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Quantification of 4-nerolidylcatechol from *Pothomorphe umbellata* (Piperaceae) in rat plasma samples by HPLC-UV

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The oral dosages formulations from Pothomorphe umbellata *(Piperaceae) can only be therapeutically evaluated after establishing 4-nerolidylcatechol (4-NRC) pharmacokinetic profile in plasma. For that purpose, a unique analytical method validation using HPLC-UV detection was developed for analysis of rat plasma samples. The animals received 10 mg/kg b.w. of 4-NRC i.v.. Analytical conditions were set with C18 column, methanol: acetonitrile: water (62:20:18) as mobile phase, and a flow rate of 1.6 mL/min. The assay was linear from 1.0 - 80.0 mcg/mL. The recovery procedure was performed by liquid-liquid extraction showing quantitative extraction (106.4±8.7%). Limit of Quantification (LOQ) was found to be 1.0 mcg/mL. Interassay precision and accuracy ranged from values of 14.4-3.0% and 24.0–0.2%, respectively. Recovery after liquid-liquid extraction was found to be 106.5 ± 3%. The method is simple and reliable with total run time of less than 15 min.*

Uniterms:
- 4-Nerolidylcatechol
- *Pothomorphe umbellata*
- HPLC-UV
- Liquid-liquid extraction

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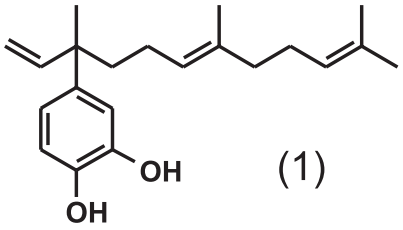
INTRODUCTION

In recent years, there has been a growing interest in the compound 4-nerolidylcatechol found in some *Piperaceae* shrubs. 4-Nerolidylcatechol (4-NRC) is the major secondary metabolite from *Pothomorphe peltata* and *Pothomorphe umbellata* traditionally used for treatment of hepatic disorders on Brazilian Southwest and Amazon area, respectively (Silva, 1926; Di Stasi *et al.*, 1994). Pharmacological studies involving quenching of lipid peroxidation have shown remarkable antioxidant activity for 4-NRC when compared with a-tocopherol (Barros *et al.,* 1996; Desmarchelier *et al.*, 1997). Corroborating these studies, 4-NRC 0.1% (w/w) was added to topic skin formulations showing UV protection and percutaneous absorption (Röpke *et al.,* 2002). Additionally, crude ethanolic extracts of *P. umbellata* leaves have demonstrated antimalarial activity (Amorim *et al.,* 1988) and absence of mutagenic effects in *Salmonella typhimurium* (Felzenszwalb *et al.,* 1987) and on micronuclear erythrocytes (Barros *et al.,* 2001). Furthermore, dimmers of 4-NRC have also shown anti-HIV activity (Gustafson *et al.*, 1992).

However, for preclinical studies it is essential to establish pharmacokinetic properties (De Smet, Rouwers, 1997; Hamburguer, Hostettmann, 1991) and assess pharmacological levels associated with the safety and efficacy of the drug. Therefore, a sensitive and reliable

analytical method must be developed to assess such properties for the compound of interest.

The chemical structure of 4-nerolidylcatechol (**1**) was first described in a hexane extract (Kijoa *et al.,* 1980), which also contained various phenylpropanoids such as apiol (Silva, Baver, 1972), dilapiol (Bernard, Tieli, 1978) and the sesquiterpene nerolidol. Since then, several other substances were identified in more polar extracts. These include some flavonoids, lignans (Bastos, 1998) and dimmers (peltatols A, B and C) of the prenylated catechol (Gustafson *et al.*, 1992).



(1)

Several analytical methods for phenols, catechols and polyphenols molecules are available. However, physicochemical properties of catechol-containing molecules show predominant water solubility (*i.e.* polyphenols) instead of liposolubility (log P=6.03), as depicted for 4-NRC (Freitas, 1999). Therefore, in response to the need for a sensitive method to quantify 4-NRC, we examined methods using liquid chromatography coupled to electrochemical detection (Cai *et al.*, 1999; Stremetzne, Jaehde, Schumack, 1997; Holmes, Eisenhofer, Goldstein, 1994; Lunte, 1987; El-Shafae, El-Domiaty, 2001) and ultraviolet detection (Romanova *et al.,* 2000; Rouseff *et al.,* 1992; Ruperez *et al.*, 1999; Castagnetta *et al.*, 1992), as primarily options. Such methods generally show suitable sensitivity for plasma samples, even when compared to more selective ones (Lee *et al.*, 1993; Stecher *et al.,* 2001). In addition, those equipments are easily handled and available in most laboratories.

Thus, the aim of this work was to develop an HPLC-UV method for preliminary quantification and assessment of biological interferences of 4-nerolidylcatechol in rat plasma after *iv* dosing.

MATERIAL AND METHODS

Materials and reagents

HPLC grade acetonitrile, methanol and hexane were purchased from Fisher Scientific (Pittsburgh, PA, USA), filtered under vacuum through 0.45 mm Teflon filter (Millipore, Bedford, MA, USA) and degassed under ultrassonication (15 min/L). 2, [6]-Di-*tert*-butyl-*p*-cresol (BHT) was purchased from Sigma (St. Louis, MO, USA). Male Wistar rats (275-325g) were purchased from Faculdade de Medicina Veterinária e Zootecnia – Universidade de São Paulo. Preparative column chromatography under vacuum and preparative radial thin-layer chromatography (Chromatotron™ model 8924) experiments used Silica gel 60 H (Merck, 63 - 210 mm) and Silica gel PF with binder (Merck, Cat.7749) as adsorbents, respectively.

Methods

Chromatographic conditions

A reverse phase column Zorbax SB C18, 4.6 X 150 mm, 5-mm (Chromatography Specialties, Mississauga, ON, Canada) with an HPLC Constametric 3200 system was coupled to UV detector (1049A WATERS) at 282 nm. Auto-sampler interface was accomplished by HP 3396 series III with a Reodyne injector loop (20 mL). Aliquots were injected and eluted with methanol-acetonitrile-water (62:20:18, v/v/v) as isocratic mobile phase mixture. Flow rate was set at 1.6 mL/min.

Purification of 4-nerolydilcatechol and its extraction from plasma

Leaves of *Pothomorphe umbellata* were collected in February 1998 at Piperaceae's garden of Instituto de Química, Universidade de São Paulo. A voucher specimen (Kato-0363) was identified by Dra. Inês Cordeiro (Universidade Federal do Paraná - Curitiba) and deposited in the herbarium of Instituto de Botânica (Secretaria do Meio Ambiente do Estado de São Paulo).

Leaves (1 kg) were dried, milled and exhaustively extracted with MeOH yielding 184.2 g of crude extract after solvent evaporation. The leaves extract (180.0 g) was resuspended in MeOH:H_2O (8:2) and filtered through a celite column (10 x 10 cm) to remove pigments and lipophilic interferents from the matrix. Solvent evaporation yielded a solid residue (84.1 g). Next, a small aliquot (10 g) was applied to a flash-filtering column on silica gel 60H (10 x 10 cm) using $CHCl_3$ as eluent. The $CHCl_3$ extract residue (6.1 g) was solubilized in hexane (20 mL), fractionated (6x) and submitted to preparative radial thin-layer chromatography (PR-TLC, 110.0 x 4.0 mm). Chromatographic conditions were flow rate of 8.0 mL/min and eluotropic gradient of hexane: CH_2Cl_2: methanol (10:5:1 up to 0:5:1) during 94 min. Separation of constituents was monitored with a UV lamp at 254 nm. Sample fractions were compared with authentic standard sample and submitted to final purification at PR-TLC (hexane:ethylacetate 80:20; 8.0 mL/min). Evaporation of

the residue yielded a pale yellow oil (1.2 g) chemically identified by ^{1}H and ^{13}C NMR, as 4-NRC. Its purity was not lower than 96% in analytical chromatographic conditions described herein.

Root extract standardization

Pothomorphe umbellata roots were cleaned, dried, milled (822.5 g) and percolated in 60% ethanol (18 L) as directed by Method A of Pharmacopeia dos Estados Unidos do Brasil (Silva, 1959). The pharmacopoeical preparation was submitted to solvent evaporation yielding a dried crude extract (78.5 g/9.5%). Standardization was carried out using five accurate weighted (0.5 mg/mL) crude extract replicates. Quantification was measured against pure 4-NRC standard solutions in methanol. Results showed 21.5 ± 3.3% of 4-NRC contents.

4-Nerolidylcatechol solutions and calibration standards

4-NRC stock solution was prepared in methanol to achieve 100.0 µg/mL, as a final concentration. Next, work solutions were prepared to give five standard points in mobile phase that were stored at –70 °C. All five work stock solutions were set in a manner such that the same aliquot amount was taken and spiked in plasma matrix (*e.g.*100 µL work solution/1 mL plasma). Subsequently, calibration standards were prepared in pooled plasma samples spiked with known amounts of the work solutions to give final concentrations of 1.0; 3.0; 20.0; 50.0; 80.00 µg/mL. Aliquots of 250 µL spiked plasma samples were transferred to borosilicate tubes using micropipettes (Gilson, USA), capped with PE plugs (Fischer Scientific, USA) and kept at – 17 °C. Thaw of samples were carry out when necessary to run daily calibration curves.

Plasma sample preparation

The 4-NRC extraction procedure from plasma samples was prepared in media containing BHT (8.0 mg/L) as antioxidant agent. 1 mL of methanol/ethanol (1:1) was added to 250 µL of plasma, followed by vortex mixing (1 minute). Next, water (500 µL) and hexane (2 mL) were added. Subsequent to sealing the tubes, horizontal agitation was performed (3 x 20 min). Fresh hexane portions were added three times followed by centrifugation. The upper (hexane) layers were removed after "snap freezing" technique under liquid nitrogen and transferred to clean borosilicate tubes. After gathering it was evaporated under N_2 stream. The residue was ressuspended in 250 µL of methanol/water (85:15). Solutions were covered with aluminum foil and left at room temperature for 10 min and 20 µL were injected in HPLC system.

Validation results

The method was validated for linearity, selectivity, recovery, accuracy, precision and stability in plasma samples. The analyte retention time in plasma and crude plant extract samples was found to be 6.3 (±0,23) min and the total run time was set at 14.5 min.

• Assessment of Linearity and Selectivity

Linearity over dynamic range (1.0 to 80.0 µg/mL) was assessed by duplicate analysis of five points, during 3-day period. 4-NRC quantification was measured by peak height response and derived from a standard curve performed in both plasma and methanol matrix (crude extract quantification). Results based on average correlation coefficient-R^2 showed great linearity (0.994±0.002).

Selectivity was determined by analyzing blank plasma samples from four different Wistar rats. Suitable 4-NRC separation from plasma constituents was shown and no coincident interferences to analyte retention time were detected.

• Recovery of 4-nerolidylcatechol

Following plasma spiking, the amount of 4-NRC recovered was estimated at all five-point calibration standards and analyzed on three different days (Table I). Pooled plasma samples were prepared based on standard technique addition. Six estimations were made on each calibration standard. Recovery was calculated as the ratio

TABLE I - Summary for recovery of added 4-nerolidylcatechol

4-NRC added concentration in plasma (mg/mL)	Average concentration (N=6) ± SD	Coefficient of Variation (%)	Recovery (%)
1	1.06±0.134	12.66	105.8
3	3.57±0.228	6.38	119.0
20	18.31±1.723	9.41	91.6
50	53.94±3.373	6.25	107.9
80	85.86±8.210	9.56	107.3

between peak height response of extracted and not extracted analyte (standard work solutions added to extracted blank plasma) from each plasma sample. The value is expressed as the ratio between average experimental and nominal concentration of the analyte, multiplied by 100%. The results summarized in Table I indicate quantitative average recovery of 106.4 ± 8.7% in plasma.

• Accuracy and precision

Analytical method accuracy and precision were assessed by daily duplicate analysis of samples containing known amounts of 4-nerolidylcatechol (1.0 up to 80.0 μg/mL), during 3-day period. These samples were prepared by the standard addition technique. Spiked plasma samples were stored at -80 °C, fractionated as single aliquots (250 μL) throughout the period of experiment.

The limit of detection (LOD) was determined using four pooled blank plasma sample spiked with work standard solutions. LOQ was set for the lowest concentration standard curve measured with acceptable accuracy and precision (SD±25.0%). LOQ was inferred as three times the LOD, which was found to be 1.0 μg/mL.

Interassay precision was determined by coefficient of variation (CV*)* obtained by the ratio between standard deviation and average mean of replicates, during 3-day validation. Sample precision was found to be in the range of 14.4-3.0%, as detailed on Table II. Intrassay precision was surely lower than interassay precision and not shown here.

Accuracy was assessed by the ratio between average relative error around the mean and added concentration, during 3-day validation experiments. The results are summarized in Table II. The accuracy relative error found for LOQ was 24% while it was between 19.7–0.2% for 3-80.0 μg/mL concentration range.

• Assessment of stability under freeze/thaw conditions

Storage conditions were evaluated by freeze-thaw stability comparing the 4-NRC amount found in samples that had been frozen (-80 °C) and thawed (20 °C) three times with fresh plasma samples. Each freeze-thaw cycle duration was no less than 12 h. The stability maintained for at least 4 weeks, based on chromatographic evidence indicating no apparent deterioration, which means not more than 2.9% difference on the amount of 4-NRC. These

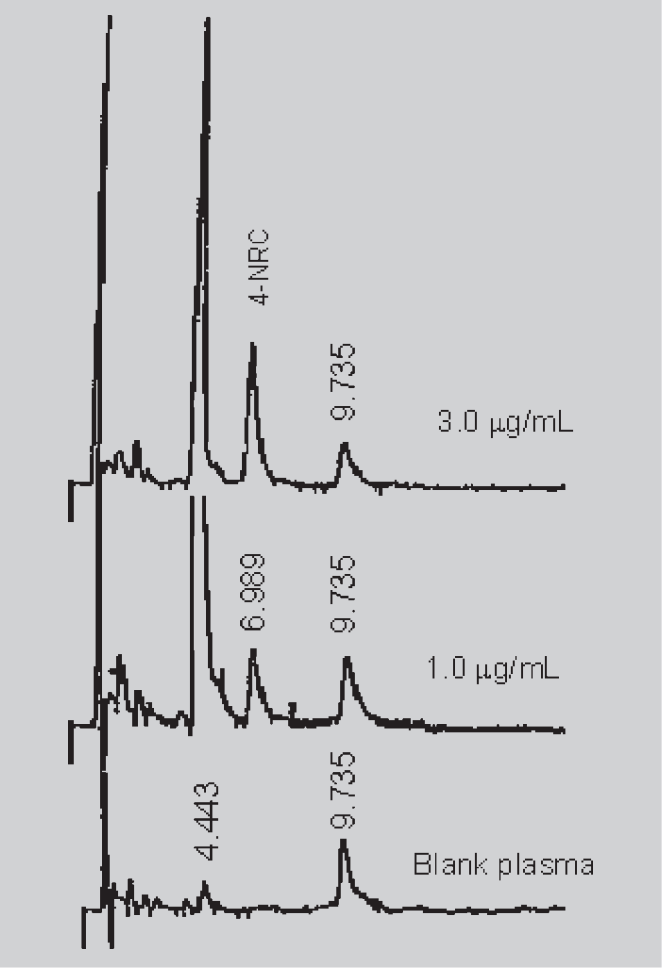


FIGURE 1 - Typical chromatogram of blank plasma (**C**) and spiked plasma with 4-nerolidylcatechol at 1,0 μg/mL de 4-NRC (**B**) and 3,0 μg/mL de 4-NRC (**A**).

TABLE II - Summary of interassay precision and accuracy of calibration curves during 3-day

	R^2	slope	intercept	4-NRC spiked plasma concentrations (μg/mL)				
				1. 0	3. 0	20. 0	50. 0	80. 0
Mean (n=9)	0. 994	4213. 47	1628.58	1. 24	3.59	17. 89	50. 87	80. 13
SD $_{interday}$	0. 002	368. 56	1676.94	17.80	0.35	1.86	3. 24	2. 39
Precision*	0. 22%	8. 75%	——	14.36%	9.75%	10.38%	6. 37%	2. 98%
Accuracy**	—-	——	——	24.0%	19.67%	10.55%	1.74%	0.16%

* CV (%)- standard deviation/mean x 100%; ** Error %- (found concentration – nominal concentration)/nominal concentration x 100%

results indicate 4-NRC is stable in rat plasma after three freeze-thaw cycles.

Pharmacokinetic data applications

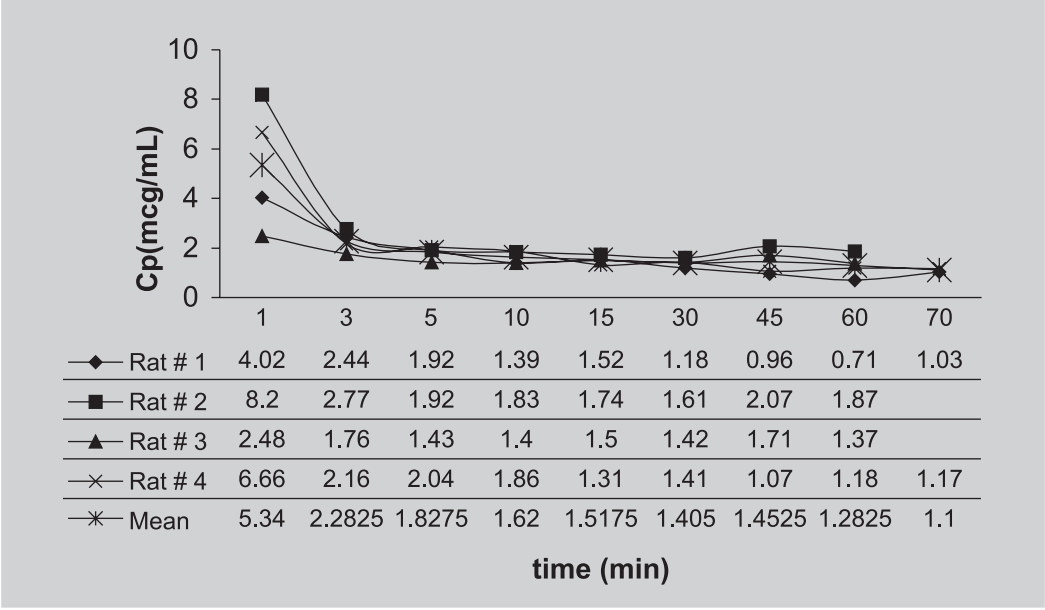
4-NRC parenteral solution (2.5 mg/mL) was prepared in 30% hydroxypropyl-β-cyclodextrin (Sigma, St Louis, USA) and filtered (Millipore , 0.45 μm). The drug was administered as *i.v. bolu*s (10 mg/kg) through dissected femoral vein (27G needle), while animal body conditions (temperature and plasma volume) were maintained under control (warming paddle and 5 U.I. heparin solution reposition). Blood samples were collected after distal and proximal isolation of carotid artery portion (thread silk, 5.O.1), following its canulation with a catheter (FEP tubing, 22 G). Sample collection (eight time points; 300 μL) was set at the following schedule time: 0.0; 2.5; 5.0; 7.5; 10.0; 15.0; 17.5; 20.0; 30.0; 45.0; 60.0; 70.0 min on heparinized eppendorf tubes. Blood samples were centrifuged (3000 rpm/2 min) and plasma was stored (–80 °C) until analysis. The 4-NRC plasma concentration obtained at each time point is presented in Figure 2.

DISCUSSION AND CONCLUSION

A simple HPLC-UV method for analysis of 4-nerolydilcatechol in plasma was developed. This method is based on liquid-liquid extraction and an isocratic reversed-phase HPLC analysis with a total run time of less than 15 min, a run time frequently targeted with ultraviolet detection.

The accuracy maximum relative error for LOQ was set at ± 25%. It can be partially justified by the absence of a suitable internal standard and also, by the 4-NRC high affinity to blood cells. This later assertion is based on additional evaluation of some bench-top conditions. Preliminary studies reinforce suspicions of high erythrocyte partition coefficient based on a high liposolubility of 4-NRC (Freitas, 1999). The assay procedure was based on literature (Boulton, Walle, Walle, 1999). Blood samples were spiked with 4-NRC (50 mg/mL) and quantified on triplicate. Analytical conditions were simulated keeping blood samples at controlled temperature (0 °C and 37 °C) and incubation time (0.0 and 60.0 min). Photoxidation during bench-top and in-process assays, including auto-sampler injection were prevented by aluminum foil protection. Results showed that blood aliquots, which were maintained at 37 °C/1 h, yielded lower 4-NRC average recovery (66.9 %) in plasma when compared to samples immediately extracted or when kept under refrigeration (0 °C/1 h). Similar findings are frequently seen on other lipophilic drug profiles (Derendorf *et al.*, 1984; Derendorf, Garret, 1983). Several methodological conditions as sample concentration, pH, temperature and protein binding (Boulton, Walle, Walle, 1999) are also important variables, but not yet controlled here. Therefore, further developments on this issue are to be carried out.

As a final point, the analytical method presented herein can be applied as an important approach to standardization of *Pothomorphe umbellata* roots extracts, previously to the determination of 4-NRC plasma level during pre-clinical phase trials. Additionally, this method



	1	3	5	10	15	30	45	60	70
Rat # 1	4.02	2.44	1.92	1.39	1.52	1.18	0.96	0.71	1.03
Rat # 2	8.2	2.77	1.92	1.83	1.74	1.61	2.07	1.87	
Rat # 3	2.48	1.76	1.43	1.4	1.5	1.42	1.71	1.37	
Rat # 4	6.66	2.16	2.04	1.86	1.31	1.41	1.07	1.18	1.17
Mean	5.34	2.2825	1.8275	1.62	1.5175	1.405	1.4525	1.2825	1.1

FIGURE 2 - Plasma concentration level of 4-NRC following *iv* bolus dosing of 10 mg/kg (2.5 mg/mL in 30% hydroxypropyl-β-cyclodextrin).

can be applied to preliminary pharmacokinetic studies in animal model leading to an important overview of the drug distribution and elimination process. Kinetica 2000™ software was used and *Vd* and *Kel* parameters were calculated. The values found after *i.v.* dosing (100 mg/kg) were 0.5 L and 0.11 min, respectively, meaning a large distribution and rapid elimination rate, as expected for highly lipophilic drugs, as for 4-NRC.

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RESUMO

Quantificação de 4-nerolidilcatecol de *Pothomorphe umbellata* (Piperaceae) em amostras de plasma de rato por CLAE-UV

O 4-nerolidilcatecol representa o metabólito secundário mais abundante de Pothomorphe umbellata *(Piperaceae), cujo uso terapêutico deve ser fundamentado após a caracterização farmacocinética do mesmo (4-nerolidicatecol). Os níveis séricos de animais tratados com 10 mg/kg do fármaco i.v. foram avaliados por validação analítica em CLAE-UV. As condições cromatográficas empregadas foram fase móvel de acetonitrila:metanol:água (62:20:18), coluna RP-18, fluxo de 1,6 mL/min e detector UV em 282 nm, com tempo total de corrida inferior a 15 minutos. Observou-se linearidade do 4-NRC em plasmas na faixa de 1,0 a 80,0 mcg/mL, com valores de precisão e exatidão entre 14,4-3,0% e 24,0–0,2%, respectivamente. O procedimento de extração líquido–líquido apresentou reprodutível e com recuperação quantitativa (106,5%±3,0%).*

UNITERMOS: 4-Nerolidilcatecol. Pothomorphe umbellata. CLAE-UV. Extração líquido-líquido.

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