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Development of acetaminophen proline prodrug

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ABSTRACT

In this research work, proline ester prodrug of acetaminophen (Pro-APAP) was synthesized and evaluated for its stability in PBS buffer at various pH and Caco-2 cell homogenate. The Pro-APAP is more stable at lower pH than higher pH, with half-life of 120 min in PBS buffer at pH 2.0, half-life of 65 min at pH 5.0, and half life of 3.5 min at pH 7.4, respectively. The half-life of Pro-APAP in Caco-2 cell homogenate is about 1 min, much shorter than the half-life in PBS buffer at pH 7.4, indicating enzymes in the cell homogenate contribute to the hydrolysis of the ester bond. Carboxypeptidase A was incubated with Pro-APAP at pH 7.4 with half-life of 3.8 min which is very close to the half life in buffer itself. This clearly indicates carboxypeptidase A is not one of the enzymes contributing to the hydrolysis of the prodrug. Physicochemical characteristics such as melting point and stability of newly synthesized prodrug were determined by MDSC technique.

Acetaminophen is widely used as a non-opiate, analgesic, and antipyretic drug. It is also known as *N*-acetyl-*p*-aminophenol (APAP) or paracetamol outside of North America. Acetaminophen is probably the most prescribed medicine in children for treatment of fever and pain. There are a couple of problems associated with acetaminophen which had been targeted by prodrug approach. First, the unpleasantly bitter taste of acetaminophen makes it difficult to be formulated as a chewable dosage form for pediatric use. The bitter taste could be masked by capping the hydroxyl group with promoieties including tetrahydropyran and ethoxyethan.[1,2] Secondly, orally administered phenols typically showed poor bioavailability and hepatic toxicity due to first-pass metabolism in the gastrointestinal tract and liver.[3] These toxic effects have been attributed to the formation of a toxic metabolite *N*-acetylquinone imine, which is detoxified by reaction with glutathione leading to glutathione depletion and cell death.[4] Esterification of acetaminophen with amino acids and peptides (promoieties) was reported as a means to overcome liver toxicity of the drug at high doses as well as to enhance aqueous solubility.[5] Transdermal delivery is another ideal approach to avoid first-pass metabolism. Drugs with phenolic functional groups are attractive candidates for topical drug delivery via prodrug approach. The phenolic OH group is very convenient to attach a wide range of promoieties. Most of the previous work on the phenols via a prodrug approach has focused on the corresponding ester or ether.[6–9] Sulfate and amino acid esters of acetaminophen were also developed as potential prodrugs.[10,11]

In this study, proline prodrug of acetaminophen (Pro-APAP) was synthesized and physicochemical characteristics were determined. The purpose was to develop a prodrug of acetaminophen that could mask the bitter taste for formulation of chewable dosage form. Compared with other amino acids ester prodrugs, such as isoleucine and valine ester prodrugs, proline ester prodrugs have shorter half lives, which is appropriate for a chewable dosage form.[12] This prodrug could also be tested as a candidate for transdermal delivery to avoid the liver toxicity caused by first pass metabolism.

Acetaminophen prodrug was synthesized as shown in Scheme 1.[13] The purity of the prodrug was determined by HPLC. Prodrug was 95% pure. The prodrug was easily separated from parent drug by HPLC. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospray ionization mass spectrometer. The observed molecular weight of the prodrug was found to be identical to that required by the structure. Structural identity was also confirmed using proton nuclear magnetic resonance spectra (^{1}H NMR). ^{1}H NMR spectra were obtained with a 300 MHz Bruker NMR spectrometer.[14]

Acetaminophen and prodrug were analyzed by reversed phase chromatography performed at room temperature. Detection wavelength was 243 nm. Samples were injected into a C18 column (Symmetry® C18 3.5 μm, 4.6 × 75 mm column) and eluted at a flow rate of 0.6 mL/min. Mobile phase consisted of 10% v/v methanol and 1% v/v glacial acetic acid in HPLC water. The HPLC equipment consisted of a Waters 717 plus autosampler, Waters 2695 separation module, Waters 2998 (Photodiode Array Detector) and Empower 2 software (Water Corporation) data handling system.

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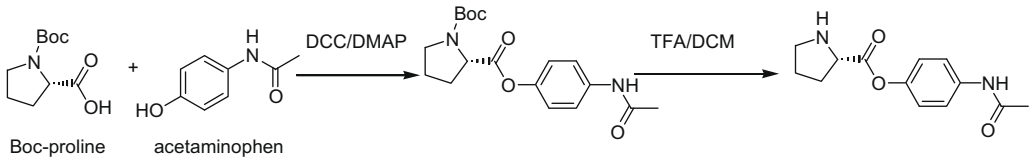

Scheme 1. Synthesis of Pro-APAP.

Table 1
Half-life of Pro-APAP in different hydrolysis media at pH 7.4 and 37 °C (n = 3) (average ± S.D.)

Hydrolysis medium	Caco-2 cell homogenate	Carboxypeptidase A	PBS pH 7.4	PBS pH 5.0	PBS pH 2.0
Half-life (min)	1.04 ± 0.01	3.88 ± 0.08	3.40 ± 0.20	68.00 ± 4.00	120.67 ± 3.06

The chemical stability of the prodrug was determined in PBS at pH 2.0, 5.0, 7.4 and at 37 °C in order to obtain the contribution of non-enzymatic hydrolysis.[15] Estimated half-lives of acetaminophen prodrug in phosphate buffers were calculated from linear regression plots of prodrug concentrations versus time and listed in Table 1. In PBS pH 2.0 medium, half-life of Pro-APAP was found to be 120.0 min which is the longest half-life in all media. In PBS pH 5.0 medium, half-life of Pro-APAP was found to be 65.0 min. While in PBS pH 7.4, half-life was found to be 3.5 min. (Figs. 1–3)

The hydrolysis study of Pro-APAP was performed in Caco-2 cell line.[16] The hydrolysis of Pro-APAP incubated with carboxypeptidase A was carried out following the instruction of the enzyme kit. Estimated half-lives of acetaminophen prodrug in Caco-2 cell homogenates and carboxypeptidase A were calculated from linear regression plots of prodrugs concentrations versus time and listed in Table 1. In Caco-2 cell homogenate, half-life of Pro-APAP was found to be 1 min. While incubated with carboxypeptidase A, half-life of Pro-APAP was found to be about 4 min. As for the enzymatic stability, acetaminophen prodrug hydrolysis rate was significantly faster in Caco-2 cell homogenates compared to pH 7.4 phosphate buffer, suggesting specific enzymes play an important role in the hydrolysis process (Figs. 4 and 5).

Differential Scanning Calorimetry (DSC) has many applications in pharmaceutical field including determination of melting point

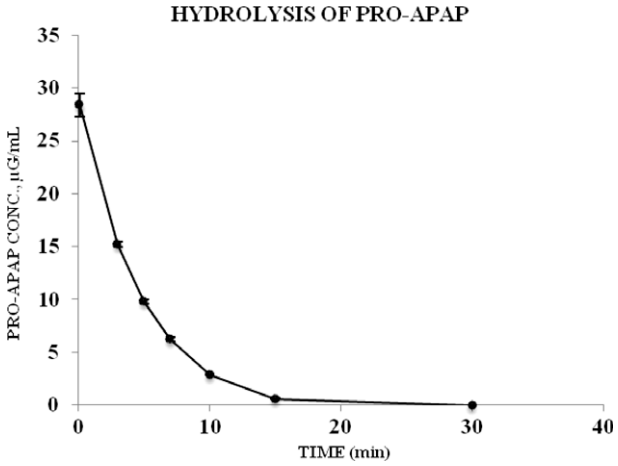

Figure 1. Hydrolysis of Pro-APAP in PBS pH 7.4 medium (n = 3).

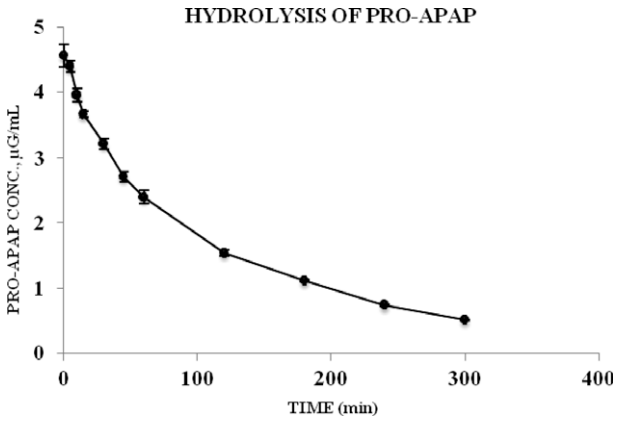

Figure 2. Hydrolysis of Pro-APAP in PBS pH 5.0 medium (n = 3).

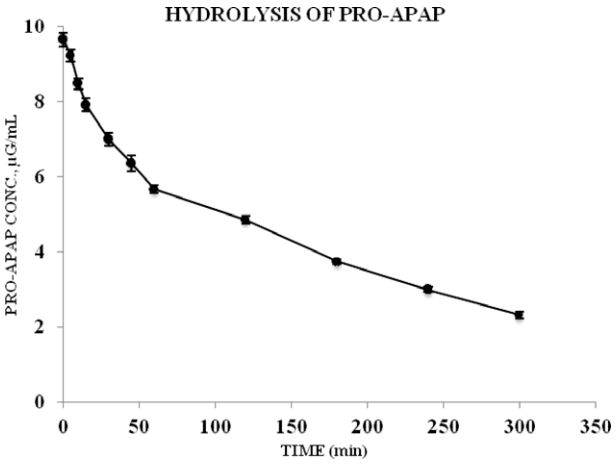

Figure 3. Hydrolysis of Pro-APAP in PBS pH 2.0 medium (n = 3).

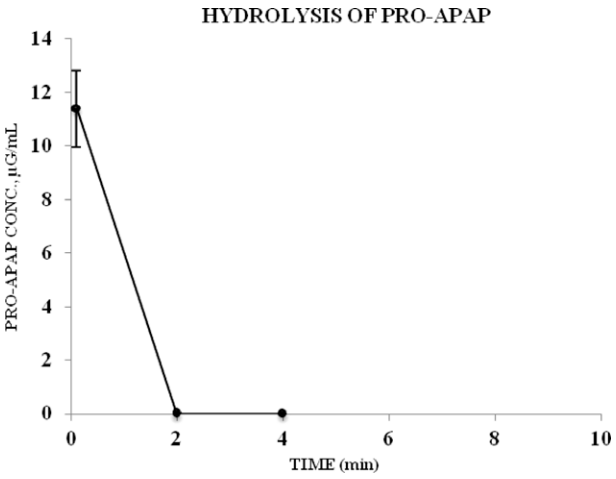

Figure 4. Hydrolysis of Pro-APAP in Caco-2 cell homogenate (n = 3).

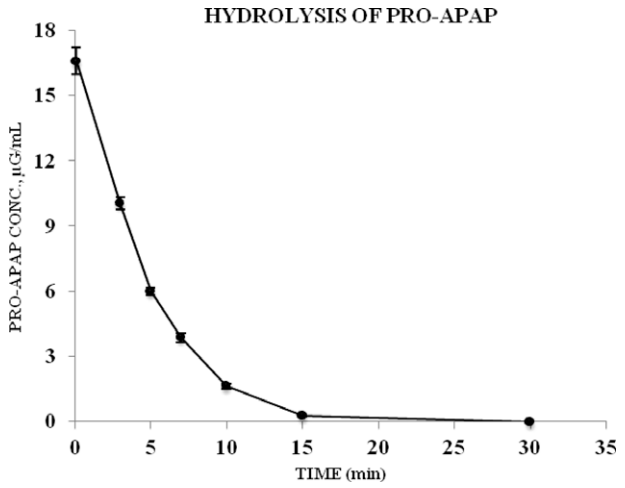

Figure 5. Hydrolysis of Pro-APAP incubated with carboxypeptidase A ($n = 3$).

of compounds, confirmation of crystalline or amorphous nature of powder, finding purity, and polymorphism of sample, etc. DSC is a thermal analysis technique that has been used to measure thermodynamic properties associated with transitions in materials as a function of time and temperature. Modulated Differential Scanning Calorimetry (MDSC) is modification of conventional DSC which provides additional information for the compound of interest. MDSC was used for physical characterization of acetaminophen, Boc-L-proline, and Pro-APAP. MDSC thermograms of these samples were recorded from 20 °C to 240 °C. Melting points of acetaminophen, Boc-L-proline, and Pro-APAP were determined. Also melting point of Pro-APAP was determined three times over a period of three months at room temperature to see its stability.[17]

Acetaminophen shows characteristic endothermic peak at 171 °C and Boc-L-proline shows peak at 140 °C as it has been reported in literature. Prodrug shows endothermic peak at 197 °C (Fig. 6). Stability results of Pro-APAP also indicate similar melting point of 197 °C with very minor variation. From overlay of thermograms of prodrug stored over the time it is clear that no degradation had occurred (Figs. 6 and 7).

In this study, proline prodrug of acetaminophen (Pro-APAP) was prepared by esterification of the hydroxyl group. This is a commonly utilized prodrug strategy to improve certain drug properties. The chemical stability of Pro-APAP was studied in PBS buffer at different pH. The stability of the prodrug in the presence of enzymes was also measured in Caco-2 cell homogenate, a cell line that is commonly used to mimic small intestine absorption. Carboxypeptidase A, a common enzyme involved in prodrug hydrolysis, was incubated with Pro-APAP to determine if it is one of the enzymes that contribute to the hydrolysis of the prodrug to acetaminophen. Some physicochemical properties such as melting point and stability of the prodrug were also determined by DSC. One purpose of this study is to develop an acetaminophen prodrug which is suitable to be formulated as chewable dosage form by masking the bitter taste of acetaminophen. The other purpose is to develop a prodrug for transdermal delivery to reduce hepatic toxicity by avoiding the first-pass metabolism.

The Pro-APAP is more stable at lower pH than higher pH, with half-life of 120 min in PBS buffer at pH 2.0, half-life of 65 min at pH 5.0 and half-life of 3.5 min at pH 7.4, respectively. This is consistent with the stability of other amino acid ester prodrugs such as valacyclovir, Ile-gemcitabine.[18] Generally, the proline ester prodrug is not as stable as some other amino acid ester prodrugs such as isoleucine and valine. The lower chemical stability of proline ester prodrug could be due to the higher pK_a of the secondary amine of proline (pK_a ~10.6). The proline is readily ionized at pH 7.4 and that makes the hydrolysis of the ester bond easier.[12,19] The half-life of Pro-APAP in Caco-2 cell homogenate is about 1 min, much shorter than the half life in PBS buffer at pH 7.4. This result indicates enzymes in the cell homogenate contribute to the hydrolysis of the ester bond. These enzymes could be esterase and peptidase which are known to hydrolyze ester bond. Carboxypeptidase A was incubated with Pro-APAP in PBS buffer at pH 7.4 with half-life of 3.8 min which is very close to the half life in buffer itself. This clearly indicates carboxypeptidase A is not one of the enzymes contributing to the hydrolysis of the prodrug. Other enzymes including esterase will be tested as converting enzymes of Pro-APAP.

Compared with other amino acids ester prodrugs, such as isoleucine and valine ester prodrugs, proline ester prodrugs have

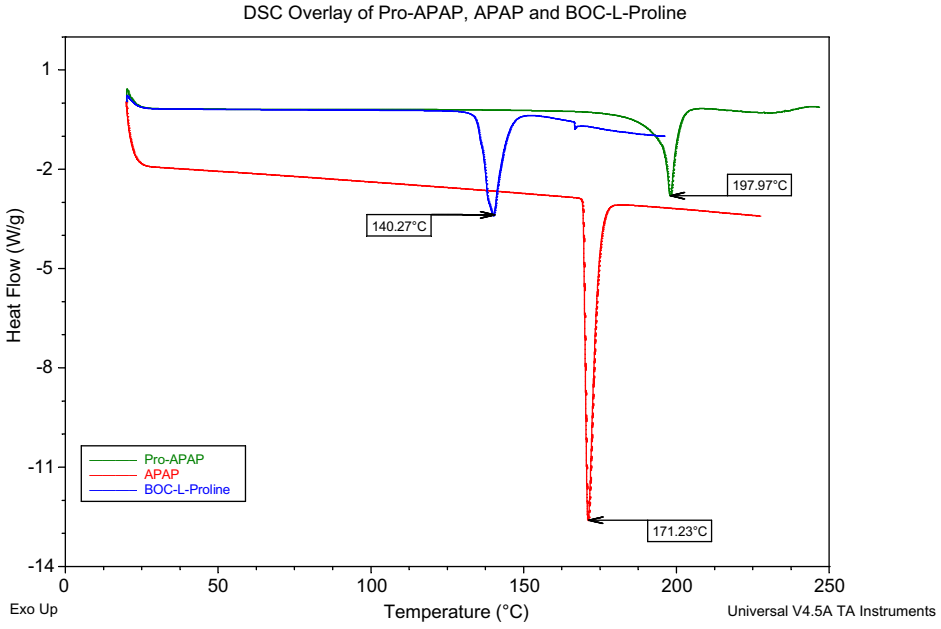

Figure 6. DSC thermogram overlay-1.

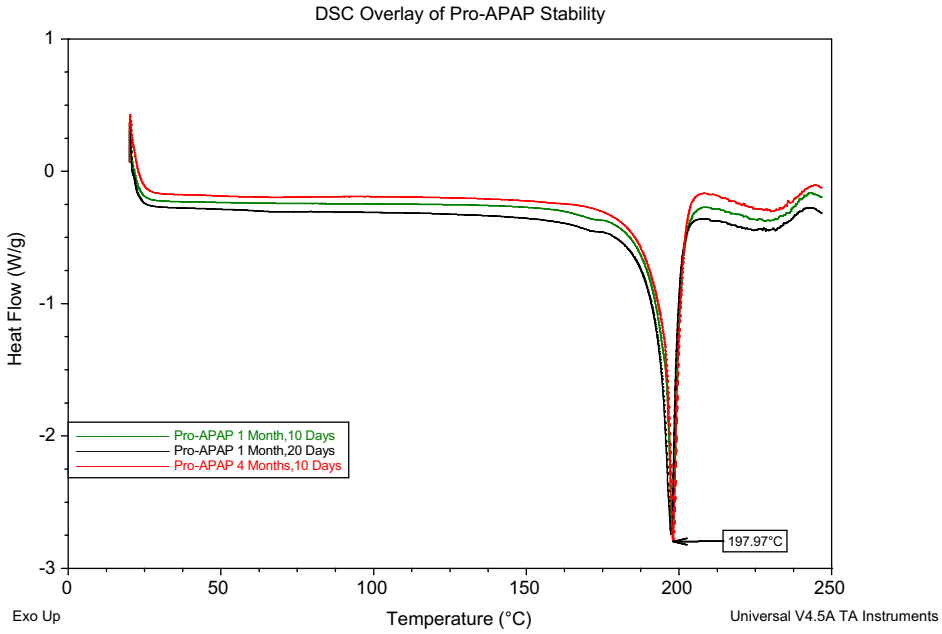

Figure 7. DSC thermogram overlay-2.

significantly shorter half lives. The half life of Pro-APAP is only about 4 min in PBS buffer at pH 7.4, which is appropriate for a chewable dosage form. After swallowing, the prodrug is expected to quickly convert into acetaminophen. Nevertheless, Pro-APAP seems to be not stable enough to be used in a transdermal formulation, because transdermal absorption is significantly longer than other delivery systems due to its lag time. Other amino acid ester prodrug of acetaminophen will be synthesized for a transdermal formulation. Amino acids selected for synthesis should cover neutral, acidic, and basic amino acids. It is reported that dipeptide ester prodrugs of acetaminophen have better stability.[5] Various dipeptide promoieties should be used to synthesize acetaminophen prodrugs for transdermal delivery.

Pro-APAP synthesized here is in the form a TFA salt. To be formulated as a chewable dosage form to mask the bitter taste of acetaminophen, the TFA salt should be converted to HCL salt first. To know if Pro-APAP is able to mask the bitterness of acetaminophen, human study is necessary for this information. The fact that the phenol group of acetaminophen, which may contribute to the bitter taste, is capped by proline-a natural amino acid, makes us believe it may be less bitter than acetaminophen.[1,2]

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13. Briefly, *N*-*t*Boc-protected proline was (1.2 equiv) added dropwisely into the mixture of dicyclohexylcarbodiimide (DCC, 1.2 equiv) and 4-dimethylaminopyridine (DMAP, 0.12 equiv) and acetaminophen (1 equiv) in dry dimethylformamide (DMF). The reaction was stirred at room temperature for 24 h. The reaction was monitored by TLC (hexane/ethylacetate, 1:1). DMF was removed under high vacuum. The residue was extracted with ethylacetate (50 mL) and washed with water (2 × 30 mL), saturated $NaHCO_3$ (2 × 30 mL) and saturated NaCl (1 × 30 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. Crude compounds were separated using column chromatography (hexane/ethylacetate, 10:1). The fractions were collected and analyzed by thin layer chromatography (TLC) for purity. Fractions from each spot were concentrated under vacuum separately. The Boc group was removed by treating the residues with 5 mL of trifluoroacetic acid (TFA) and 5 mL of dichloromethane (DCM) for 4 h. After evaporating DCM and most TFA (95%), cold ether was added to precipitate out the pure compounds. After removing ether, the residues were reconstituted with water and lyophilized. White powder was obtained as final product in TFA salt form.
14. *Spectroscopic data and yield of Pro-APAP*: Pro-APAP: yield, 70%; ^{1}H NMR (DMSO): 1.90 (s, 3H, CH_3), 2.11 (m, 2H, proline CH_2CH_2), 2.30 (m, 2H, proline CH_2CH_2), 3.57 (m, 2H, proline N–CH_2), 4.54 (t, 1H, α-H), 7.13 (d, 2H, $CH_{benzine}$–O), 7.65 (d, 2H, $CH_{benzine}$–N); ESI-MS: 249 $(M+H)^+$.
15. The hydrolysis study was carried out in 96-well plates. PBS buffer (230 μL) was placed in triplicate wells and the reactions were started with the addition of acetaminophen prodrug and incubated at 37 °C. At various time points, 40 μL aliquots were removed and added to 40 μL of 10% ice-cold TFA. The mixtures were centrifuged and filtered through a 0.45 μm filter for 10 min at 2000 rcf and 4 °C. The filtrate was then analyzed by HPLC.
16. The cell line was cultured at 37 °C with 5% CO_2 and 90% relative humidity. Caco-2 cells were cultured in 80% minimum essential medium (MEM) with 20% FBS. Split confluent culture 1:4 to 1:6 every 3–5 days using trypsin/EDTA. Cell homogenate were prepared when the cells were 90% confluent. After trypsinization, the cells were washed three times with pH 7.4 PBS buffer and re-suspended in pH 7.4 PBS (10 mM). To prepare cell homogenate, 1% Triton-X 100 was added in PBS solution and vortexed vigorously. The cell suspension was centrifuged at 18,000 rpm for 30 min at 4 °C. The supernatant was used in hydrolysis study and to determine protein content. Total protein was quantified with the BioRad Protein Assay using bovine serum albumin as standard. The protein content was adjusted to approximately 1000 μg/mL by appropriate dilutions before being used in hydrolysis studies.
17. MDSC Q100 series was used in this study to measure melting points of acetaminophen and Pro-APAP. MDSC test of Pro-APAP was repeated up to 3 months to see the stability of the prodrug. About 4–6 mg powder was sealed in aluminum hermetic pan with lid and loaded on MDSC to run this experiment. Melting point of Boc-L-proline was also determined.
The following is the procedure for conducting MDSC thermogram of these samples: (1) Data storage: Off; (2) Equilibrate at 20.00 °C; (3) Data storage: On; (4) Ramp 10.00 °C/min to 250.00 °C; (5) End of method.
In Step 4, maximum temperature was modified as per the known theoretical melting point of sample to be analyzed. Boc-L-proline has melting point of near to 135 °C, so in this case maximum temperature was set at 200.00 °C.
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