Tetra-crowned porphyrin as P450 biomimetic model for carbamazepine oxidation

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Abstract
A substituted porphyrin bearing four crown ether units, H₂(TCP), was synthesized from the reaction between (5,10,15,20-tetra(o-aminophenyl)porphyrin) and the acyl derivative of the ether (4-carboxy-18-crown-6). The free-base porphyrin was characterized by C, N, and H elemental analysis; UV-vis and IR spectroscopies; and ¹H NMR. The corresponding ironporphyrin, Fe(TCP)Cl, was obtained via iron insertion into H₂(TCP). Fe(TCP)Cl was employed as catalyst for carbamazepine (CBZ) oxidation by iodosylbenzene (PhIO), 3-chloroperoxybenzoic acid (m-CPBA) or sodium hypochlorite (NaOCl), in methanol or in a biphasic water/dichloroethane system. The crowned ironporphyrin proved to be a highly efficient and selective catalyst for CBZ epoxidation even in the biphasic dichloroethane /H₂O system, with no need for an additional phase transfer agent.

Keywords: Porphyrins, crown ether, oxidation, carbamazepine, catalysis

Introduction
There has been considerable research interest in the synthesis of porphyrins with various substitutents in view of their ability to function as models that mimic several biofunctions such as heme oxygenation and cytochrome activity.¹ The design of model compounds depends on the judicious choice of the substituent and the peripheral position to be substituted. Minor changes in the basic structure of the macrocyclic ring bring significant changes to the properties of porphyrins, a fact that has been widely employed in the design of a great variety of compounds,
for purposes as specific as the preparation of metalloenzyme models and the synthesis of porphyrin complexes for application in analytical and electroanalytical chemistry, cancer photodynamic therapy, energy converters, catalysis, among others.\textsuperscript{2-6}

Covalently-linked porphyrin dimers, sterically crowded porphyrins and porphyrins substituted with organic moieties with acceptor functionalities have been studied to elucidate molecular mechanisms involved in energy transfers, reversible oxygenations and light-induced electron transport, respectively.\textsuperscript{7,8}

Crowned porphyrins result from the covalent binding of at least one crown-ether unit to a porphyrin. This type of macromolecule has attracted much attention because both entities exhibit very special properties concerning the complexation of either metal ions or organic molecules.\textsuperscript{6,9,10}

From a catalytic point of view, however, porphyrins functionalized with crown ethers have been little studied. Results indicate that when the crown ethers lie close to the metal site, they can interact with the substrate, orienting it toward the catalytic site. This orientation, coupled with the steric effect imposed by the presence of the ether, may result in high selectivity for the catalytic reaction.\textsuperscript{11} When two or more crown ether units are located on the same side of the porphyrin plane, they can act as clamps that orient and direct the substrate toward the cavity of the active site.\textsuperscript{12} The presence of a polyether attached to a porphyrin may also aid solubilization of the water-soluble oxidant and/or substrate in the organic medium, avoiding the need for a phase transfer agent.\textsuperscript{13}

Carbamazepine (CBZ), $5H$-dibenz[b,f]azepine-5-carboxamide, is an anticonvulsant used in clinical practice as a first-line treatment for generalized tonic-clonic and partial seizures.\textsuperscript{14} This anticonvulsant is a substrate suitable for epoxidation studies and was first used by Meunier in studies \textit{in vitro} employing water-soluble metalloporphyrins as catalyst. Over the last two decades, 33 CBZ metabolites have been isolated and identified in the urine of patients on an oral dose.\textsuperscript{15-17} Of these metabolites, carbamazepine 10,11-epoxide (CBZ-EP) is the most important from a clinical point of view.

In previous work we reported the synthesis and characterization of a porphyrin covalently bound to a crown ether unit through the \textit{ortho}-phenyl ring of the precursor 5-mono(o-aminophenyl)-10,15,20-triphenylporphyrin. The catalytic activity of its corresponding ironporphyrin in the oxidation of alkenes by sodium hypochlorite was also investigated.\textsuperscript{13} In the present work we have investigated the catalytic activity of the ironporphyrin 5,10,15,20-tetrakis[1,4,7,10,13,17-hexoxacyclohexadecane-4-amide benzo] 5,10,15,20-tetraphenylporphyrin iron(III) chloride, $[\text{Fe(TCP)Cl}]$ (Figure 1), in the oxidation of carbamazepine by several oxidants such as iodosylbenzene, \textit{m}-CPBA, and sodium hypochlorite. The catalytic activity of this system was compared with that of the 5-mono[1,4,7,10,13,17-hexoxacyclohexadecane-4-amidebenzo]-10,15,20-triphenylporphyrin iron(III) chloride, $\text{Fe(MCP)Cl}$ (Figure 1), and of the corresponding precursor amino ironporphyrins in order to evaluate the crown ether effect on catalytic activity.
**Results and Discussion**

The [5,10,15,20-tetra(o-aminophenyl)] porphyrin (Figure 1) was selected as the precursor complex for the synthesis of the crowned porphyrin $H_2(TCP)$ because it presents NH$_2$ groups capable of forming a covalent bond with the crown ether derivative. In the resulting compound, the crown ether units bind to the porphyrin through four points, namely the ortho position of the phenyl rings, and they remain close to the central cavity of the porphyrin.

The crowned porphyrin 2, $H_2(TCP)$, was obtained via formation of a peptide bond between the [5,10,15,20-tetra(o-aminophenyl)] porphyrin and the acyl chloride derivative of the 4-carboxybenzo-18-crown-6 ether (1). Formation of the crowned porphyrin was monitored by TLC, by comparing its retention factor ($R_f = 0.54$) with that of the tetra-amine substituted
porphyrin \((R_f = 0.76)\), using methanol:acetone 1:1 as eluent. The high number of oxygen atoms on the ring of \(\text{H}_2\text{(TCP)}\) explains the lower mobility of this crowned porphyrin on TLC plates.\(^\text{18}\)

The UV-vis spectrum of the crowned porphyrin \(\text{H}_2\text{(TCP)}\) displayed a Soret band at 418 nm, bands in the visible region at 512, 550, 592 and 650 nm, being similar to the UV-vis spectrum of the parent amino-substituted porphyrin. The UV-vis spectrum of \(\text{H}_2\text{(TCP)}\) also displayed the typical crown ether bands at 230, 266 and 296 nm (Figure 2). The binding of four crown ether units to the porphyrin did not lead to significant changes in the energy of the orbitals involved in the porphyrin electronic transitions.

![UV–vis spectra of the crowned porphyrin \(\text{H}_2\text{(TCP)}\) in dichloromethane.](image)

**Figure 2.** UV–vis spectra of the crowned porphyrin \(\text{H}_2\text{(TCP)}\) in dichloromethane.

The IR spectrum of the tetra-crowned porphyrin displayed the bands due to the ether vibrations \(\text{Ar-O-C}\) and \(\text{C-O-C}\) at 1285 \(\text{cm}^{-1}\) and 1120 \(\text{cm}^{-1}\), respectively. A strong band at 1620 \(\text{cm}^{-1}\) was assigned to the axial deformation of the \(\text{C=O}\) group on the amide, while a sharp band at 3330 \(\text{cm}^{-1}\) was attributed to the internal \(\text{N-H}\) of the porphyrin ring.

The crowned ironporphyrin was easily obtained by reacting the free-base porphyrin with iron(II) chloride. The resulting \(\text{Fe(TCP)Cl}\) was initially purified by column chromatography on alumina, followed by recrystallization in dichloromethane/pentane. The signal at -2.76 ppm
(singlet, 2H) due to N-H (pyrrole) in the $^1$H NMR spectrum of the free-base porphyrin disappeared in the spectrum of Fe(TCP)Cl, indicating successful iron insertion. Other characteristic signals of the porphyrin were observed at 3.25-3.98 (80H, OCH$_2$ of the crown ether), 8.8 (4H, CONH), 9.32 (8H, pyrrole), 8.4-7.23 (28H, aromatic ring), which is in agreement with the proposed structure.

**Catalytic studies on carbamazepine oxidation**

The main metabolic pathway undergone by carbamazepine in biological systems is its oxidation by cytochrome P450 enzymes, which generates carbamazepine 10,11-epoxide (CBZ-EP). The latter compound is then hydrolyzed to carbamazepine trans-diol (CBZ-DiOH) by the enzyme epoxide hydrolase (Figure 3)$^{6,14,17,19,20}$.

![CBZ-EP and CBZ-DiOH](image)

**Figure 3.** Carbamazepine (CBZ) and its major metabolites CBZ-EP and CBZ-DiOH.

Carbamazepine oxidation catalyzed by metalloporphyrins has already been investigated in homogeneous medium$^{21,22}$ and supported systems.$^{23}$ However, to the best of our knowledge, studies using crowned metalloporphyrins as catalysts for the oxidation of this drug have not yet been reported.

The catalytic activity of the iron(III) tetra-crowned porphyrin, Fe(TCP)Cl, in the oxidation of carbamazepine by either iodosylbenzene, $m$-CPBA and sodium hypochlorite was evaluated. The results were compared to those obtained with the parent tetra-amino-substituted ironporphyrin, Fe(T$_{NH_2}$PP)Cl, and the previously synthesized mono-crowned porphyrin, Fe(MCP)Cl (Figure 1)$^{13}$.

The catalytic oxidation results in homogeneous medium are presented in Table 1. We observed that the crowned ironporphyrins proved to be highly efficient and selective toward CBZ-EP and no other product was obtained. Knowing that CBZ-EP is the main metabolite generated during carbamazepine metabolism by P450 *in vivo*, these metalloporphyrins are good biomimetic models of this enzyme.
Table 1. Yields of carbamazepine epoxide from oxidations by various oxidants in homogeneous medium (methanol) catalysed by ironporphyrins

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Oxidant</th>
<th>Yield of CBZ-EP&lt;sup&gt;b&lt;/sup&gt; (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fe(MCP)Cl</td>
<td>PhIO</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>FeM&lt;sub&gt;NH2&lt;/sub&gt;TPP</td>
<td>PhIO</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Fe(MCP)Cl</td>
<td>m-CPBA</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>FeM&lt;sub&gt;NH2&lt;/sub&gt;TPP</td>
<td>m-CPBA</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>Fe(TCP)Cl</td>
<td>PhIO</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>FeT&lt;sub&gt;NH2&lt;/sub&gt;PP</td>
<td>PhIO</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>Fe(TCP)Cl</td>
<td>m-CPBA</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>FeT&lt;sub&gt;NH2&lt;/sub&gt;PP</td>
<td>m-CPBA</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Catalyst:substrate:oxidant molar ratio = 1:40:35; blank reactions (absence of catalyst).

<sup>b</sup>CBZ-EP not detected. <sup>c</sup>% yields based on initial CBZ.

The crowned ironporphyrins (FeMCP)Cl and (FeTCP)Cl are very efficient catalysts for carbamazepine oxidation compared with the precursor amino ironporphyrins, showing the beneficial effect of the crown ether substituents (Table 1). These substituents act in two ways. The first mode consists of an associative effect with the porphyrin, favoring the approach of both the oxidant and the substrate to the catalytic center, as well as the interaction between the reactants and the catalytic site, probably through hydrogen bond interactions. A similar but much smaller effect has been previously observed in the oxidation of cyclooctene and cyclohexene catalyzed by the mono-crowned ironporphyrin. The second mode consists of the steric effect imposed by the presence of the ether units, which avoids the bimolecular destruction of the ironporphyrin. This was evident from the comparison between the UV-vis spectra of the crowned ironporphyrin and the amine-substituted ironporphyrin after the oxidation reactions. In the reactions with the mono- and tetra-amine-substituted ironporphyrins, solution bleaching was observed after reaction completion and the spectrum revealed the disappearance of Soret band, indicating total destruction of the catalysts. In the reactions with the mono- and tetra-crowned ironporphyrins, though, the Soret band was maintained after the reactions, indicating that the crown ether units protected the catalyst from self-oxidation.

When four units of crown ethers are linked in the same porphyrin ring, they can act as clamps that orient and direct the substrate toward the cavity where the active site is located, thus leading to higher yields. This effect can be observed by comparing the catalytic results achieved with Fe(TCP)Cl <i>versus</i> those obtained with Fe(MCP)Cl (Table 1 and Table 2, entries 1 <i>versus</i> 5, 3 <i>versus</i> 7).

The best oxidant for the Fe(TCP)Cl-catalyzed epoxidation of carbamazepine is m-CPBA. The formation of the epoxide as main product indicates that the [Fe<sup>IV</sup>O(P•⁺)] species is the active oxidant, generated through heterolysis of the peracid, which is favored in the presence of...
the electron-withdrawing substituents on the phenyl group of this oxidant.\textsuperscript{24,25,26} Fe(TCP)Cl produced better yields compared with Fe(MCP)Cl (Table 1, entries 3 and 7).

The catalytic results obtained when NaOCl and \textit{m}-CPBA were used as oxidant in a biphasic water/dichloroethane system give evidence of another beneficial effect of the crown ether substituent (Table 2).

### Table 2. Yields of 10,11-carbamazepine epoxide from oxidations by various oxidants in biphasic systems (dichloroethane/water) catalysed by ironporphyrins

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst\textsuperscript{a}</th>
<th>oxidant</th>
<th>Yield of CBZ-EP\textsuperscript{b} (%)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fe(MCP)Cl</td>
<td>NaOCl</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>FeMN\textsubscript{2}TPP</td>
<td>NaOCl</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Fe(MCP)Cl</td>
<td>\textit{m}-CPBA</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>FeMN\textsubscript{2}TPP</td>
<td>\textit{m}-CPBA</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Fe(TCP)Cl</td>
<td>NaOCl</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>FeTN\textsubscript{2}PP</td>
<td>NaOCl</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Fe(TCP)Cl</td>
<td>\textit{m}-CPBA</td>
<td>81</td>
</tr>
<tr>
<td>8</td>
<td>FeTN\textsubscript{2}PP</td>
<td>\textit{m}-CPBA</td>
<td>8</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Catalyst:substrate:oxidant molar ratio = 1:40:35; blank reactions (absence of catalyst).
\textsuperscript{b}CBZ-EP not detected. \textsuperscript{c} % yields based on initial CBZ.

The crowned ironporphyrins are able to catalyze the oxidation of CBZ even in the biphasic system, leading to epoxide yields as high as 81\%, with no need for a phase transfer agent (Table 2, entry 7), contrary to other biphasic systems using metalloporphyrins and this oxidant, described previously.\textsuperscript{27} These results show that the crown ether unit may also aid solubilization of the aqueous oxidant in the organic phase where the substrate is found. In this way, these substituents promote interaction of the oxidant with CBZ and catalyst. Lower yields (< 6\%) were obtained when the amine-substituted ironporphyrin and NaOCl were employed in the same conditions (Table 2 entries 2 and 6).

When NaOCl was used as oxidant, the weak hypochlorous acid generated spontaneously in the aqueous phase in the reaction conditions (NaOCl pH 10) is distributed between the aqueous phase and the dichloroethane phase containing the substrate and the crowned ironporphyrin. HOCl is the oxidizing species, and the crown ether could favor formation of the HOCl/Fe-porphyrin complex through a “pull” effect as described by Banfi \textit{et al.}\textsuperscript{11} for a similar system. The basicity of the crown ether oxygen atoms might facilitate the cleavage of the O-H bond (Figure 4) thus facilitating formation of the iron-oxo porphyrin \pi-cation radical [Fe\textsuperscript{IV}O(P\textsuperscript{+}+)] species responsible for oxygen transfer to the substrate. Similarly, the H\textsuperscript{+} ion of \textit{m}-CPBA promotes interaction with the crown ether unit (Figure 4), which facilitates O-O bond heterolysis and
results in the formation of the [Fe$^{IV}$O($P^+$)] species, resulting in the epoxide product in good yields.

![Figure 4](image-url)

**Figure 4.** Scheme for [Fe$^{IV}$O($P^+$)], species formation through the ‘pull’ effect, adapted from Banfi et al.$^{11}$

Also, when NaOCl is used as oxidant, the ion Na$^+$ can be complexed with the units of the crown ether, thus dragging the oxidant near to the catalytic site. This increases the concentration of the ion OCl$^-$ that can substitute the axial (chloride) ligand, improving the catalysis. The higher number of crown ether units in Fe(TCP)Cl increase this effect, leading to high epoxide yields (Table 2, entry 1 *versus* 5).

**Conclusions**

An ironporphyrin linked to four crown ether units was obtained and it proved to be a highly efficient and selective catalyst for CBZ epoxidation even in the biphasic dichloromethane/H$_2$O system, with no need for an additional phase transfer agent. Knowing that CBZ-EP is the main metabolite generated during carbamazepine metabolism by P450 *in vivo*, this metalloporphyrin is a good biomimetic model of this enzyme. We also observed that the catalytic activity of the metalloporphyrin depends on the number of crown ether units attached to the porphyrin. When four crown ethers are linked to the same porphyrin ring, they can act as clamps that orient and direct the substrate toward the cavity of the catalytic site. This effect will be explored with other substrates in order to obtain more regioselective oxidations.
Experimental Section

General Procedures. UV-vis spectra were obtained in a Hewlett-Packard 8452A diode array spectrometer. Infrared spectra were obtained in an FTIR 1600 Perkin Elmer spectrometer. \textsuperscript{1}H NMR spectra were recorded on a Bruker DRX400, 9.4 Tesla (400 MHz for proton frequency) equipment, in a BBO 5 mm probe at 298 K, using CDCl\textsubscript{3} as solvent. TMS was used as reference. The analytical HPLC analyses were performed on a SHIMADZU liquid chromatograph equipped with an LC-10AS solvent pump, an SPD-M 10A VP spectrophotometric detector (\( \lambda = 210 \text{ nm} \)) coupled to a CTO-10A VP column oven, and an SCL-10A VP system controller. Separation of carbamazepine and the oxidation product was carried out in a Lichrospher 100RP – 18 column, with a particle size of 5\( \mu \)m (125mm x 4mm), supplied by Merck. The analytical column was protected by a Lichrospher guard column (4 mm x 4 mm).

Materials

Unless otherwise stated, all compounds were purchased from Aldrich or Merck and were of analytical grade. Methanol was refluxed over a magnesium and iodine mixture, and stored over 4 Å molecular sieves after distillation. Dichloromethane was distilled twice over sodium sulfate and stored over 4 Å molecular sieves. Iodosylbenzene was prepared from iodobenzene in two steps, following the method described by Saltzmann and Sharefkin.\textsuperscript{29} Its purity (97%) was determined by iodometric titration. Kiesegel 60 Merck (230-400 mesh) silica gel was used for column chromatography. Aluminum backed silica gel 60 F254 plates (Merck) were employed for thin layer chromatography (TLC). The free base [5,10,15,20-tetra(o-aminophenyl)porphyrin] was used as purchased from Mid Century.

Activation of 4-carboxybenzo-18-crown-6 ether (1). SOCl\textsubscript{2} (10 mL) was distilled over quinoline and linseed oil twice,\textsuperscript{30} and added to the 4-carboxybenzo-18-crown-6 ether (500 mg, 1.4 \( \times \) 10\textsuperscript{-3} mol). The system was refluxed at 80 °C for 5 h. Excess SOCl\textsubscript{2} was removed in vacuum, giving 450 mg of the corresponding acyl chloride derivative of the crown ether.

Synthesis of the crowned porphyrin, H\textsubscript{2}(TCP) (2). The porphyrin 5,10,15,20-tetrakis(o-amino)porphyrin, H\textsubscript{2}(T\textsubscript{NH2}PP) (80 mg, 1.2 \( \times \) 10\textsuperscript{-4} mol) was dissolved in previously distilled and dried dichloromethane (250 mL), and the acyl chloride derivative of the crown ether 1 (421 mg, 1.2 \( \times \) 10\textsuperscript{-3} mol) was added to the resulting solution. The mixture was stirred at 30 °C for 24 h, and the solution became green. The solvent was removed in vacuum, and a violet crystalline solid was obtained. This solid was dissolved in chloroform (50 mL) and this organic phase was washed three times with deionized water, to remove excess crown ether. The solvent was removed in vacuum. The solid was purified on an alumina column, using a methanol/acetone 1:1 solvent mixture as eluent. The crowned porphyrin was eluted as the second band. After solvent removal, the crowned porphyrin 2 was recrystallized from a dichloromethane/pentane mixture. The solid was filtered off, yielding 296 mg of the solid porphyrin (71%). UV-vis: \( \lambda \text{max}, \text{nm} \left( \varepsilon \times 10^3 \text{ L. mol}^{-1}.\text{cm}^{-1} \right) \) 230 (284.6), 266 (257.4), 296 (184.7), 418 (301.2), 512 (9.3), 550 (3.7), 592
IR cm⁻¹ (KBr): 3425(CONH), 3330(NH), 1585, 1590(Ar), 1620(C=O), 1635(C=N), 1285(ArOC), 1120(COC). Anal. calcd. for C₁₁₂H₁₂₂N₈O₂₈, %: C, 66.32; H, 6.06; N, 5.52; O, 22.09. Found: C, 66.89; H, 6.42; N, 5.72; O, 22.58. ¹H NMR (CDCl₃): δ, ppm -9.32 (8H, pyrrole H), -2.76 (2H, pyrrole) 8.5 (4H, CONH) 3.25-3.98 (80H, OCH₂ crown ether), 8.4-7.23 (28H, ArH).

Synthesis of the crowned ironporphyrin, Fe(TCP)Cl (3). Dimethylformamide (20 mL) was placed in a round-bottomed flask (100 mL) and argon through it for 30 min. Porphyrin 2 (200 mg, 9.6 × 10⁻⁵ mol) and FeCl₂·4H₂O (300 mg, 1.5 × 10⁻³ mol) were added to the flask, and the mixture was refluxed for 22 h, under magnetic stirring. The reaction was monitored by TLC and UV-vis spectroscopy. After reaction completion, the solvent was removed in vacuum, and the resulting solid was recrystallized from dichloromethane/pentane. After filtration, 124 mg (5.8 × 10⁻⁵ mol) of the pure ironporphyrin were obtained (62%). UV-vis: λ, nm (ε × 10³ L. mol⁻¹.cm⁻¹) 264 (265.8), 294 (176.3), 424 (251.4), 518 (15.2), 590 (4.5), 656 (2.3).

Carbamazepine oxidations

In a typical experiment, reactions were carried out in a 3 mL vial containing a screw cap. The crowned ironporphyrin (6.0 × 10⁻⁷ mol) was stirred with 5.0 mg carbamazepine (2.1 × 10⁻⁵ mol) in methanol or dichloroethane/water 1:1 (2 mL) before the oxidant (2.4 × 10⁻⁵ mol - m-CPBA, NaOCl, PhIO) was added. Reactions were run for 2 h under magnetic stirring at room temperature, at a catalyst/oxidant/drug molar ratio of 1:40:35. After the desired time, an aliquot of 50 µL of the reaction mixture was withdrawn and exposed to a hot air flow for solvent removal. Then, carbamazepine and the possible oxidation products were solubilized in 1 mL mobile phase (Milli-Q water/ acetonitrile 70:30), and the reaction products were analyzed by high performance liquid chromatography (HPLC). The products were analyzed by comparison of their retention times with those of authentic samples.

The optimal condition for analysis of carbamazepine and its metabolites was obtained by means of a C18 column and the mobile phase used consisted of Milli-Q water/acetonitrile 70:30 (v/v), flow rate at 1 mL/min, detection was done at 210 nm. The isocratic system was operated at ambient temperature and required less than 15 min of chromatographic time.

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References