Short Report

Acetaminophen Prodrug: Microwave-Assisted Synthesis and *in vitro* Metabolism Evaluation by Mass Spectrometry

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Propacetamol is an acetaminophen prodrug of intravenous administration used to control fever and pain of perioperative period in multimodal analgesia therapy. After injection, it is completely converted by plasma esterases into *N,N*-diethylglycine and acetaminophen, its active metabolite whose mechanism of action is the inhibition of prostaglandin synthesis. Herein, we report an improved protocol for the synthesis of propacetamol hydrochloride that allows the isolation of the active pharmaceutical ingredient (API) with high purity and yield. In addition, the *in vitro* metabolism of propacetamol in a microssomal reaction was evaluated by ion trap tandem mass spectrometry.

Keywords: microwave, drug synthesis, prodrug, propacetamol, acetaminophen, mass spectrometry

Introduction

In 1959, Harper introduced the term "latentiation" to describe a chemical modification in a drug that could regenerate the parent active substance after metabolism.[1] Since then, prodrugs have been highly explored to overcome several biologic barriers in drug delivery such as absorption, distribution and enzymatic metabolism.[2]

In drug development programs, difficulties such as low solubility, premature degradation, short half-life and other pharmacokinetic and pharmacodynamic parameters can also be tackled with a prodrug approach.[3] However, in order to become a prodrug candidate, a molecule must present some features such as biologic inactivity, absence of toxicity, simple synthetic route, a biolabile drug-carrier conjugate and ensure effective concentration of the drug in the target tissues.[4]

Hydrolysis activated prodrugs correspond to 49% of available compounds in the market.[5] Due to their ability to enhance drug's balance in water solubility, lipophilicity, permeability through biologic membranes and nontoxicity, esters are the most used prodrugs.[6-9] Additionally, amino acid-based prodrugs have found a broad range of applications since they allow parenteral administration of low soluble drugs such as metronidazole to patients unable to receive oral medication.[10]

Propacetamol hydrochloride (**2**) is a prodrug of intravenous administration used to control fever and pain of perioperative period in multimodal analgesia therapy.[11] After injection, it is completely converted by plasma esterases into *N,N*-diethylglycine and acetaminophen (**1**), its active principle whose mechanism of action is the inhibition of prostaglandin synthesis.[12-14] During an overdose situation, acetaminophen is converted to *N*-acetil-*p*-benzoquinone imine (NAPQI) (**3**) by biotransformation mediated by P450 enzymes, mainly CYP2E1. This toxic metabolite is highly reactive and responsible for hepatic injury.[15]

Although widely used, in the literature there is a restricted number of publications dealing with the synthesis of propacetamol.[16-22] In pioneering work, Cognacq[19] prepared propacetamol hydrochloride through the reaction of diethylamine with 4-acetamidophenyl-2-chloroacetate. Moreover, Devarajan-Ketha and Sloan[20] synthesized the same compound by coupling acetominophen with diethylglicine in the presence of dicyclohexyl carbodiimide (DCC).

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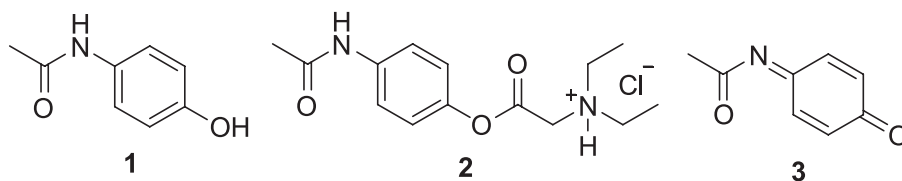
**Figure 1.** Structures of acetaminophen (**1**), propacetamol (**2**) and toxic metabolite NAPQI (**3**).

Herein, we wish to report an improved protocol for the synthesis of propacetamol hydrochloride that allows the isolation of the active pharmaceutical ingredient (API) with high purity and yield. Moreover, the *in vitro* metabolism of **2** in rat liver microsomes was investigated using ion trap tandem mass spectrometry.

Results and Discussion

We initiated our work by examining the synthesis of target compound **2** in two reaction steps from acetaminophen (**1**) using 4-acetamidophenyl 2-chloroacetate (**4**) as a key intermediate. Following this strategy, our initial goal was to study the esterification of **1** with chloroacetyl chloride (**5**). Although this reaction has already been reported in the literature using several reaction conditions,[21,22] best results were obtained when the reaction was performed at –15 °C using triethylamine as a base, leading to **4** as only product in 89% conversion, Scheme 1. Recrystallization of the crude product in ethanol afforded pure compound **4** in 53% yield (purification of **4** using flash column chromatography yielded the same product in 64% isolated yield).
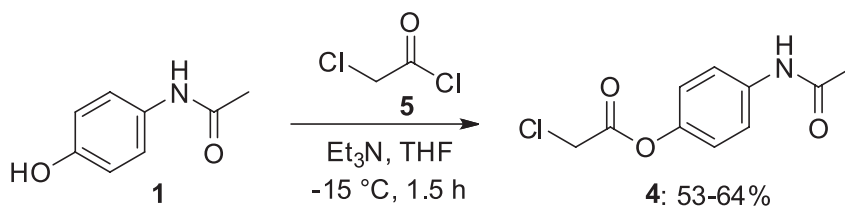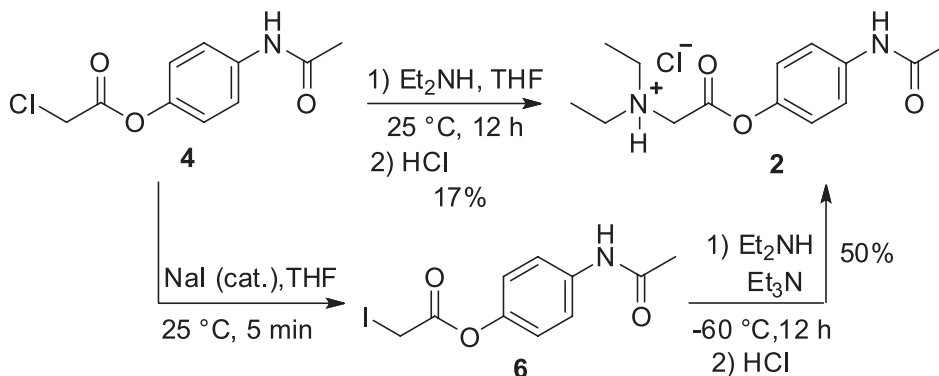
Subsequently, the synthesis of propacetamol hydrochloride was studied through the nucleophilic substitution of **4** with diethylamine. After screening for optimal reaction conditions, the use of catalytic amount of sodium iodide generated the more reactive intermediate **6** *in situ*. This was shown to be essential to obtain the desired compound **2** in reasonable yield of 50% after a crystallization step. In contrast, in absence of the iodide salt compound **2** was isolated in only 17% yield due to a competitive attack to the carboxyl group (Scheme 2).

Over the last years, a number of studies have been demonstrating the advantages of replacing the traditional heating methods with microwave irradiation. Thus, microwave heating has become a popular method for reducing reaction times, obtaining cleaner reactions and improving product yields.[23-26] Interestingly, it has been reported that heating **4** with excess of diethylamine at 40-50 °C for 2 hours allows isolation of **2** in 40 to 44% yields.[19] Therefore, we envisioned that the use of microwave irradiation could allow propacetamol obtainment in high yield from **4** in absence of an iodide salt.

It is well established that choosing the appropriate solvent is essential for the success of a microwave-assisted

Scheme 1.

Scheme 2.

synthesis, because of its ability to absorb electromagnetic energy and covert into heat. This ability is correlated with the loss tangent (tanδ) value (energy dissipation factor), where higher values indicate a greater penetration and conversion of the electromagnetic energy into heat.[27-29] Thus, we began this study by varying different solvents such as methyl *tert*-butyl ether (MTBE), acetone, acetonitrile, dimethylformamide (DMF) and tetrahydrofuran (THF) at the same temperature and reaction time (Table 1, entries 1-6). As observed, although having the lowest loss factor of the examined solvents (tanδ 0.047), THF was found to be the best solvent allowing propacetamol hydrochloride **2** to be isolated in 91% yield after the crystallization step (entry 6). Interestingly, when diethylamine was used as both reactant and reaction solvent compound **2** was obtained in 62% yield (entry 5).

In order to find the best reaction condition for synthesizing **2** we have also investigated the use of different amounts of diethylamine as well as other reaction temperatures (Table 1, entries 7-12). To our delight, compound **2** was obtained in 98% isolated yield when the reaction mixture was heated in the microwave under 10 min at 120 °C (entry 9). To date, the yield obtained in this reaction is very superior to the ones described in the literature for approaching **2**. Moreover,

the developed protocol has also some extra advantages such as the absence of catalyst, low solvent volume and short reaction time.

The antipyretic activity of produced **2** was evaluated through a pharmacologic study using male Wistar rats. Thus, animal groups were initially treated with acetaminophen (300 mg kg^{-1}) and propacetamol hydrochloride in different dosages (150 to 600 mg kg^{-1}). After thirty minutes of drug or prodrug administration, lipopolysaccharide (LPS) was injected to the animals as an inflammatory stimulus to cause fever.[30] Profiles of the temperature variations in function of the time have shown that the fever inhibition using **2** was successfully achieved using 600 mg kg^{-1} dosage (see Supplementary Information section). As observed in Figure 2, the temperature variation in the acetaminophen treated group was similar to the one treated with propacetamol hydrocloride. Therefore, the 600 mg kg^{-1} dosage of **2** was equivalent to acetaminophen (300 mg kg^{-1}) to inhibit fever until five hours after administration which is agreement with literature data that predicts the efficient dosage of propacetamol as twice the acetaminophen.[31]

We have also studied the *in vitro* metabolism of **2** in rat liver microsomes using ion trap tandem mass spectrometry.[32] To the best of our knowledge, this strategy

Table 1. Screening of reaction conditions for the microwave-assisted synthesis of **2**
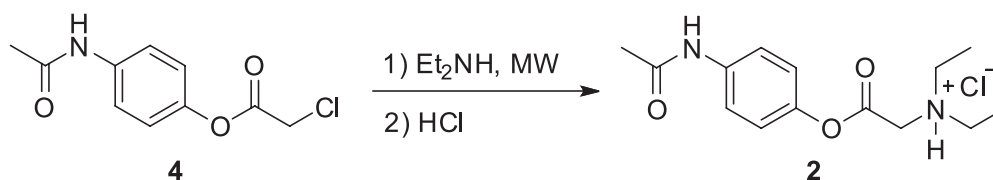
entry[a]	Et$_2$NH / mL	Temperature / °C	time[b] / min	Solvent	Yield[c] / %
1	3.5	70	40	MTBE[d]	42
2	3.5	70	40	acetone	35
3	3.5	70	40	acetonitrile	52
4	3.5	70	40	DMF[e]	81
5	3.5	70	40		62
6	3.5	70	40	THF[f]	91
7	3.5	100	8	THF[f]	70
8	3.5	120	4	THF[f]	77
9	1	120	10	THF[f]	98
10	1	150	5	THF[f]	73
11	1	200	2	THF[f]	76
12	0.5	120	15	THF[f]	94
13	0.25	120	21	THF[f]	72
14	0.2	120	32	THF[f]	71

[a]0.5 mmol of **4** were used; [b]time necessary for full conversion of the starting material; [c]isolated yield; [d]MTBE: methyl *tert*-butyl ether; [e]DMF: dimethylformamide; [f]THF: tetrahydrofuran.
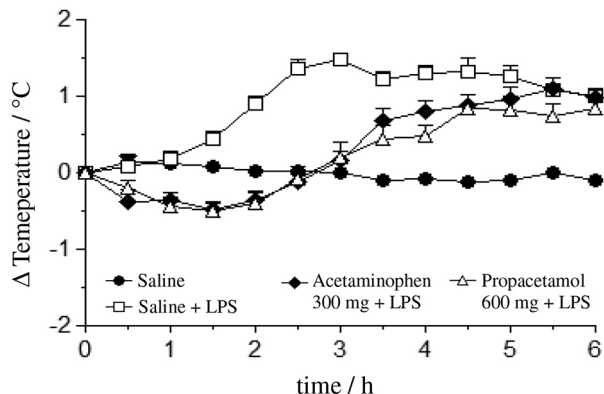
Figure 2. Comparison of body temperature variation in function of time between acetaminophen 300 mg kg^{-1} dosage and propacetamol 600 mg kg^{-1} dosage.

has never been used to evaluate the metabolism of propacetamol. Thus, once the microsomal reaction was completed, the standard compounds and reaction extracts were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Figure 3). As observed in the LC-MS chromatogram, standard compounds acetaminophen, propacetamol and

4-acetamidophenyl acetate have shown signals at retention time (Rt) 9.8, 6.6 and 18.8 min, respectively (Figure 3a).

Chromatograms (Figures 3b and 3c) show the obtained results of the microsomal reaction using propacetamol hydrocloride. Both chromatograms of the control reaction (absence of cofactors) and the test reaction employing rat liver microsomes have presented only one peak at the Rt 9.8 min, which corresponds to acetaminophen (m/z 152.02). Similar results were obtained using acetaminophen and 4-acetamidophenyl acetate substrates (see Supplementary Information section). These results showed that the hydrolitic cleavage of the propacetamol and the more hydrophobic derivative 4-acetamidophenyl acetate side chain, prepared through the esterefication of acetaminophen with acetic anhydride, are microsome and co-factor independent processes.

An *et al.*[33] and Ma *et al.*[34] have detected the paracetamol reactive metabolite **3** by trapping this short half-live intermediate with glutathione. Interestingly, when the microsomal reaction of **2** was performed in the presence of this trapping agent neither the expected adduct nor different derivatives were detected by ion trap tandem mass
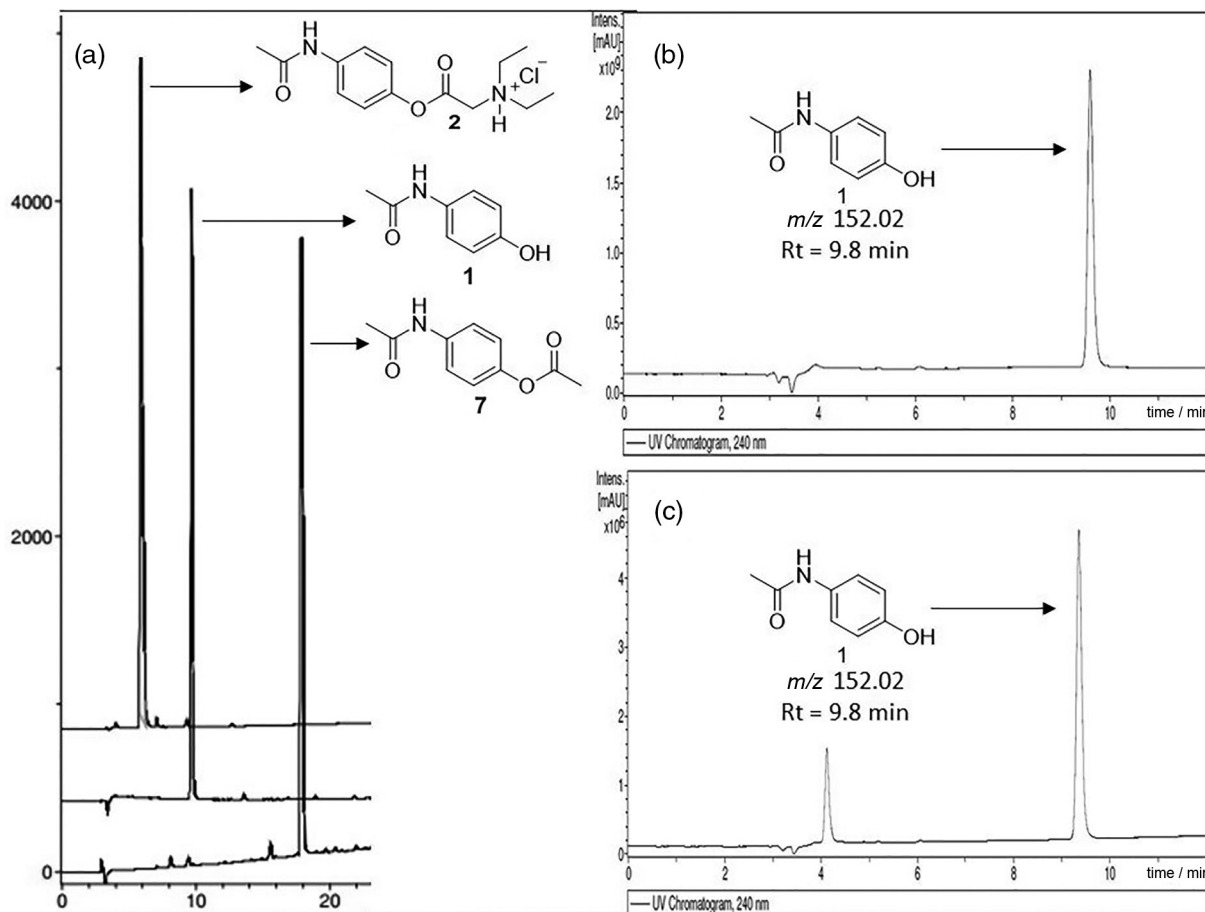
Figure 3. Chromatograms of isolated analytes (a) propacetamol hydrochloride (**2**) (m/z 265.13, Rt 6.6 min), acetaminophen (**1**) (m/z 152.02, Rt 9.8 min) and 4-acetamidophenyl acetate (**7**) (m/z 194.02, Rt 18.8 min). Chromatograms of microssome reaction products: (b) control tube; (c) test tube.

spectrometry, which indicates that the acetaminophen first phase metabolism did not occur in the microsomal reaction studied.

Conclusions

In summary, we have reported an improved protocol for the synthesis of propacetamol hydrochloride in high yield and purity using microwave irradiation in the key step. Moreover, the antipyretic activity of this active pharmaceutical ingredient was confirmed by an *in vivo* pharmacologic essay. Although oxidative derivatives were expected, the ion trap tandem mass spectrometry evaluation of the *in vitro* metabolism of propacetamol and the more hidrofobic derivative 4-acetamidophenyl acetate in rat liver microsomes indicates acetaminophen as only metabolite. Such results update the available data for acetaminophen prodrugs by providing information to help understanding the drug release by enzymatic metabolism.

Experimental

Synthesis of compounds

4-Acetamidophenyl-2-chloroacetate (**4**)

To a round-bottom flask containing acetaminophen (15 mmol, 2.26 g), THF (45 mL) and triethylamine (22.5 mmol, 2.28 g, 3.14 mL) chloroacetyl chloride (18 mmol, 2.03 g, 1.44 mL) was added dropwise and the reaction mixture was stirred at –15 °C for 1.5 h. After that, the formed precipitate was removed by filtration. Ethyl acetate (200 mL) was added to the filtrate, which was washed with water (3 × 150 mL), saturated solution of sodium bicarbonate (2 × 150 mL) and brine (150 mL). The organic solution was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The residue was recrystallized from dry ethanol to yield compound **4** as a white solid (53%). Purification of the crude product using flash silica column chromatography (hexanes/ethyl acetate, 7:3) yielded compound **4** in 64% isolated yield; mp 184-185 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.04 (s, 3H, CH_3), 4.67 (s, 2H, CH_2), 7.10 (d, 2H, *J* 8.9 Hz, Ph-H), 7.61 (d, 2H, *J* 8.9 Hz, Ph-H), 10.04 (s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 23.9, 41.3, 119.9 (2C), 121.6 (2C), 237.3, 145.3, 166.6, 168.3; MS *m/z* (rel. int.): 227 (10), 151 (43), 109 (100), 108 (21), 80 (14), 43 (30), 42 (11).

4-Acetamidophenyl acetate (**7**)

To a round-bottom flask containing acetaminophen (3 mmol, 0.45 g), 4-dimethylaminopyridine (DMAP) (0.25 mmol, 0.091 g) and THF (30 mL), acetic anhydride (3.6 mmol, 0.37 g, 0.34 mL) was added and the reaction mixture was stirred for 2 h at 25 °C. After that, the formed precipitate was removed by filtration. Ethyl acetate (80 mL) was added to the filtrate, which was washed with water (2 × 80 mL), saturated solution of sodium bicarbonate (2 × 80 mL) and brine (150 mL). The organic solution was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography (hexanes/ethyl acetate, 2:8) to yield compound **7** as a white solid (95%); mp 151-152 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.04 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 7.04 (d, 2H, *J* 8.9 Hz, Ph-H), 7.59 (d, 2H, *J* 8.9 Hz, Ph-H), 10.00 (s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.8, 23.9, 119.8 (2C), 121.9 (2C), 136.9, 145.6, 168.2, 169.3; MS *m/z* (rel. int.): 193 (5), 151 (40), 109 (100), 108 (11), 80 (14), 53 (11), 43 (50).

4-Acetamidofenil-2-iodoacetate (**6**)

To a round-bottom flask containing sodium iodide (30 mmol, 0.46 g) and acetone (60 mL), 4-acetamidophenyl-2-chloroacetate (**4**) (28 mmol, 0.638 g) was added and the reaction mixture was stirred at 25 °C for 22 h. After that, the formed precipitate was removed by filtration. Ethyl acetate (120 mL) was added to the filtrate, which was washed with water (3 × 100 mL), saturated solution of sodium bisulfite (2 × 100 mL) and brine (100 mL). The organic solution was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography (hexane/ethyl acetate, 7:3) to yield compound **6** as a yellow solid (80%); mp 114-115 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.14 (s, 3H, CH_3), 3.90 (s, 2H, CH_2), 7.04 (d, 2H, *J* 8.9 Hz, Ph-H), 7.50 (d, 2H, *J* 8.9 Hz, Ph-H), 7.66 (s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ –5.9, 24.6, 121.0 (2C), 121.4 (2C), 136.1, 146.7, 167.9, 168.6; MS *m/z* (rel. int.): 319 (5), 151 (98), 109 (100), 108 (31), 80 (17), 43 (41), 42 (15); HRMS (FTMS + pESI) *m/z*, calcd. for $C_{10}H_{11}NO_3I$ [M]+: 319.9784; found: 319.9781.

2-(4-Acetamidophenoxy)-*N,N*-diethyl-2-oxaethan-1-aminium chloride (**2**)

Method A (classic): triethylamine (9 mmol, 0.91 g, 1.3 mL) and diethylamine (7.2 mmol, 0.52 g, 0.7 mL) were added at –60 °C to a flask containing 4-acetamidophenyl-2-chloroacetate (**4**) (6 mmol, 1.36 g), sodium iodide (22 mol%, 0.19 g) and THF (10 mL). Under stirring, the reaction temperature was allowed to gradually increase to 20 °C. After 12 h reaction, the precipitated was removed by filtration and the filtrate concentrated under reduced pressure. The yellow oil was dissolved in acetone and allowed to react with excess of concentrated hydrochloric

acid (35-37%) to yield compound **2** as a white solid (50%) after filtration.

Method B (microwave-assisted reaction): microwave irradiation reaction were carried out using a dedicated single-mode microwave reactor (Monowave 300, Anton Paar, Graz Austria) able to provide 850 W maximum continuous microwave power in combination with an efficient magnetic stirring system. For all reactions described in Table 1, the reaction temperature was monitored by an internal fiber-optic temperature probe (ruby thermometer). Diethylamine (9.67 mmol, 1 mL) was added to a vial containing 4-acetamidophenyl-2-chloroacetate (**4**) (0.5 mmol, 0.113 g) and THF (4 mL). The microwave reactor was programmed to work at 120 °C for 10 min. After completed reaction, the precipitated was removed by filtration and the filtrate concentrated under reduced pressure. The yellow oil was dissolved in acetone and allowed to react with excess of concentrated hydrochloric acid (35-37%) to yield compound **2** as a white solid (98%) after filtration; mp 209-210 °C; ^{1}H NMR (400 MHz, D_2O) δ 1.35 (t, 6H, *J* 7.3 Hz, CH_3), 2.13 (s, 3H, CH_3), 3.39 (q, 4H, *J* 7.3 Hz, CH_2), 4.44 (s, 2H, CH_2), 7.20 (d, 2H, *J* 9.0 Hz, Ph-H), 7.47 (d, 2H, *J* 9.0 Hz, Ph-H); ^{13}C NMR (100 MHz, D_2O) δ 8.3 (2C), 22.7, 49.5 (2C), 52.4, 121.6 (2C), 123.1 (2C), 135.6, 146.0, 166.3, 172.9; HRMS (FTMS + pESI) *m/z*, calcd. for $C_{14}H_{21}N_2O_3$ $[M]^+$: 265.1547; found: 265.1552.

Antipyretic evaluation

Experiments were conducted on 72 male Wistar rats weighing 180-200 g. They were housed individually at 24 ± 1 °C under a 12:12 h light-dark cycle (lights on at 06:00 h) with free access to food and tap water until the night before the experiment when only water was made available. Each animal was used only once. The study was previously approved by the Animal Research Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (Protocol No. 019/2012). After lipopolysaccharide (LPS) injection, the animals were divided in groups and underwent to pre-treatment with different dosage propacetamol (150, 300, 450 and 600 mg kg^{-1}). The rectal temperature was measured in conscious and unrestrained rats every 30 min for 6 h by gently inserting a vaseline-coated thermistor probe (model 402 coupled to a model 46 telethermometer, Yellow Springs Instruments, Yellow Springs, OH, USA) 4 cm into the rectum, without removing the rats from their cages. Experimental measurements were conducted in a temperature-controlled room at 27 ± 1 °C, within the thermoneutral zone for rats. Baseline temperatures were

determined three times, and at 30 min intervals before intraperitoneal injection of LPS. The profiles of body temperature variation in function to time were plotted in Figure S12 (see Supplementary Information section).

In vitro metabolism evaluation by mass spectrometry

Rat liver microsomes (RLM, pooled from 6 animals) were obtained by differential high-speed centrifugation according to a previously published procedure.[10] All care and handling of the animals was performed with the approval of the Ethical Committee from the University of São Paulo (14.1.721.53.6), according to the Guiding Principles for Research Involving Animals and Human Beings from the American Physiological Society.

Microsomal incubation conditions

Incubations were performed with the reconstituted rat liver microsomes in 10 mL amber tubes, using a shaking water bath at 37 °C. Microsomal sample preparation consisted of a cofactor solution, rat liver microsomes, phosphate buffer (pH 7.4, 0.25 mol L^{-1}) and the substrate (acetaminophen or propacetamol or 4-acetamidophenyl acetate), in a total volume of 1 mL. Cofactor solution consisted of NADP$^+$ (0.25 mmol L^{-1}), glucose-6-phosphate (5 mmol L^{-1}) and glucose-6-phosphate dehydrogenase (0.5 units) in Tris-HCl buffer (Tris-HCl 0.05 mol L^{-1}, KCl 0.15 mol L^{-1}, pH 7.4). After 5 min pre warmed at 37 °C, the metabolic reaction was initiated by the addition of the rat liver microsomes. To stop metabolic reaction after 90 min, it was added 4 mL of ethyl acetate. Control incubations were performed in the absence of cofactor solution and in the absence of microsomal preparation. The difference between 'with' and 'without' NADPH was considered as CYP450-mediated metabolism. The sample preparation was carried out according to sub-section Sample preparation.

Sample preparation

A liquid-liquid extraction (LLE) procedure was applied to extract the substrate (acetaminophen or propacetamol or 4-acetamidophenyl acetate) from the rat liver microsomes. The extraction was initiated by adding 4 mL of ethyl acetate in the microsomal preparation sample. Next, the samples were shook for 15 min (Vibrax VXR agitator, IKA, Staufen, Germany) and centrifuged for 5 min at 2860 ×g (Hitachi CF16RXII, Himac, Tokyo, Japan). The supernatant was collected (3 mL) and let to evaporate to dryness under a gently stream of compressed air. After that, the residue was

reconstituted in 100 μL of the mobile phase and 20 μL was injected into the chromatography system.

Determination of the metabolites

To investigate the formation of metabolites, the samples were analyzed using a Shimadzu (Kyoto, Japan) high performance liquid chromatography (HPLC) coupled to an amaZon-SL ion trap (IT) Bruker Daltonics® (Billerica, USA) after the *in vitro* metabolism. The HPLC comprising a LC-20AD solvent pump unit, a CTO-20A column oven, a DGU-20A3 online degasser, a CBM-20A system controller and a SPD-M20A (200 to 800 nm) diode array detector. Injections were performed automatically (20 μL) through a 100 μL loop SIL-20A HT. The separation of substrate and its metabolites was performed at 25 °C using a Phenomenex Luna C18 column (250 mm × 4.60 mm internal diameter, 5 μm particle size). The mobile phase was comprised of water (solvent A) and methanol (solvent B) both with 0.1% formic acid, and was pumped at a flow rate of 1 mL min^{-1}. The gradient elution program was performed as follows: 0 min 10% (B), 0-40 min 10-100% (B), 50 min 100% (B), 51-60 min 10% (B).

The mass spectrometer source parameters were set as follow: capillary voltage at 3.5 kV, end plate offset at 500 V. Nitrogen (N_2) was used as nebulizing (50 psi) and drying gas (9 L min^{-1}, 300 °C). Full-scan MS and MS/MS spectra were obtained by scanning *m/z* from 50 to 500. The electrospray ionization (ESI) source was operated in the positive ionization mode. The data were acquired using amplitude of 0.3 V. The data were processed through Bruker Compass Data Analysis 4.1 software (Bremen, Germany).

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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