chromatographic profile. The leaves and roots contained the highest concentrations of piperlonguminine and the stems and leaves showed the most concentrations of piperovatine. **Conclusion:** This HPLC method is suitable for routine quantitative analysis of amides in extracts of *Piper ovatum* and phytopharmaceuticals containing this herb.

Key words: High-performance liquid chromatographic, *Piper ovatum*, piperlonguminine, piperovatine, validation

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INTRODUCTION

The genus *Piper* is the most important member of the family Piperaceae, and encompasses more than 700 species, distributed worldwide.[1] Approximately 266 species of *Piper* can be found in Brazil.[2] Various species of *Piper* are used in traditional medicine and as food-flavoring and pest-control agents.[3-5]

Phytochemical investigations of different *Piper* species and plant parts have led to the isolation of numerous active components, including alkaloids, amides, pyrones, dihydrochalcones, flavonoids, phenylpropanoids and lignans.[6] *Piper ovatum* Vahl (Piperaceae), an herbaceous plant occurring throughout Brazil, is popularly known as "joão burandi" or "anesthetic." It is used in traditional medicine for the treatment of inflammations and as an analgesic.[3] Hydroalcoholic extracts of leaves, piperovatine and piperlonguminine from *P. ovatum* showed the greatest inhibitory activity of topical inflammation induced by croton oil.[7] The amides piperovatine and piperlonguminine have been isolated from other species of *Piper*.[8-14] Recent phytochemical investigations have traced the piscicidal, oral 'local anesthetic' and saliva-producing (sialogogic) properties of this species to the isobutyl-amide, piperovatine.[15] This compound induces dramatic increases in intracellular calcium concentration.[14] Piperlonguminine shows antitumor effects, inhibits the expression of the amyloid precursor protein (APP) gene that plays an important role in Alzheimer's disease, and shows significant monoamine oxidase (MAO) inhibitory activities.[16-18] The amides piperovatine and piperlonguminine from *P.*

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ovatum leaves, and showed MIC values of 15.6 and 31.2μg/mL to *B. subtilis* and 3.9μg/mL to *C. tropicalis,* and low toxic effects to Vero cells and macrophages and the essential oil was active against *C. tropicalis*.[19]

The mixture of piperovatine: piperlongumune (2:3) showed important antiprotozoal activity against the amastigote and promastigote forms of *L. amazonensis*, and it produced morphological changes in promastigotes and amastigotes at 0.9μg/mL and 24μg/mL (50% growth inhibition concentration), respectively, including intense cytoplasmic vacuolization, mitochondrial swelling, and mitochondrial damage, as revealed by transmission electron microscopy.[20]

The analysis of the amides piperovatine and piperlonguminine of *P. ovatum* by HPLC is not much explored. The HPLC method is gaining increasing importance for qualitative and quantitative analysis of plant extracts, and is useful for quality control of phytochemicals. However, validated quality-control methods need to be developed, because validation of analytical procedures is an important part of the registration application for a new drug. Besides the regulatory requirements, the performance and reliability of the control test procedure are essential to effective quality control of drugs. Therefore, validation should be regarded as part of an integrated concept to ensure the quality, safety and efficacy of pharmaceuticals.[21,22] Therefore, the aim of the present study was to develop and validate a chromatographic HPLC method for qualitative and, mainly, quantitative analysis of amides in different parts of *P. ovatum*.

MATERIALS AND METHODS

Plant material

Piper ovatum Vahl leaves, stems and roots were collected in Monte Formoso, state of Minas Gerais, Brazil, in July of 2007, and were identified by Dra. Elsie Franklin Guimarães. A voucher specimen (HUM 10.621) was deposited in the herbarium of the Department of Botany, University of Maringá, Paraná, Brazil. The leaves, stems and roots of *P. ovatum* were dried at 35°C in a circulating air oven and were triturated in a knife mill (Usi-ram®) before extraction.

Extraction and purification of the amides from leaves

To purify the amides, the extract of *P. ovatum* was prepared by exhaustive maceration of the leaves (150.0g) in ethanol: water (90:10 *v/v*) at room temperature at dark room. The extract was filtered, concentrated under vacuum at 40°C to obtain and lyophilized, yielding 37.5g. Subsequently, the extract of the leaves (20.0g) was chromatographed in a vacuum silica-gel column and eluted with hexane, dichloromethane: ethyl acetate (1:1 *v/v*), ethyl acetate and methanol. Next, the dichloromethane: ethyl acetate fraction (8.0g), positive to Dragendorff's test, was rechromatographed on a silica gel 60 (70-230 mesh) column chromatograph using hexane, hexane: dichloromethane (98:2, 95:5, 90:10, 80:20 and 50:50 *v/v*), dichloromethane, dichloromethane: ethyl acetate (98:2, 95:5, 90:10, 80:20 and 50:50 *v/v*), ethyl acetate and methanol, afforded 108 fractions. Subsequently, the fraction 23-38 (282.0mg) was rechromatographed on a Sephadex LH 20 with ethyl acetate, obtaining 50 fractions. Fractions 15-32 (23mg) and 42-50 (38mg) were identified as piperovatine (1) and piperlonguminine (2) respectively, by analyses of spectral data of ^{1}H, ^{13}C NMR, mass spectrometry and by comparison of data from the literature.[7,23]

Structure elucidation of amides

The structures of the amides piperovatine (1) and piperlonguminine (2) were identified by comparing nuclear magnetic resonance (NMR) data with those in the literature [Figure 1].[7,23] NMR spectra were recorded on a Bruker DRX-400 spectrometer at 300MHz (1H, COSY) and 75.5MHz (13C, DEPT) using chlroform deuterated solvent (CDCl3). They were obtained with TMS as the internal standard and constant temperature of 298 K. In addition, a mass spectrum was obtained by a (EI) Shimadzu gas chromatography coupled with mass spectrometry GC/MS 17A QP 5000 mass spectrometer equipped with a DB5 column (30 m; 0.32mm).

Preparation of extracts

For HPLC analyses, the leaves, stems and fruits (10.0g) of *P. ovatum,* were used to prepare the crude extracts. The

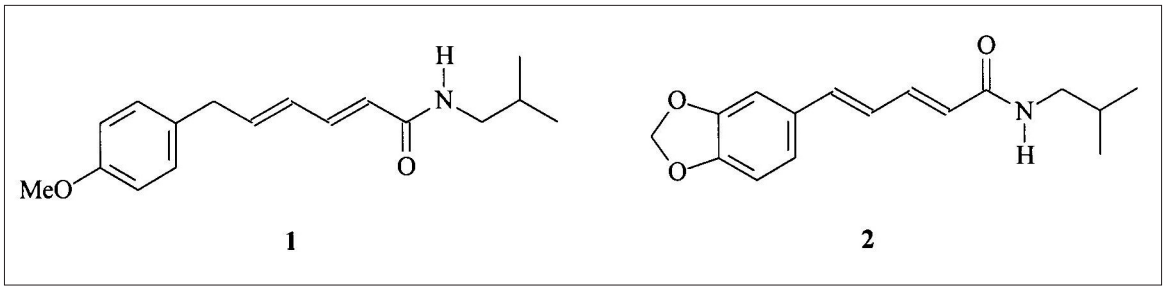

Figure 1: Structure of the amides piperovative (1) and piperlonguminine (2) from *Piper ovatum* leaves (at column width)

extracts was prepared by maceration in ethanol: water (9:1, *v*/*v*, 100mL) at room temperature for 5 days at dark room. The extracts were filtered, evaporated under vaccum at 40°C and lyophilized.

HPLC analysis

Reagents and chemicals

Acetonitrile (HPLC grade from OmniSolv EM Science, Gibbstown, NJ), ultrapure water (Milli-Q system, Millipore, Bedford, USA) and acetic acid (analytical grade, Merck, Darmstadt, Germany) were used for the mobile phase preparation. Methanol (HPLC grade from OmniSolv EM Science, Gibbstown, NJ) was used for samples preparation. The piperlonguminine (2) was used as external standard and the piperovatine (1) were only used as reference to the corresponding peak in the sample extracts.

Sample preparation

To obtain the stock solutions, piperovatine, piperlonguminine and the crude extracts of the leaves, stems and roots from *P. ovatum* were prepared in methanol at concentration of 1000 μg/mL. The solutions were filtered through 0.45 mm membrane filter (Millipore).

Instrumentation and chromatographic conditions

The analysis for HPLC were carried out using a GILSON liquid chromatography equipped with quaternary pump (Pump 321), automatic injector valve (234) with loop of 20μL, degasser (865), on oven CTO-10Avp and a UV/visible detector model 152, controlled for Software BOWTER. In the chromatographic analysis were used reverse phase column Metasil ODS, 5μm, 150.0 x 4.6mm, kept in oven to the ambient temperature. The separation was carried out in a gradient system, using as mobile phase a mixture of acetonitrile (A), and water (B) and 1.0 % acetic acid. A gradient elution used was 0–30min, 0-60% A; 30–40min, 60% A, with flow of 1mL/min at room temperature. The detenction of substances was carried out in 280nm and the running time was 40 min. The conditions were previously tested and optimized. The sample injection volume was 20μL. Three determinations were carried out for each sample. The statistical analyses of the data were performed by Statistic 6.0 Software (Statsoft Inc., Tulsa, OK, USA)

Validation parameters

Linearity

The linearity of the calibration curve for the piperlonguminine (**2**) was determined by the external standard method. Stock standard solution at a concentration of 1000μg/ml was diluted in methanol yielding concentrations of 31.25, 62.5, 125, 250 and 500μg/mL. Three determinations were carried out for each solution. The calibration curves were obtained by plotting the peak area of the piperlonguminine versus the concentration of the standard solutions. The statistical parameters of the calibration curve as slope, intercept and correlation coefficient were calculated by linear regression analysis.

Precision

The repetibility of the method was evaluated on the same day while the intermediate precision was determined for two non-consecutive days. The standard solution was analyzed at three concentrations (31.25, 125 and 500μg/mL). Three determinations were carried out for each solution. The precision was expressed as relative standard deviation (R.S.D%) of the concentrations of piperlonguminine.

Limit of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curve of the standard piperlonguminine. LOD was calculated according to the expression $3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression $10\sigma/S$.

Accuracy

The accuracy was evaluated with the recovery test by analyzing the mixture prepared by adding of the piperlonguminine solution at the three concentration levels (31.25, 125 and 500 μg/mL) to extract of the leaves of *P. ovatum* (1000μg/mL) containing known amount of this compound. Three determinations were carried out for each solution. The percentage recovery was calculated by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by hundred.

Stability of the analyte during analysis

The stability was evaluated with standard solutions and sample solutions that were stored at 4°C and at room temperature during 72 h. The solutions were analyzed every 24 h.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

The fingerprint assay method for extract of *P. ovatum* extracts was established in our previous work.[19] To develop a HPLC method for the analysis of amides in *P. ovatum* extracts, several parameters were optimized to select the proper conditions. A gradient was chosen that allowed good separation of amides within a short analysis time. To optimize the mobile phase, different compositions of acetonitrile in water containing 1% acetic acid were tested. The mobile phase using the solvents acetonitrile (A) and

water (B) with an elution gradient of 0–30 min, 0-60% A; 30–40 min, 60% A, was shown to be adequate to obtain better resolution of the peaks for the compounds. Addition of acetic acid decreased the peak tailing of the piperovatine and piperlonguminine, and was essential to improve the resolution of the chromatogram. The flow rate of 1.0 mL/min allowed good separation, with an analysis time of 40 min. Separation was further improved keeping the column at room temperature. The maximum absorption of the amides was found to be 280nm, and this wave length was chosen for the analysis. The Figures 2 a-c shows the chromatograms of the crude extracts. Peak 1 with a retention time of 23.50 min was identified as piperovatine. Peak 2 with a retention time of 24.46 min can be assigned to piperlonguminine.

Validation

For the validation of the analytical method, the guidelines of the International Requirements for Registration of Pharmaceuticals for Human Use were followed.[24] Piperlonguminine was used as the standard, because it is the majoritary compound present in *P. ovatum* extracts obtained of the roots and leaves [Figure 2].

Linearity

Results obtained in validation demonstrated an excellent linear relationship between peak area and concentration of piperlonguminine in the range 31.25–500 µg/mL, as confirmed by the correlation coefficient of 0.998. The validating parameters of the calibration curve, including linearity range, slope, intercepts and correlation coefficients obtained by linear regression analysis, are described in Table 1.

Precision

The precision of the method was evaluated in terms of repeatability and intermediate precision, by performing three repetitive analyses for each concentration level (31.25, 125 and 500µg/mL). The repeatability test showed R.S.D. values lower than 4.6%, and the intermediate precision, evaluated on 2 non-consecutive days, showed R.S.D. between 2.67% and 4.80% [Table 2]. These results were considered satisfactory, because the majority of phytochemicals have R.S.D. values lower than 6%, according to the literature.[25]

Limit of detection and quantification

The limit of detection, defined as the lowest concentration of piperlonguminine in a sample that can be detected but not necessarily quantified under the stated experimental

Table 1: Linearity parameters for the calibration curve of piperlonguminine

Parameters	Piperlonguminine
Linearity range (µg/mL)	31.25-500
Slope (a)	2.229.313
Intercept (b)	- 1.611.068
(r^2)	0.998

r^2, correlation coefficient

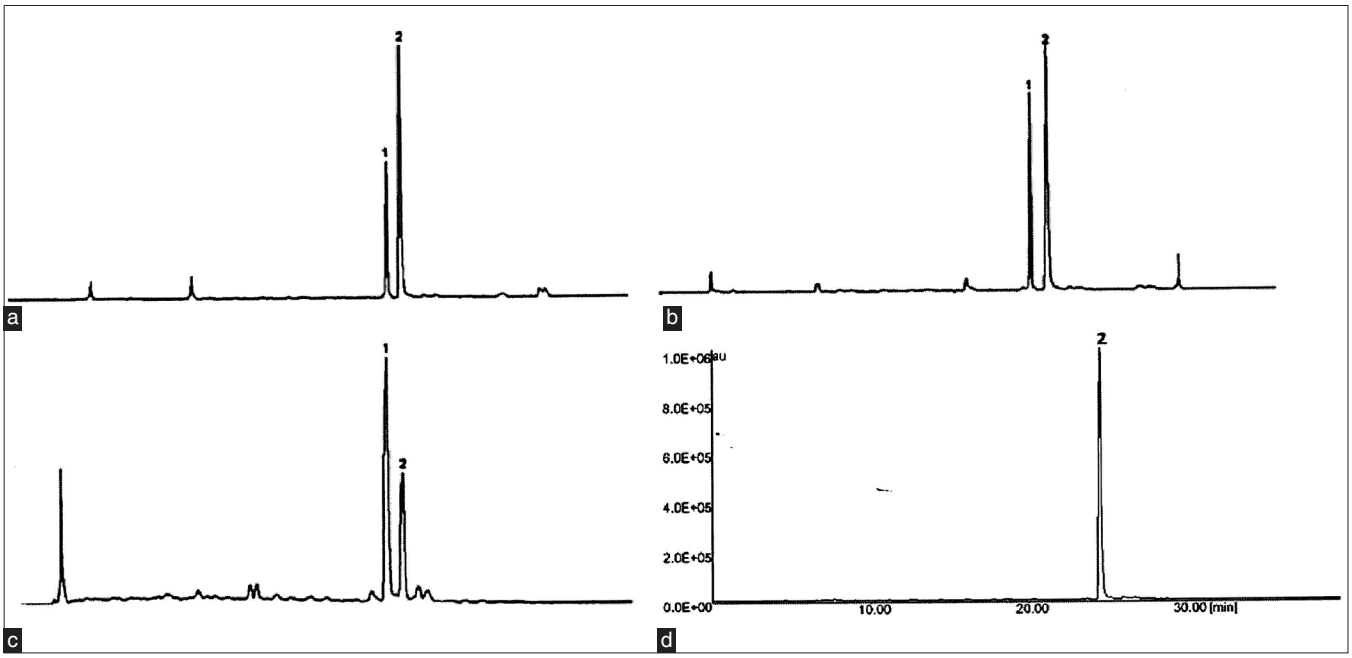

Figure 2: Chromatogram obtained by HPLC. Chromatograms of the hydroalcoholic extracts of *P. ovatum* obtained from roots (a), leaves (b) and stems (c) *Piper ovatum*; where the piperovatine (1), piperlonguminine (2). (d) Chromatogram of the standard piperlonguminine (RT = 24.46 min.). Chromatographic conditions: Metasil ODS column; mobile phase: acetonitrile: water 0% of acetonitrile for 60 % (0-30 min.) and acetonitrile: water 60:40 (v/v) (30-40 min.) with 1% acetic acid; flow rate: 1.0mL/min; room temperature; detection: 280 nm (at full page width)

Table 2: Repeatability and intermediate precision data for the determination of piperlonguminine by HPLC

Compound	Concentration (µg/mL)	Repeatability (R.S.D%)	Intermediate precision (R.S.D%)
Piperlonguminine	31.25	1.98	2.67
	125	3.21	4.80
	500	4.58	4.67

R.S.D. relative standard deviation. For each sample $n = 3$

Table 3: Results of the recovery test for piperlonguminine of the extract from *P. ovatum* leaves

Compound	Spiked concentration (µg/mL)	Recovery (%) (mean ± S.D)	Mean ± S.D	R.S.D (%)
Piperlonguminine	31.25	101.47 ± 1.21	103.78 ± 2.53	2.44
	125	103.38 ± 1.73		
	500	106.49 ± 0.89		

S.D. standard deviation; R.S.D. relative standard deviation. For each sample $n = 3$

Table 4: Quantification of piperovatine and piperlonguminine in hydroethanolic extracts of the leaves, stem and root of *P. ovatum* by HPLC

Material	Piperlonguminine (%)	Piperovatine[a] (%)
Leaves	1.64 ± 0.43	1.25 ± 0.16
Stems	0.75 ± 0.14	1.22 ± 0.13
Roots	2.05 ± 0.69	0.95 ± 0.12

$p<0.05$, $n=3$. [a]Calculated as piperlonguminine

conditions, was 1.21µg/mL. The limit of quantification, defined as the lowest concentration of piperlonguminine in a sample that can be determined with acceptable precision and accuracy, was 4.03µg/mL.

Accuracy

The accuracy of the method was evaluated by means of the recovery test. Table 3 shows the recovery data, which were obtained by the relationship between the amount of added standard and the amount detected. The method produced a mean recovery of 103.78% of the concentration, confirming the accuracy of the method. In this analysis, a recovery between 70 and 120% is acceptable.[26]

Stability of the analyte during analysis

The analytes in solution did not show any appreciable change in chromatographic profile for at least 72 h. No degradation products were observed, confirming the stability of the samples under the conditions employed.

Analysis of leaves, stems and roots extracts of *P. ovatum*

The retention times of the piperlonguminine (2) and piperovatine (1) standards were used to identify the corresponding peaks in the extracts of *P. ovatum*. Peak 1 was identified as piperovatine (1), with a retention time of 23.50 min., and peak 2 (24.46 min) as piperlonguminine (2) [Figure 2]. For determination of the piperlonguminine content in the extracts of *P. ovatum,* the regression equation y = 2.229.312.76 x - 1.611.067.58 was used. The concentrations of piperovatine were expressed as those of piperlonguminine. Figures 2a-c shows the chromatograms of the extracts of *P. ovatum* obtained from roots, leaves, and stems, respectively.

The extracts showed the same chromatographic profile [Figures 2a-c], but there were differences in the concentrations of the amides in the different parts of the plant [Table 4]. The leaves and roots contained the highest concentrations of piperlonguminine (2), and the stems and leaves showed the most concentrations of piperovatine (1); the difference was significant ($P < 0.05$).

Thus, HPLC method validated allowed the detection and quantification of amides in different parts of *P. ovatum*. The validation procedure demonstrated that the method showed linearity, precision and accuracy in the range studied. This procedure confirms that the technique developed provides a reliable analysis of the amides, and is appropriate for the quality control of extracts and phytopharmaceutical preparations produced with *P. ovatum*.

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