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# New Pioglitazone Metabolites and Absence of Opened Ring Metabolites in New *N*-substituted Thiazolidinedione

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**Nonstandard abbreviations:** ADME, absorption, distribution, metabolism, and excretion; $CL_h$, hepatic clearance; $CL_{int}$, intrinsic clearance; ESI, electrospray ionization; GQ-11, 5-(indol-3-ylmethylene)-3-(4-methylbenzyl)-thiazolidine-2,4-dione; HLM, human liver microsome; LC-HRMS, liquid chromatography coupled to high resolution mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; PPARγ, peroxisome proliferator-activated receptor gamma; RLM, rat liver microsome; $t_{1/2}$, half-life; TZD, thiazolidinedione;

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## Abstract

Thiazolidinediones are drugs used to treat type 2 diabetes mellitus; however, there remain several safety concerns regarding the available drugs in this class. Therefore, the search for new thiazolidinedione candidates is still ongoing; metabolism studies play a crucial step in the development of new candidates. Pioglitazone, which is one of the most commonly used thiazolidinediones, and GQ-11, a new *N*-substituted thiazolidinedione, were investigated in terms of their metabolic activity in rat and human liver microsomes, in order to assess their metabolic stability and to investigate their metabolites. Methods for preparation of samples were based on liquid-liquid extraction and protein precipitation. Quantitation was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the metabolite investigation was performed using Ultra-Performance Liquid Chromatography (UPLC) coupled to a hybrid quadrupole-time of flight (Q-Tof) mass spectrometer. The predicted intrinsic clearance of GQ-11 was 70.3 and 46.1 mL/kg/min for rats and humans, respectively. The predicted intrinsic clearance of pioglitazone was 24.1 and 15.9 mL/kg/min for rats and humans, respectively. The pioglitazone metabolite investigation revealed two unpublished metabolites (M-D and M-A). M-A is a hydration product, which may be related to the mechanism of ring opening, and the toxicity of pioglitazone. The metabolites of GQ-11 are products of oxidation; no ring opening metabolite was observed for GQ-11. In conclusion, in the same experimental conditions, a ring opening metabolite was observed only for pioglitazone. The resistance of GQ-11 to ring opening is probably related to *N*-substitution in the thiazolidinedione ring.

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## Introduction

Thiazolidinediones (TZDs) are drugs used to treat type 2 diabetes mellitus. They are also called glitazones, and the most commonly used compounds include troglitazone, rosiglitazone, and pioglitazone (Saltiel and Olefsky, 1996; Naim et al., 2017). TZDs are a class of oral insulin sensitizing drugs, whose mechanism of action involves activation of peroxisome proliferator-activated receptor gamma (PPARγ) (Hauner, 2002; Stumvoll, 2005). Troglitazone was the first launched thiazolidinedione, but it was withdrawn from the market due to severe and sometimes fatal hepatotoxicity (Scheen, 2003). Rosiglitazone is available in the United States, whilst in Europe, its approval was withdrawn due to a significant increase in myocardial infarction (Alemán-González-Duhart et al., 2016); hepatotoxicity was also reported (Al-Salman et al., 2000; Forman et al., 2000). Pioglitazone (Figure 1A) use in the United States is restricted as it may cause urinary bladder cancer (Alemán-González-Duhart et al., 2016). In addition, hepatotoxicity cases have occurred (Maeda, 2001; Marcy et al., 2004), including one fulminant hepatic failure (Chase and Yarze, 2002). Given these adverse reactions, the search for safer thiazolidinediones is still ongoing. Several new candidates have been reported in the literature (Imran et al., 2007; Naim et al., 2017). Most of these candidates possess substitutions in the carbon 5 of the thiazolidinedione ring only. However, substitutions in the nitrogen at position 3 have shown good pharmacological activities in models of diabetes (Mourao et al., 2005) and atherosclerosis (Cesar et al., 2015). Mourão et al. (2005) published a study on the synthesis of 5-benzylidene and 5-acridinylidene derivatives, both of which were *N*-3 substituted; these compounds showed hypoglycemic activity in mice. Many other authors have used similar approaches with promising outcomes (da Costa Leite et al., 2007; Barros et al., 2010; Araújo et al., 2011; Amato et al.,

2012; Santin et al., 2013a; Santin et al., 2013b; Cesar et al., 2015; Rudnicki et al., 2016; Silva et al., 2016).

Metabolism studies are an important step in the development of new compounds (Kostiainen et al., 2003). In addition, due to toxicity concerns, investigation of possible metabolites is preferably performed soon in the development process, avoiding delays in the later phases (Brandon et al., 2003; Masimirembwa et al., 2003; Beuck et al., 2011). This type of research and information is crucial, since active metabolites are one of the main causes for unexpected safety issues in the research and development of new drug candidates (Baillie et al., 2002; Leclercq et al., 2009). With the recent publication of the Guidance for Metabolite Safety Testing (FDA, 2008), metabolite investigations have become even more important (Baillie, 2008; Leclercq et al., 2009). Considering thiazolidinediones, it was proposed that the ring-opened may be related to formation of reactive metabolites of troglitazone (Kassahun et al., 2001). This ring opening mechanism is common to other thiazolidinediones (Alvarez-Sánchez et al., 2006). Shen et al. (2008) first published the ring opening of pioglitazone, but only in dog microsomes, whilst Baughman et al. (2005) described these metabolites in rat and human microsomes. In another study, it was observed that *N*-3 substituted compounds were non-toxic (Tang et al., 2015). Thus, there is a growing need to elucidate the correct metabolic profile of thiazolidinediones, which will in turn contribute to clarification of the safety profile of these drugs. The aim of this study was to investigate metabolism properties of 5-(indol-3-ylmethylene)-3-(4-methylbenzyl)-thiazolidine-2,4-dione (GQ-11, Figure 1B), including metabolic stability and metabolite formation, using human and rat microsomes, and to compare these findings to those of pioglitazone.

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## Material and Methods

### Chemicals and reagents

The compound 5-((1H-indol-3-yl)methylene)-3-(4-methylbenzyl)thiazolidine-2,4-dione (GQ-11) was synthesized at the Laboratory of Design and Drug Synthesis (LPSF) of the Federal University of Pernambuco (Recife, Brazil). The thiazolidine derivative was synthesized by the nucleophilic Michael addition of substituted 3-benzyl-thiazolidine-dione on substituted cyanoacrylate, to obtain thethiazolidine-2,4-diones (GQs) (Mourao et al., 2005; da Costa Leite et al., 2007). Pioglitazone, Jacobsen catalyst, formic acid, hydrogen peroxide, $MgCl_2$, glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St Louis, MO, USA). β-Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Mexico City, Mexico). Human liver microsomes (code HMMC-PM) and rat liver microsomes (code RTMC-PL) were purchased from Thermo Scientific (Waltham, MA, USA). Water was purified by a Milipore system.

### Microsomal Incubation and Processing of Samples

Human liver microsomes (HLM) or rat liver microsomes (RLM) were thawed and diluted to appropriate protein concentrations (0.5 mg/mL) with 100 mM potassium phosphate buffer (pH 7.4 at 37°C). Incubations were started by the addition of NADPH (1 mM) and compounds in two concentrations: 2 µM for metabolic stability and 10 µM for metabolite investigation. Final incubation volume was 1 mL. Incubations were carried out in triplicate in a 12-well plate. In the metabolic stability

study, aliquots (100 µL) were collected at times points 0, 5, 10, 20, 40, 60, and 90 min. For metabolite investigation, time point 0 was collected as the control sample, while time point 60 min was the test sample. Incubation was stopped by starting the sample processing.

For the investigation of metabolic stability, sample incubation media (100 µL) processing started by the addition of ethyl acetate (1 mL) and internal standard (10 µL). The internal standard was rosiglitazone at 10 µg/mL for pioglitazone analysis, and 100 µg/mL for GQ-11 analysis. Next, the mixture was vortex-mixed for 30 s. It was then centrifuged for 5 min at 4000 x g, and the organic phase (900 µL) was transferred to a new clean 1.5 mL microtube, which was evaporated to dryness using a MiVac concentrator (Genevac, Ipswich, Reino Unido) at 50ºC for 40 min. The extract was resuspended with 50 µL of mobile phase and 30 s of vortex-mixing. This mixture was transferred to a vial with an insert, and was placed in the sample manager until injection in the liquid chromatography-tandem mass spectrometry (LC-MS/MS). For the investigation of metabolites, the aforementioned procedure, without internal standard, was used in addition to another protein precipitation procedure, resulting in two extracts for each sample. The protein precipitation consisted of adding 100 µL of acetonitrile to 100 µL of incubation media, followed by vortex-mixing and centrifugation at 15000 x g for 15 min. Next, the supernatant was separated and transferred to max recovery vials to be injected in the Ultra-Performance Liquid Chromatography (UPLC) coupled to a hybrid quadrupole-time of flight (Q-Tof) mass spectrometer.

**LC-MS/MS quantitation**

Quantitation of pioglitazone and GQ-11 was performed on an LC Alliance 2695 (Waters, Milford, Massachussetts, USA) coupled to a mass spectrometer triple quadrupole Quattro Micro (Micromass, Wilmslow, UK) equipped with an electrospray ionization (ESI) interface. Data were acquired with MassLynx 4.1 (Waters, Milford, Massachussets).

Chromatographic separation was performed using a Symmetry (Waters, Milford, Massachussets) C18 column (50 x 2.1 mm, 3.5 µm) protected by a 5 x 2.1 mm guard column of the same stationery phase, both at 35ºC. The flow rate was 0.3 mL/min. Injection volume was 10 µL. For pioglitazone, separation was achieved using isocratic mobile phase consisting of acetonitrile: water (80:20), whilst for GQ-11, mobile phase was composed of acetonitrile and 2 mM ammonium acetate (80:20).

Mass spectrometer parameters for the analysis were as follows. Positive ion mode was used for pioglitazone and negative ion mode was used for GQ-11. ESI source and desolvation temperatures: 120°C and 200°C, respectively; capillary voltage: 4.5 kV for pioglitazone and 3.0 kV for GQ-11. Nitrogen was the desolvation gas (800 L/h) and argon was the collision gas. Analysis was performed working in multiple reaction monitoring (MRM) mode. In the pioglitazone analysis, the transition 357>134 was used for pioglitazone and 358>94 was used for rosiglitazone. Cone voltage was 35 V for both, and collision was 23 and 40 (arbitrary units) for pioglitazone and rosiglitazone, respectively. In the GQ-11 analysis, the transitions were 347>172 for GQ-11 and 356>313 for rosiglitazone. Cone voltages were 33 V for

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GQ-11 and 25 V for rosiglitazone. Collision energy was 23 and 19 (arbitrary units) for GQ-11 and rosiglitazone, respectively.

**Metabolic stability calculations**

GQ-11 and pioglitazone concentrations were used to build the concentration versus time plot. Next, *in vitro* microsomal half-life ($t_{1/2}$), predicted intrinsic clearance ($CL_{int}$), and predicted hepatic clearance ($CL_h$) were calculated according to Obach (1999). For human microsomes, the assumptions were 45 mg of microsomes per gram of liver, 20 g of liver per kg of body weight, and 21 mL/min/kg (1470 mL/min) as human hepatic blood flow (Obach, 1999), while for rats the assumptions were 45 mg of microsomes per gram of liver, 45 g of liver per kg of body weight (Lu et al., 2006), and 55 mL/min/kg (220 mL/min) (Liu et al., 2015) as rat hepatic blood flow.

**Biomimetic oxidation study using the Jacobsen catalyst**

Reactions were carried out in a 2 mL microtube at room temperature for 24 h, and under mechanical stirring. Hydrogen peroxide was the oxygen donor and Jacobsen catalyst was the responsible for the catalysis. The molar ratio was 1:80:20 for catalyst/oxidant/drug. In order to achieve that proportion, the mixture was constituted by $6.0\times10^{-7}$ mol of catalyst, $4.8\times10^{-5}$ mol of oxygen donor, and $1.2\times10^{-5}$ mol of drug in the reaction medium. The reaction medium was acetonitrile ($CH_3CN$). At the end of the reaction, agitation was stopped, and the reaction mixture was withdrawn. The products were analyzed by liquid chromatography coupled to high resolution mass spectrometry.

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**LC-HRMS analysis**

Investigation of metabolites was performed using the Acquity H-Class UPLC® system (Waters, Milford, Massachussets) coupled to a G2-S hybrid quadrupole-time of flight (Q-Tof) mass spectrometer (Waters, Milford, Massachussets) equipped with an ESI source operating in positive ion mode. Data acquisition was carried out with MassLynx 4.1 (Waters, Milford, Massachussets).

The separation was performed on an Acquity HSS SB C18 column (2.1 x 100 mm; 1.7 µm) protected by a HSS VanGuard® guard column (2.1 x 5 mm; 1.7 µm) at 35°C. The gradient mobile phase solvents were water (A) and acetonitrile (B), both containing formic acid 0.1% at a flow rate of 0.3 mL/min. The initial mobile phase composition was maintained at 90% A for 1 min, changed linearly to 10% (1–8 min) and held for 1 min, then returned to the initial condition at 10 min, and kept until 12 min (total running time), for the chromatograph column equilibrium. The injection volume was 5 µL.

The mass spectrometer ESI source capillary voltage was 4 kV. The temperature of the source was set at 150°C, and the desolvation temperature was set at 350°C. The cone voltage was 20 V. Nitrogen was used as the cone and desolvation gas. The cone gas flow was 50 L/h, and the desolvation gas flow was 550 L/h. Leucine-enkephalin was used as the lockmass generating a reference ion in positive mode at (*m/z* 556.2771$[M+H]^+$), and introduced by a lockspray at 20 µL/min for accurate mass acquisition. For control and test sample analysis, data were collected using $MS^E$ in the scan range 50 to 600 Da. Function 1 with low energy was used to monitor precursor ions, whilst function 2 with high energy (ramp collision

energy from 20 to 60 V) was used to monitor product ions. After each pair of control and test samples, data dependent MS/MS experiments were performed for selected precursor ions using ramp collision energy from 10 to 40 (arbitrary units). Data acquisition was performed using MassLynx 4.1 software (Waters Corp., Milford, USA).

**Data analysis**

The $MS^E$ data were analyzed using Metabolynx XS™, an extension of MassLynx software (Waters Corp., Milford, USA). The mass defect filter was enabled at 25 mDa and the analysis time was 1-11 min. The mass window was 0.02 Da, the absolute area of the peak was 30 (p.a.u.), the spectrum was above the relative intensity of 2%, and the maximum tolerance of mass error was set as 5 ppm. The prediction rules of elemental composition were defined as follows: atom numbers of carbon, hydrogen, oxygen, nitrogen, and sulfur were set to ranges of 0–100, 0–120, 0–60, 0–10, and 0–6, respectively. After processing, a new run of the test sample was performed using the metabolite peaks found as precursor ions in the MS/MS experiment, in order to confirm the fragmentation pattern observed in the $MS^E$ acquisition mode.

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## Results

### Microsome metabolic stability

Pioglitazone and GQ-11 were quantitated and their concentrations were converted to remaining percentage, and then plotted against time. The *in vitro* metabolism parameters can be seen in Table 1. The free-fraction in blood was not accounted for scaling the clearance values to body weight.

### Mass spectrometry analysis of parent drugs

The analysis of parent compounds GQ-11 and pioglitazone was performed first, in order to obtain their fragmentation pattern. The high resolution mass spectrometry analysis of GQ-11 and pioglitazone showed the $[M+H]^+$ of both compounds with *m/z* 349.1015 and 357.1273 (1.1 and 1.7 ppm), respectively. The MS/MS analyses of pioglitazone and GQ-11 are shown in Figure 2. Most of the product peaks were related to structure fragments. As can be observed, the base fragment peaks were 134 for pioglitazone and 105 for GQ-11. This is important as changes in the *m/z* of specific parts of the molecule may be related to the expected metabolic reaction. In addition, other fragments may arise after metabolic structure modifications.

### Pioglitazone metabolites

Pioglitazone was incubated with human and rat microsomes to obtain its phase I metabolites, and was compared to GQ-11 metabolite formation under the

same conditions. Several metabolites of pioglitazone have been described in the literature (Shen et al., 2008; Uchiyama et al., 2010), and most of them were observed in the current study (Figure 3 and Table 2).

*Well-established pioglitazone metabolites*

Most of the observed metabolites were those reported in literature (Baughman, 2005; Shen et al., 2008; Uchiyama et al., 2010), and were confirmed by exact mass, with error less than 5 ppm (Table 2); $MS^2$ product ions were in agreement with their structures.

The metabolites formed by opening of the thiazolidinedione ring at 4.9 (M-B) and 5.79 min (M-C) were first described by Uchiyama et al. (2010) as S9P1 and M11, respectively. Uchiyama et al. (2010) suggested that the formation of S9P1 and M11 occurred with the *N*-glucuronide metabolite (M7) as precursor, since they observed the formation of S9P1 and M11 in a S9 metabolism experiment system containing S-adenosyl-L-methionine, and using M7 as the substrate. However, the conversion was not dependent on S-adenosyl-L-methionine or human liver S9. In our study, we have confirmed the occurrence of these metabolites, but without the *N*-glucuronide metabolite M7. This suggests that these metabolites may also be formed by another pathway.

*New pioglitazone metabolites*

Two new pioglitazone metabolites were observed in the current study, a new hydroxylated metabolite at 5.9 min (M-D), and another metabolite resulting from hydration reaction at retention time 4.31 min (M-A). The former was only observed in the RLM, while M-A was observed in both species. The base product ion of M-D was at *m/z* 150 (Figure 4), indicating that an oxygen was added in the pyridine ring or in

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the carbon adjacent to the ether function. The fragmentation pattern suggests that the substitution site is aliphatic and not aromatic, however, it is not a definitive proof, further studies may confirm this observation. The metabolite formed by hydrolysis (M-A) of the thiazolidinedione ring may work as an intermediate between M-B and M-C. The absence of the metabolite M-A (4.31 min) in previous studies may be explained by its proximity to the third isotope of the major hydroxylated metabolite (4.24 min). The protonated M-A ($C_{19}H_{23}N_2O_4S^+$) has an *m/z* of 375.1379. The protonated hydroxylated metabolite of 4.24 min ($C_{19}H_{21}N_2O_4S^+$) was observed at the *m/z* of 373.1222, but its third isotope peak (4.5% intensity) appeared at *m/z* of 375.1180. This peak (*m/z* 375.1180) had an intensity as high as the major isotope of M-A. Therefore, to see it, it was necessary to close, as much as possible, the *m/z* window to filter the interfering signal. The metabolite M-A has not been previously described; thus, its detailed spectrum is shown in Figure 4. The fragmentation pattern is similar to that of the *N*-glucuronide metabolite described by Uchiyama et al. (2010). Our findings suggest an alternative pathway for formation of the opened ring metabolites M-B and M-C, via hydrolysis of the imide in the thiazolidinedione ring leading to formation of an *S*-carbamothioate and a carboxylic acid.

**GQ-11 metabolites**

The new thiazolidinedione was incubated with human and rat microsomes to obtain its phase I metabolites. The main objective was to observe if opened ring metabolites, similar to those reported for glitazones, may occur under the same conditions. All possible reactions previously related to thiazolidinediones were inputted before data processing. Further, metabolites predicted by the software

Metabolizer v.15.12.14 (Chemaxon, Budapeste, Hungria) were added for data processing. Five metabolites were found (Table 3) as oxidized products of GQ-11 (Figure 5).

The GQ-11 metabolite M1 was only found in the human liver microsome incubation. M2 was higher in RLM than in HLM, while M3, M4, and M5 showed similar patterns. Three other peaks were observed as possible metabolites (retention times 5.35, 5.77, and 6.04 min), but they could not be confirmed, and they were only found in RLM. Figure 6 shows the product ions spectra and the proposed structures of the five observed metabolites.

In order to find more evidence for the formation of the non-aromatic metabolites of GQ-11, a reaction using the Jacobsen catalyst-$H_2O_2$ oxidation system was employed. This allowed retention time comparison and better observation of the $MS^2$ products for the metabolites M1 and M2. Still, it was possible to select the in source fragment of M2 at *m/z* 347, which is formed by loss of water in the hydroxylation site. Figure 5 shows the chromatograms of the reaction products M1 (C) and M2 (D). The signals of reaction products in the $MS^2$ spectra matches the signals observed in the $MS^2$ spectra of the microsome incubation sample (Supplemental Figure 1).

The metabolite M1 has the characteristic fragment of the benzylic portion of the parent molecule (*m/z* 105), indicating that the oxidation was not in that portion; however, neither of the other fragments were similar to those of the parent molecule. Still, the fragment *m/z* 132 was compatible with the presence of the oxygen in the indole portion of the molecule. However, the findings that support the occurrence of M3, M4, and M5, suggest that the oxygen is in the pyrrole ring of the indole portion

(Figure 6 A). Also, the Jacobsen catalyst did not performs oxidation in the aromatic ring, and once M1 was formed in this reaction (Figure 5 C), this is evidence that M1 is not an aromatic hydroxylation.

The highest intensity fragment of M2 (Figure 6 B) was in the *m/z* 121, suggesting that the oxidation occurred in the benzylic portion of the molecule. The presence of the fragment *m/z* 347 indicates the loss of *m/z* 18, probably as a consequence of water loss, which is common for aliphatic alcohols. The reaction with Jacobsen catalyst was able to form M2 (Figure 5 D), and the $MS^2$ fragments were in agreement with those of the microsome incubation system (Supplemental Figure 1). Once 347 [$M^+$] have been formed as an in source fragment, it was used to help understand the structure of M2 by setting it as a parent ion to go through collision induced dissociation. The main finding was the absence of *m/z* 121 observed for the parent ion 365 and the rise of the *m/*z 104, corroborating the loss of neutral water in the parent ion. On the other hand, most of the other signals were similar between parent ions 347 and 365, also suggesting that the modification was in the benzylic portion.

The metabolites M3, M4, and M5 showed very similar fragmentation patterns (Figure 6 C, D, and E). The product ions matched the product ions of the parent molecule plus 16 units, with the exception of the *m/z* 105, indicating that the oxidation was not in the benzylic part of the molecule. This similar pattern indicates that the metabolites are very similar, since the change in the position of the oxidation had no effect in the fragmentation mechanism of its peers. The only explanation for this is that the oxidation occurs in the aromatic portion of the indole ring, which is far from the fragment locations, and would have the same effect in the molecule as a whole.

The absence of M3, M4, and M5 in the Jacobsen catalyst reaction brings more evidence that they are aromatic hydroxylations.

## Discussion

Thiazolidinediones such as pioglitazone are very useful for treatment of diabetes. An investigation of the metabolic stability of GQ-11 and pioglitazone was performed in rat and human liver microsomes. Pioglitazone was found to have an intrinsic clearance around three-fold higher than previously published (Xiao et al., 2015). The new candidate was less stable than pioglitazone in both microsome species. Considering these findings, it is important to highlight that there is a need for monitoring of metabolite formation. As expected, the predicted clearance for rats was higher than the predicted clearance for humans (Mahmood et al., 2003). The higher clearance of GQ-11 may lead to a half-life shorter than that of pioglitazone (8.3 h) (Jaakkola et al., 2005). On the other hand, GQ-11 is more lipophilic (Log P 5.1) than pioglitazone (Log P 2.7) (Gimenez et al., 2010), which may help to extend its half-life *in vivo* by a change in volume of distribution.

The use of thiazolidinediones is limited due to their toxicity, and some authors have related this toxicity to the formation of thiazolidinedione ring opening metabolites in troglitazone (Kassahun et al., 2001), which is a common pathway for other thiazolidinediones (Baughman, 2005; Alvarez-Sánchez et al., 2006; Shen et al., 2008). These opened ring metabolites have been previously reported (Uchiyama et al., 2010), and two of them (M-B and M-C) were observed in our work. In addition, a new hydration metabolic product (M-A) of pioglitazone was observed, which may be an intermediary in the formation of M-B and M-C. The new proposed hydration metabolite M-A eluted very close (4.31 min) to the retention time of the hydroxyl

metabolite M-IV (4.25 min), and was almost hidden because of this. Once the intensity of M-A was much smaller than the intensity of M-IV, it would be impossible to see it due to the presence of the *m/z* 375 from the isotope pattern of the hydroxylated metabolite (M-IV *m/z* 373). Without the chromatographic separation, the $MS^2$ fragments of *m/z* 375 from M-A would be suppressed by the $MS^2$ fragments of *m/z* 375 from M-IV (monoisotopic *m/z* 373). Although not reported before for pioglitazone, it would be predicted that hydrolysis in the imide function of the thiazolidinedione ring would occur. This is similar to what happens in the phthalimide ring of thalidomide with the formation of its *N*-(o-carboxybenzoyl)glutamic acid imide metabolite (Lu et al., 2003). Another hydrolysis of imide was observed in aniracetam, which has a lactam ring, resulting in a cleaved product (Ogiso et al., 1998).

The other unpublished metabolite observed in the current study is proposed as the result of oxidation in the carbon adjacent to the ether group (Figure 4), which is not related to opening in the thiazolidinedione ring; therefore, it may not be related to general toxicity. Indeed, some hydroxylation and oxidation products of pioglitazone, such as the metabolites M-III, M-IV, and M-II to a minor degree, share its hypoglycemic activity (Eckland and Danhof, 2000; Shen et al., 2008).

Based on the crucial role of thiazolidinediones in the treatment of diabetes, and given the risk associated with the use of the available drugs of this class (Al-Salman et al., 2000; Maeda, 2001; Scheen, 2001; Scheen, 2003; Marcy et al., 2004; Alemán-González-Duhart et al., 2016), the development of new safer thiazolidinediones is of current interest (Cesar et al., 2015; Rudnicki et al., 2016; Silva et al., 2016; Naim et al., 2017; Thangavel et al., 2017). Since glitazones do not cause liver damage in laboratory animals, comparing these to new thiazolidinediones in order to research the mechanism of toxicity can be misleading (Patel et al., 2012).

Further, it has been shown that thiazolidinediones with the *N*3-carboxymethyl substituent are nontoxic (Tang et al., 2015), which may be related to their metabolic stability due to avoidance of the ring opening, since compounds with this feature are more stable than their peers. Considering the aforementioned properties of pioglitazone metabolites, it is reasonable to assume that the absence of opening ring metabolites and the presence of hydroxylated metabolites for GQ-11 represent a promising profile for drug development.

Jacobsen catalyst is known to be able to react similarly to cytochrome P450 in the presence of an oxygen donor. Examples of this property can be found in literature, where it was able to react with drugs (Leod et al., 2007; Mac Leod et al., 2008) and natural products (De Santis Ferreira et al., 2012; Niehues et al., 2012; Rocha et al., 2014) to confirm or predict metabolism products. Therefore, the biomimetic system using Jacobsen catalyst and $H_2O_2$ was successfully applied to obtainment of the non-aromatic oxidation products of GQ-11 in order to bring more evidence for the proposed metabolite structures.

There was only one metabolite signal found in human liver microsomes that was not found in rat liver microsomes; therefore, it will be necessary to look for the metabolite M1 in preclinical studies of GQ-11, since the metabolites identified only in human microsomes may arise safety concerns (FDA, 2008). Despite the uniqueness of M1, the similarity between the metabolite profiles in laboratory animals and humans may be enough to avoid the need to perform preclinical toxicological studies for the preformed metabolites, unless the formation of one of these metabolites surpasses 10% of total drug related exposure (area under curve). This is another important property for potentially successful new drug candidates (Baillie, 2002; FDA, 2008).

In summary, two new metabolites of pioglitazone were reported, one of them being a hydration product due to hydrolytic cleavage of the thiazolidinedione ring. The *in vitro* metabolic characteristics of the new thiazolidinedione GQ-11 were also described for the first time. Five new metabolites for GQ-11 were found, all of them were products of oxidation reactions. The profile in human and rat liver microsomes was similar, with only one small intensity metabolite being exclusively formed in human microsomes. Hydrolysis of the thiazolidinedione ring of GQ-11 was not observed, which suggests it is potentially less hazardous than the clinically available glitazones. This is probably a consequence of the substitution in the nitrogen at position 3 of the thiazolidinedione ring. Further metabolism studies with other N3-substituted thiazolidinediones may support this conclusion.

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## Authorship Contribution

Participated in research design: Campos, Pitta, Peccinini, and Pontarolo.

Conducted experiments: Campos, Cerqueira, Silva, Franchin, and Galdino-Pitta.

Contributed new reagents or analytic tools: Campos, Pitta, Peccinini, and Pontarolo.

Performed data analysis: Campos.

Wrote or contributed to the writing of the manuscript: Campos, Cerqueira, Silva, Franchin, Galdino-Pitta, Pitta, Peccinini, and Pontarolo.

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## Footnotes

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## Figure Legends

**Figure 1.** Structures of pioglitazone (A) and the new thizolidinedione GQ-11 (B).

**Figure 2.** Product ions spectra of pioglitazone (top) and GQ-11 (bottom) under collision ion dissociation energy ramp of 10-40 units. The first analyzer was set to *m/z* 357 for pioglitazone and *m/z* 349 for GQ-11.

**Figure 3.** Overlay chromatogram of pioglitazone metabolites M-A, MIX, M-B, M-III, and M-C after incubation in (A) human liver microsomes and (B) rat liver microsomes, and pioglitazone metabolites M-VII, M-IV, M-VIII, M-II, and M-D in (C) human liver microsomes and (D) rat liver microsomes.

**Figure 4.** Representative spectra and proposed fragmentation pattern of the metabolite M-A (top) $[M+H]^+$ at *m/z* 375 ($C_{19}H_{22}N_2O_4S^+$) in human liver microsomes (HLM) and the metabolite M-D (bottom) $[M+H]^+$ at *m/z* 373 ($C_{19}H_{20}N_2O_4S^+$) in rat liver microsomes (RLM).

**Figure 5.** Profile of the observed metabolites of GQ-11 after incubation in (A) human liver microsomes, (B) rat liver microsomes, and after reaction using the Jacobsen catalyst-$H_2O_2$ system (C and D).

**Figure 6.** Representative spectra and proposed structures of the oxidation metabolic products of GQ-11, M1 (A), M2 (B), M3 (C), M4 (D), and M5 (E).

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**Table 1**. *In vitro* metabolism parameters of pioglitazone and GQ-11, both at 2 μM, incubated with 0.5 mg/mL microsome protein.

| Compound | Species | $k^a$ ($min^{-1}$) | $t_{1/2}{}^b$ (min) | $CL_{int,predicted}{}^c$ (mL/kg/min) | $CL_{h,predicted}{}^d$ (mL/kg/min) |
|---|---|---|---|---|---|
| **Pioglitazone** | Rat | 0.008 ± 0.001 | 93.2 ± 13.7 | 30.5 ± 4.0 | 19.6 ± 1.7 |
| | Human | 0.009 ± 0.001 | 78.7 ± 8.2 | 15.9 ± 1.6 | 9.0 ± 0.5 |
| **GQ-11** | Rat | 0.0174 ± 0.007 | 44.7 ± 18.3 | 70.3 ± 28.7 | 30.0 ± 5.6 |
| | Human | 0.026 ± 0.0001 | 27.1 ± 0.1 | 46.1 ± 0.3 | 14.4 ± 0.02 |

[a] decay rate constant; [b] half-life; [c] predicted intrinsic clearance; [d] predicted hepatic clearance was obtained disregarding binding values.

**Table 2**. Pioglitazone metabolites using ultra-performance liquid chromatography coupled to hybrid quadrupole-time of flight after human and rat liver microsome incubation.

| Metabolite[a] | M+H (*m/z*) | Error (ppm) | RT[b] (min) | Product ions (*m/z*) | RLM[c] | HLM[d] |
|---|---|---|---|---|---|---|
| **M-II (M10)** | 373.1221 | -0.2 | 4.58 | 133, 150, 210, 238, 239, 284, 355 | + | + |
| **M-III (M13)** | 371.1075 | 2.4 | 5.65 | 148 | + | + |
| **M-IV (M5)** | 373.1231 | 2.5 | 4.24 | 132, 150, 355 | + | + |
| **M-VII (M9)** | 373.1225 | 0.9 | 4.13 | 119, 150, 240, 256 | | + |
| **M-VIII** | 373.1222 | 0.1 | 4.49 | 268, 313 | + | + |
| **M-IX (M12)** | 355.1120 | 1.1 | 4.58 | 133, 210, 238, 239, 284 | | + |
| **M-B (S9P1)** | 332.1320 | 0 | 4.90 | 119, 134, 286 | + | + |
| **M-C (M11)** | 346.1464 | -3.7 | 5.79 | 119, 134, 254 | + | + |
| **M-D** | 373.1215 | -1.8 | 5.90 | 93, 138, 150, 357 | + | |
| **M-A** | 375.1373 | -1.4 | 4.31 | 134, 286, 332 | + | + |

[a] Metabolites (M) labeled as previously described by Shen et al. (2008) (roman numbers) or with our label (capital letters); in brackets is the label used by Uchiyama et al. (2010); [b] retention time; [c] rat liver microsome; [d] human liver microsome. Plus (+) sign indicates the presence of the metabolite in the respective microsome system.

**Table 3.** GQ-11 metabolites using ultra-performance liquid chromatography coupled to hybrid quadrupole-time of flight after human and rat liver microsome incubation.

| Metabolites | M+H (*m/z*) | Error (ppm) | RT[a] (min) | Product ions (*m/z*) | RLM[b] | HLM[c] |
|---|---|---|---|---|---|---|
| **M1** | 365.0944 | -4.3 | 4.04 | 105, 132 | | + |
| **M2** | 365.0948 | -3.2 | 4.20 | 121, 173, 319, 347 | + | + |
| **M3** | 365.0960 | 0.1 | 4.87 | 105b, 189, 245, 273, 294, 322 | + | + |
| **M4** | 365.0964 | 1.2 | 4.97 | 105b, 189, 245, 273, 294, 322 | + | + |
| **M5** | 365.0977 | 4.8 | 5.23 | 105b, 189, 245, 273, 294, 322 | + | + |

[a] retention time; [b] rat liver microsome; [c] human liver microsome. Plus (+) sign indicates presence of the metabolite.

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# Figures

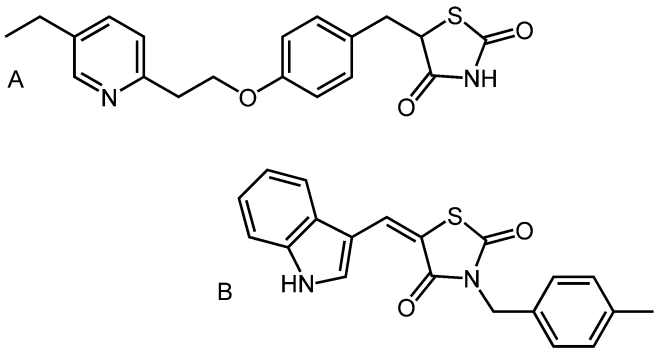


**Figure 1.**

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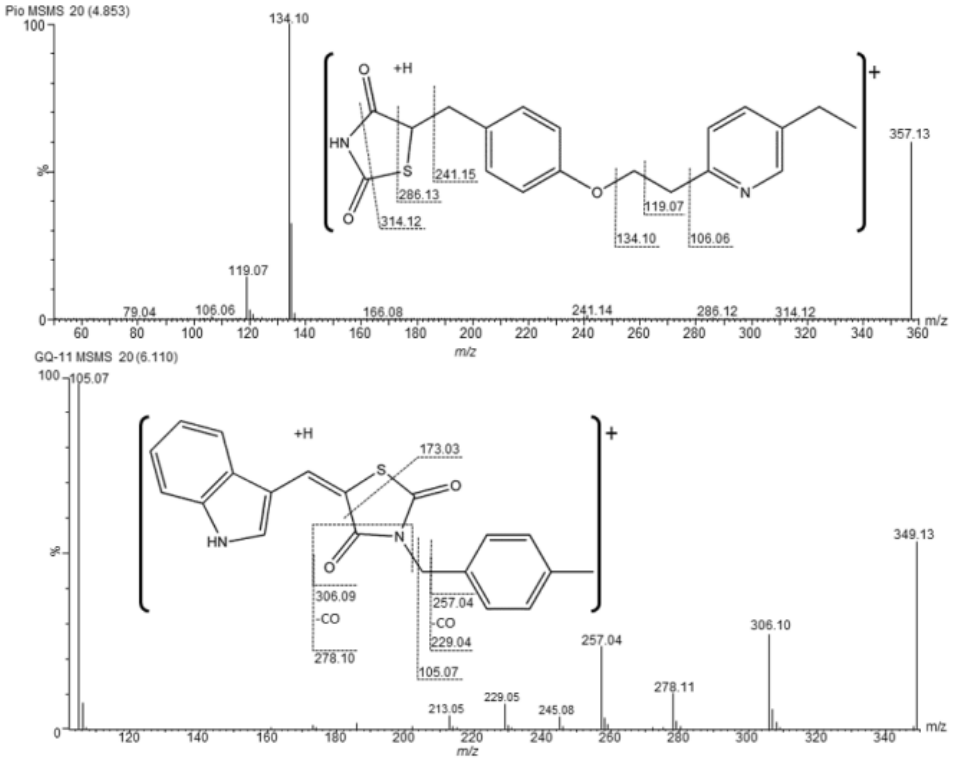


**Figure 2.**

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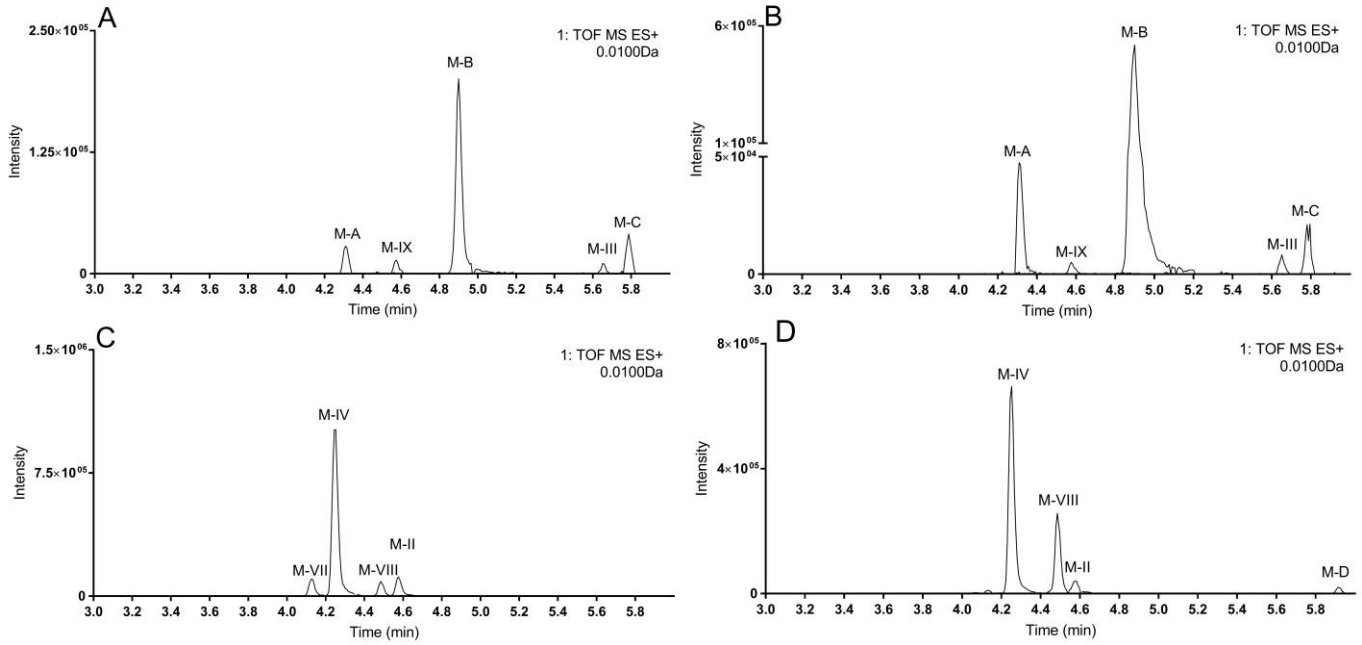


**Figure 3.**

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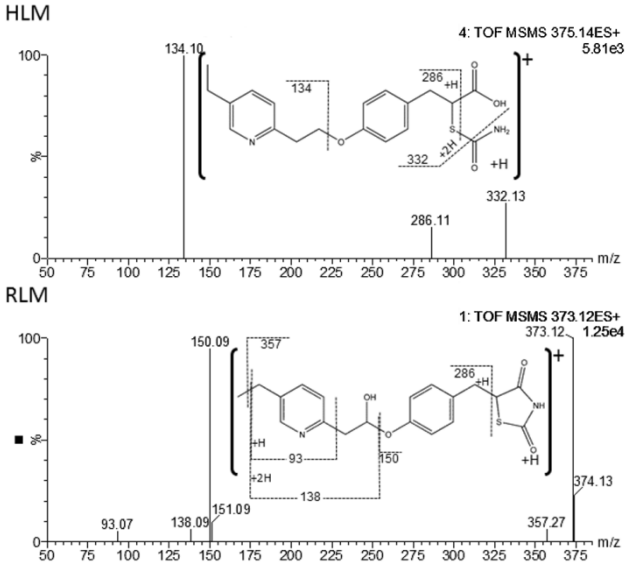


**Figure 4.**

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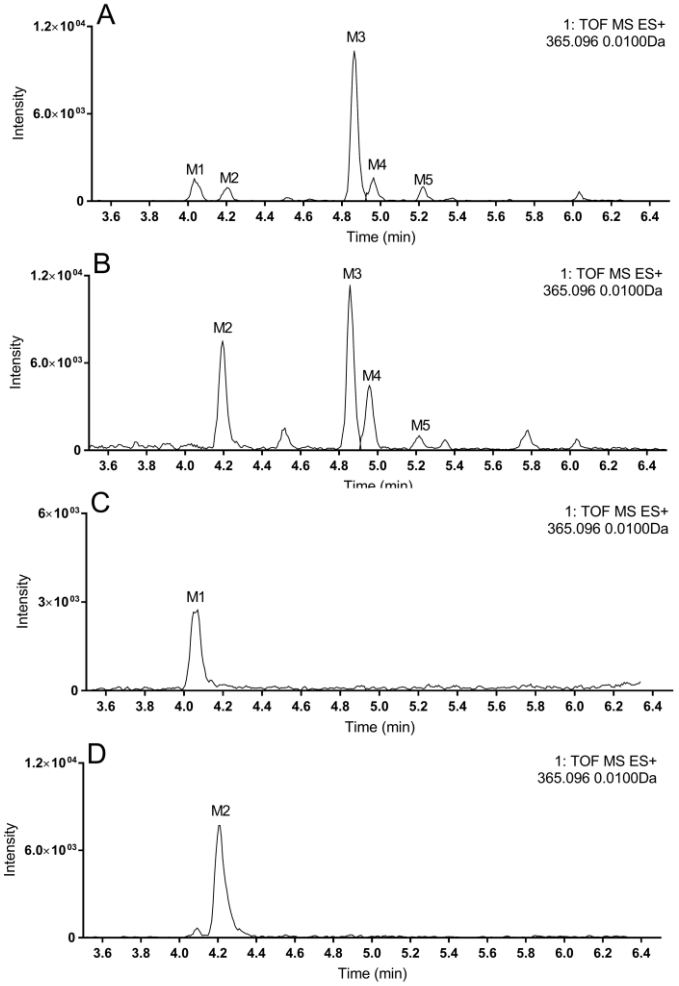


**Figure 5.**

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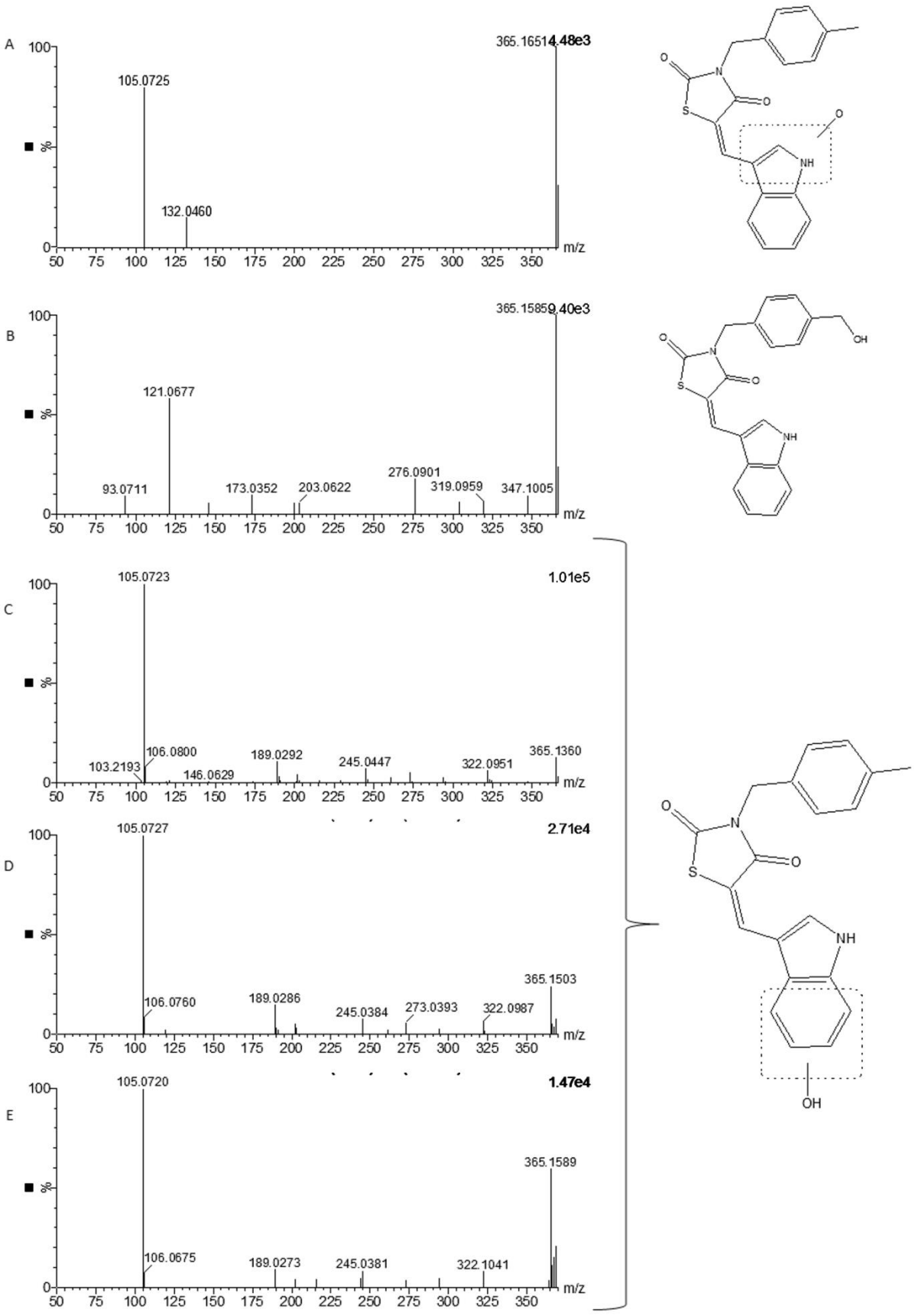


**Figure 6.**