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Formulation and biopharmaceutical evaluation of bitter taste masking microparticles containing azithromycin loaded in dispersible tablets

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ABSTRACT

The objective of this study was to prepare and evaluate some physicochemical and biopharmaceutical properties of bitter taste masking microparticles containing azithromycin loaded in dispersible tablets. In the first stage of the study, the bitter taste masking microparticles were prepared by solvent evaporation and spray drying method. When compared to the bitter threshold (32.43 µg/ml) of azithromycin (AZI), the microparticles using AZI: Eudragit L100 = 1: 4 and having a size distribution of 45 – 212 µm did significantly mask the bitter taste of AZI. Fourier transform infrared spectroscopy (FTIR), and proton nuclear magnetic resonance spectroscopy (¹H-NMR) proved that the taste masking of microparticles resulted from the intermolecular interaction of the amine group in AZI and the carbonyl group in Eudragit L100. Differential scanning calorimeter (DSC) analysis was used to display the amorphous state of AZI in microparticles. Images obtaining from optical microscopy and scanning electron microscopy (SEM) indicated the existence of microparticles in regular cube shape with many layers. In the second stage, dispersible tablets containing microparticles (DTs-MP) were prepared by direct compression technique. Stability study was conducted to screen pH modulators for DTs-MP, and a combination of alkali agents (CaCO₃: NaH₂PO₄, 2:1) was added into DTs-MP to create microenvironment pH of 5.0 to 6.0 for the tablets. The disintegration time of optimum DTs-MP was 53 ± 5.29 seconds and strongly depended on the kinds of lubricant and diluent. The pharmacokinetic study in the rabbit model using liquid chromatography tandem mass spectrometry showed that the mean relative bioavailability (AUC) and mean maximum concentration (Cₘₐₓ) of DTs-MP were improved by 2.19 and 2.02 times, respectively, compared to the reference product (Zithromax®, Pfizer).

Keywords: Azithromycin · bitter taste masking · microparticles · dispersible tablets · stability · bioavailability
1. Introduction

Azithromycin (AZI) is a broad-spectrum antibiotic with strong antibiotic activity. The drug is commonly indicated to treat or prevent many different kinds of infections (e.g., respiratory infections, skin infections, ear infections, and sexually transmitted diseases). AZI is a subgroup of macrolide antibiotics and has a similar antibacterial mechanism of action to that of erythromycin, an older macrolide antibiotic. However, AZI has a 15-membered ring and a methyl-substituted nitrogen replacing the 9A carbonyl group [1]. For this reason, AZI has several advantages including fewer gastrointestinal (GI) side effects and a longer half-life ($T_{1/2} = 68h$) as compared to erythromycin [1, 2]. The long half-life of azithromycin is very beneficial to patients, especially those who are children and older adults because of the minimum frequency of administration required and the improved compliance of patients to treatment [1].

Azithromycin is marketed in several oral dosage forms including film-coated tablet, capsule, extended-release oral suspension, and granules for suspension in sachet [3, 4]. To successfully develop these oral dosage forms, the manufacturers need to overcome the general limitations of macrolide antibiotics including extreme bitter taste and instability in the gastric medium [5]. The film-coated tablet is commonly used to mask the taste of AZI. However, this dosage form is not quite suitable for the pediatric patients or infants since it is not easy for these subjects to swallow tablets. Consequently, a palatable oral suspension containing AZI has been commercialized for pediatric medications. However, this dosage form faces up to some disadvantages such as unpleasant taste (especially to pediatric patients), physical instability, imprecise dosage and high transportation cost.

The addition of sweeteners and flavors to suspension has been used as the most popular approach to obscure the unpleasant taste of suspension. Choi et al. [6] investigated the taste masking effect of several sweeteners such as neohesperidin dihydrochalcone, sucrose, sacralose, and aspartame. Acetaminophen, ibuprofen, tramadol hydrochloride and sildenafil citrate were model drugs (all at 20 mM). The bitterness value of free drugs and combinations of drug and sweeteners was investigated by a multichannel taste sensor system (an electronic tongue). The authors concluded that the taste masking of the drugs varied with the concentration and type of sweeteners. However, molar amounts and ionic structure of sweeteners played a more important role in taste masking than their sweetening potency. Even though being effective, this method does not work with active pharmaceuticals having
an extremely bitter taste like azithromycin [7] [8], since the compounds still can dissolve in the saliva and have an impact on the taste receptors. Furthermore, as azithromycin dissolves in upper parts of the gastrointestinal tract, it will interact with the bitter receptors or motilin receptors located in the upper GI tract and elicit responses such as an intense lingering aftertaste or GI side effects (nausea, vomiting, and diarrhea). Consequently, the aim of taste masking microparticles containing AZI is to prevent interactions of the dissolved drug with the receptors in the mouth, throat, or stomach.

Several approaches applying to inhibit the contact of dissolved AZI with the saliva include the application of physical barrier on the drug/the dosage form or modification of AZI solubility [7] [9] [10]. The physical barrier of dosage forms can be carried out using polymeric coating processes in fluid bed systems or conventional coaters [11] [12] [13]. By using new fluid-bed coating approach, Stange formulated the bitter taste masking granules containing naproxen sodium. Eudragit E was effective in inhibiting the drug release to a level that is under the threshold bitter value, and an appropriate taste masking for more than 5 min was guaranteed [12]. However, the taste masking coating of drug or dosage forms may break during compression to prepare dispersible tablets (DTs). Thus, modification of AZI solubility is more suitable to prepare tablets [7] [9] [10]. Bora et al. [14] used the spray-drying technique to develop the taste-masking microspheres of an intensely bitter drug, ondansetron hydrochloride (OSH). The bitter taste threshold value of OSH was determined. Chitosan, hydroxypropyl methylcellulose (methocel E15 LV), and poly(meth)acrylates (Eudragit E100) were used as polymeric carriers for preparation of microspheres. The results indicated that methocel could not mask the taste of OSH at any drug–polymer ratios. Meanwhile, the Eudragit microspheres showed their taste masking effect at 1:2 drug–polymer ratio and chitosan microspheres took effect at the ratio of 1:1. Similarly, this approach has been proved effective with azithromycin using ethylcellulose or glyceryl behenate as hydrophobic carriers for taste masking microspheres. However, while being effective, microspheres using hydrophobic carriers also shows limitations concerning drug dissolution processes in GI tract. The pharmacokinetic profile and bioavailability of drug using these microspheres may change or reduce when the dissolution rate of AZI decreases. Accordingly, application of polymers with pH-dependent solubility as carriers for microspheres may be other choices for taste masking of compounds [15] [16] [17]. Since such polymers keep drugs insoluble in the saliva, they neither dramatically alter the solubility of compounds in the absorption window in GI tract nor reduce the bioavailability of drugs. Until now, there is short of study about
taste masking of AZI using polymers with pH-dependent solubility as carriers for microparticles. Thus a study about the impact of polymers with pH-dependent solubility on the taste masking and relative bioavailability of AZI is essential.

Besides the unpleasant taste of compounds, application of oral suspension to azithromycin does come with other challenges such as physical instability, imprecise dosage, and high transportation cost. Development of dispersible tablets (DTs) is a potential approach to overcome these shortcomings of oral suspension [18] [19] [20] [21] [22] [23]. Currently, there are not any commercial DTs containing azithromycin or the drug’s taste masking microparticles. Thus, a replacement of conventional oral suspension by DTs containing the taste masking microparticles will expand the choices for pediatric medications.

Dispersible tablets (DTs) are defined as those disintegrating into a homogenous dispersion within 3 minutes in water before administration [24]. After coming in contact with water, the fast disintegration accelerates the rapid loss of tablet structure and releases smaller drug carriers (microspheres, granules, powder particles, etc.) [25] [26] [27], which makes it easier to swallow [1]. The disintegration of DTs before swallowing can bring about some outstanding advantages such as elimination of the potential risk of aspiration and choking in children and older adults when using big monolithic dosage forms, convenient administration, and ease of use for the pediatric and geriatric population. As a result, DTs now attract increasing attention from scientists and pharmaceutical companies [25]. Several commercial products have been approved by the Food and Drug Administration (FDA) such as Zofran® Zydis® (Ondansetron, GlaxoSmithKline), Risperdal® M-Tab (Risperidone, Janssen), Ascotop® melting tablet (Zolmitriptan, AstraZeneca), Zomig®-ZMT (Zolmitriptan, AstraZeneca) [26].

Different techniques have been employed to prepare dispersible tablets namely direct compression, sublimation, molding, mass extrusion, melt granulation and so on [27]. In this research, direct compression was chosen to prepare dispersible tablets containing microparticles (DTs-MP) because of its relatively easy handling, widespread knowledge, and cheap manufacturing process [18]. The rapid disintegration of DTs-MP upon contact with the saliva requires incorporation of superdisintegrants in the compressed masses [18] [28]. Besides, because a macrolide antibiotic like AZI is unstable in gastric medium [1, 5], pH modulators were also screened and incorporated into the DTs for stability improvement of the drug. The aim of this study was to formulate bitter taste masking microparticles containing
azithromycin and incorporate these microparticles into dispersible tablets. The obtained DTs-MP was used to compare the relative bioavailability in the rabbit model with the reference product, Zithromax® (Pfizer, U.S.A).

2. Materials and methods

2.1. Materials

Azithromycin (AZI) was obtained from Hebei Dongfeng Pharmaceutical Co., Ltd (China). Poly(meth)acrylates (Eudragit EPO, E100, L100, S100), and hydrophilic fumed silica (Aerosil® 200) were supported by Evonik Co., Ltd (Germany). Quinine hydrochloride, roxithromycin were purchased from Sigma-Aldrich (U.S.A). HPLC-grade methanol was purchased from J.T. Baker (U.S.A). Water was purified by reverse osmosis. Sodium croscarmellose (Disocel), microcrystalline cellulose (Avicel PH 101) were purchased from Mingtai Chemical Co., Ltd (Taiwan). Sodium lauryl sulfate, polyvinylpyrrolidone K30 (PVP K30) were purchased from BASF Chemical Co., Ltd (Germany). All other reagents were of analytical grade commercial products and purchased from Beijing Chemical Reagent Factory (China).

2.2. Animals

New Zealand white rabbits, each weighed between 2 and 2.5 kg, were obtained from the National Institute of Drug Quality Control (Vietnam) for the pharmacokinetic (PK) test. They were kept in a clean room at a temperature of 25 ± 2°C with a 12-h light/dark cycle. The relative humidity was 55±15% with air ventilation frequency of 15–20 times/h. All rabbits were fed with water and commercial diet. The protocol of the animal study was approved by the Animal Care and Use Committee of the Hanoi University of Pharmacy, Vietnam.

2.3. Preparation of azithromycin microparticles

Microparticles were prepared by the two methods of spray drying and solvent evaporation. Regarding spray drying method, AZI and polymer were dissolved in a mixture of ethanol: dichloromethane (1:1, v/v) and filtered through a 125 µm sieve. To reduce the viscosity of this solution, polymer concentration was fixed around 50 mg/ml. The filtrate was sprayed at the speed of 10 ml/min in a spray drier (Buchi, Switzerland, Mini Spray Dryer B-290) in which the nozzle diameter was 0.75 mm, and inlet temperature was 80°C.
To prepare microparticles by a solvent evaporation method, AZI and polymer were also dissolved in the mixture of ethanol: dichloromethane (1:1, v/v) and the polymer concentration was 300 mg/ml. The organic solvents were evaporated at 60°C in a heated bath for 6 hours under stirring and in a static oven for the next 24 hours. The obtained product was then ground by a ball milling machine (Retsch, Germany, Mixer Mill MM 200) and sieved through suitable sieves to harvest microparticles. The microparticles were finally put in plastic bags and kept in a desiccator before analysis.

2.4. Characterization of azithromycin microparticles

2.4.1. Bitter taste masking of microparticles

The bitterness value of AZI was determined based on the appendix of the British Pharmacopoeia 2013 [4]. Simply speaking, the bitterness value of a drug, i.e. the reciprocal of the dilution of an extract or a liquid that still had a bitter taste, was evaluated by comparing with that of quinine hydrochloride (the standard), which was set at 200000. The experiment was conducted with six volunteers (3 males and 3 females). To make up for individual differences in evaluating bitterness, a correction factor (k) for each volunteer was determined.

**Determination of the correction factor**

Prepare a series of dilutions of quinine hydrochloride in water. Determine the dilution with the lowest concentration that still had a bitter taste. Take 10.0 mL of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution was not found to be bitter, spit it out and waited for 1 min. Rinse the mouth with water. After 10 min, use the next dilution in order of increasing concentration. Calculate the correction factor k for individual volunteer using the expression:

\[ K = \frac{n}{5} \]

Where \( n \) = the number of milliliters of the stock solution in the dilution of lowest concentration that was judged to be bitter.

**Determination of the bitterness value of AZI**

Dissolve 0.1 g AZI in 1000 mL distilled water to have a stock solution of AZI. Prepare a series of dilutions of AZI in water from the stock solution of AZI in 10 mL distilled water.
Determine the dilution with the lowest concentration that still had a bitter taste. Take 10.0 mL of the tested solution in order of increasing concentration.

\[
\text{The bitterness value of AZI} = \frac{1000000 \cdot K}{X}
\]

Where \( X \) was the lowest volume of tested solution that still had a bitter taste and \( K \) was the correction factor.

\[
\text{The bitterness threshold (µg/ml)} = \frac{1000000}{\text{The bitterness value}}
\]

The bitter taste masking of microparticles was determined by the bitter taste threshold of AZI. The experiment to determine the taste masking effect of microparticles was modified from the method developed by Kharb et al. [29]. Accordingly, microparticles equivalent to 600 mg AZI were put into glass tubes where 10 mL of distilled water was then added to each. The volume of distilled water (10 mL) was used to mimic the real condition when the patient used the reference product (Zithromax oral suspension). The tubes were mixed for 30 seconds using a vortex mixer and filtered through membranes 0.45 µm (Sartorius, Germany, Model Minisart RC 25). The drug concentration in the filtrate was analyzed by high-performance liquid chromatography (HPLC) and compared to the bitter taste threshold. If the drug concentration in the filtrate was smaller than the bitter taste threshold, the microparticles were considered to have completely masked the bitter taste of AZI.

The HPLC system consisted of an isocratic pump (Agilent, U.S.A., Model G1311C), a manual injector (Agilent, U.S.A., Model G1328C), a column thermostat (Agilent, U.S.A., Model G1316A), and a multi-wavelength detector (Agilent, U.S.A., Model G1315D). Detector output was integrated and digitalized using the Agilent ChemStation software (Agilent, U.S.A., Model 1200 Series HPLC system). The column used was a C18 (Zorbax SB, 4.6×250 mm, 5 µm particle size, Agilent, U.S.A.). The detector was set at 215 nm. The mobile phase containing methanol: distilled water: concentrated ammonia solution at a ratio of 80: 19.9: 0.1 was delivered at 1 mL/min at room temperature. The injection volume was 100 µL and the total run time for a sample was about 10 min.

2.4.2. Particle size and particle size distribution

The particle size was determined using Malvern Mastersizer (Malvern Instruments Limited, U.K, Mastersizer 3000). The microparticles were directly dispersed into about 500
mL of distilled water for 30 seconds before sample examination. The laser obscuration was set from 0.1 to 20%. The particle size was expressed as the mean volume diameter in µm.

2.4.3. Differential scanning calorimeter (DSC) analysis

The thermal properties of samples were determined by differential scanning calorimeter (Mettler Toledo, Germany, DSC 1). An empty aluminum pan was used as a reference. The analysis of samples was conducted with sealable aluminum pans using approximately 10 mg sample. A continuous flow of nitrogen at 50 ml/min was used for the measurement. The samples were heated from 30 to 250°C at a rate of 10°C/min. Analysis of the results was conducted using the STAR – Software version 10.00 (Mettler Toledo, Germany, DSC 1).

2.4.4. Fourier transform infrared spectroscopy (FTIR)

FT-IR spectra were obtained on a Fourier transform infrared spectroscopy (Bruker Optics, Germany, Model IFS-66/S) using the potassium bromide (KBr) disk method. One to two milligrams of the sample were mixed with 150mg of spectra-grade KBr and pressed into a disk that was 12mm in diameter using a Carver hydraulic press (Carver, U.S.A., Model 3912). Samples were analyzed from 600-4000cm⁻¹ with an instrument resolution of 0.1cm⁻¹.

2.4.5. Proton nuclear magnetic resonance spectroscopy (¹H-NMR)

¹H-NMR experiments were conducted to investigate the possibility of the intermolecular interaction between drug and polymer. The ¹H-NMR spectra of the samples were taken at 25°C on a Bruker Avance 500 MHz spectrometer equipped with 5 mm TCI HCN Z gradient cryoprobe. Spectra were processed by Bruker Topspin 2.1 software and analyzed by CARA 1.8.4 software. A sample containing drug and polymer was dissolved at an optimum ratio in CD₃OD at a concentration of 20 mg/ml. This solution was added to a suitable glass tube which was then placed into to Bruker NMR Spectrometer.

2.4.6. Morphology

The morphology of the microparticles was examined by both scanning electron microscopy and optical microscopy. The former, which required the microparticles be fixed on a plate using adhesive tape and coated with gold for 240s, was carried out using a scanning electron microscope (Hitachi, Japan, FESEM S4800). For the latter, the microparticles were placed directly on lamella and immediately observed by optical microscopy (Olympus, U.S.A, Olympus CX 31). Images of microparticles were taken by a
camera (ToupTek Photonics Co., Ltd, China, Camera ToupCam 3.1MP) connected to the optical microscopy.

2.5. Preparation of dispersible tablets containing azithromycin microparticles

2.5.1. Stability kinetics and screening of pH modulators

The kinetic stability of AZI in different pHs (solution of 0.1 N hydrochloric acid pH 1.0, phosphate buffer saline pH 2.0, 3.0, 4.0 and 5.0) was determined under the two conditions. First, a series of AZI solution (2 mg/ml) was prepared by completely dissolving AZI in different pHs for 30 seconds that was referred to as initial time \( t_0 \). After predetermined interval times \( t \), 5 ml of the medium was withdrawn and immediately analyzed by a validated HPLC method. This was called stability test without neutralization step.

Second, a similar stability test of AZI in these pHs was also conducted; however, the withdrawn samples were immediately neutralized by 5 mL of 0.2 M Na₂HPO₄ solution. The neutralized products were also analyzed by HPLC method. The degradation rate constant \( K \) and the time required for the drug to decrease its initial amount by 10% \( (t_{90\%}) \) followed pseudo-first-order kinetic behavior and were determined by the equation:

\[
K = \frac{1}{t} \ln \frac{C_0}{C} \quad \text{(min)}
\]

\[
T_{90\%} = \frac{\ln \left(\frac{10}{9}\right)}{K} \quad \text{(min)}
\]

Where \( C_0 \) was the initial drug concentration and \( C \) was the drug concentration in time \( t \).

Based on the stability test, a suitable pH range was chosen. The addition of pH modulators to adjust the medium pH was necessary, and the kinds of pH modulator were screened by modification of titration method developed by Lo et al. [30]. Briefly, the pH modulator was dissolved in 10 mL distilled water and mixed with 40 mL gastric medium (0.1 N hydrochloric acid). The pH value was determined after 5 minutes. After that, the previous solutions were added with a further 10 mL of 0.1 N hydrochloride acid. These solutions were allowed to equilibrate for 5 minutes before reading of pH values. Finally, diagrams displaying the relationship between pH values and volume of additional gastric medium were drawn.

The effect of pH modulators on the dissolution rate of AZI from microparticles was studied using the dissolution apparatus type 2 (Vankel Varian, U.S.A, Model 7010).
quantity of 500 ml dissolution medium of 0.1 N hydrochloric acid was used for the first 2 hours. After 2 hours, the dissolution medium was changed to pH 6.8 phosphate buffer saline by the addition of 10 mL solutions of NaOH (5M) and 10 mL solutions of pH 6.8 phosphate buffer saline (1M). The temperature was maintained at 37 ± 0.5°C. The rotation speed was 100 rounds per minute (rpm). Ten milliliters of aliquot were withdrawn at predetermined time intervals of 5, 30, 60, 120, 125, 150, 180 and 240 minutes and filtered through membranes 0.45 µm (Sartorius, Germany, Model Minisart RC 25). Five mL of filtrate was immediately neutralized by 5 mL solutions of 0.2 M Na₂HPO₄ and analyzed by HPLC method. The dissolution profile of the reference product (Zithromax) was also investigated under the same dissolution apparatus and dissolution conditions. However, the dissolution medium was changed to pH 6.8 phosphate buffer saline right after 30 minutes in a dissolution medium of 0.1 N hydrochloric acid. The sampling times for the dissolution test of Zithromax were 5, 15, 30, 35, 60, 90, and 120 minutes.

2.5.2. Preparation of dispersible tablets

Dispersible tablets containing microparticles were prepared by direct compression method. The main compositions of the tablet were the microparticles, lubricants, superdisintegrant, a suspension stabilizer, pH modulators and diluents. The microparticles and additional excipients were sieved through a 250-µm sieve and equally mixed in a plastic bag and then pressed by a single punch tableting machine (Korsch, Germany, Model VFD007S21A) to form dispersible tablets. The total weight and diameter of these tablets were 650 mg and 13 mm, respectively. The microparticles consisting of AZI and Eud L100 (1:4) had the size distribution of 45 – 212 µm. Regarding the amount of lubricant, the screening ranges of Aerosil, Talc, and magnesium stearate were 0 – 0.5%, 0 – 1% and 0.5 – 1%, respectively. Sodium croscarmellose (Disocel) was fixed as the only superdisintegrant in the tablets with the screening range of 1 – 5%. The investigated levels of pH modulators (CaCO₃: NaH₂PO₄ = 2:1) were 0, 50, 75 and 100%. The diluent including Avicel, lactose SD or mannitol, was added to fulfill the quantity of the tablets. To stabilize the suspension which was formed after the dispersion of DT-MP in water, 1% sodium lauryl sulfate was fixed in the compositions of the tablet. The tablets were prepared with the two levels of crushing strength including 30 – 40 N and 90 – 95 N. The crushing strength of the tablets was measured by a crushing strength tester (Pharmatest, Germany, Type PTB 511E). The disintegration time of tablets and dissolution rate of AZI were used as the output factors for the screening of excipients.
Besides, the effect of breaking forces on the disintegration time and friability of DT-MP was also determined.

2.6. Characterization of dispersible tablets containing azithromycin microparticles

2.6.1. Disintegration time studies

A method for evaluating the disintegration time of DT-MP was modified from one described by Gohel et al. [31]. Specifically, a volume of 10 mL distilled water was added to a petri dish (10-cm diameter) with a tablet in the center. This experiment was conducted in a static condition. Disintegration time was determined at the point at which the tablet disintegrated completely into fine particles and was recorded by stopwatch.

2.6.2. Friability test of dispersible tablets

The friability of the tablets was determined by a friability tester (Pharmatest, Germany, Type PTF20E) based on the guidance in British Pharmacopoeia. Briefly, twenty DTs-MP were carefully dedusted by sieving these tablets through a sieve of 710 µm. These tablets were then accurately weighed (M₁), placed in the drum of the machine, and rotated at 25 rpm for 100 times. Finally, the rotated tablets were again dedusted and accurately weighted (M₂).

\[
Friability (\%) = \frac{M_1 - M_2}{M_1} \times 100
\]

2.6.3. In vitro release studies

The dissolution rate of AZI from samples into the medium was modified from the method developed by Hu [32]. The dissolution apparatus type 2 (Vankel Varian, U.S.A, Model 7010) was used in this study. A quantity of 500 ml dissolution medium of pH 6.8 phosphate buffer saline was used for 2 hours. The temperature was maintained at 37 ± 0.5°C. The rotation speed was 100 rounds per minute (rpm). Ten milliliters of aliquot were withdrawn at predetermined time intervals of 0.25, 0.5, 1, 1.5, 2 hours and filtered through membranes 0.45 µm (Sartorius, Germany, Model Minisart RC 25). The medium was replenished with 10 ml of fresh medium each time. Withdrawn samples were analyzed using HPLC method.

2.7. Pharmacokinetics studies in rabbits

The animal study was approved by the Local Animal Use Committee. Six male rabbits, each weighed 2 – 2.5 kilograms, were used for the pharmacokinetics study. The rabbits,
which were divided into 2 groups of three, were kept in fasting condition one night before the day of the experiment. The two samples were optimal DT-MP and azithromycin 200mg/5ml powder for oral suspension (Zithromax®. Pfizer). The optimal DT-MP containing 100 mg AZI was completely dispersed in 25 ml distilled water, and the reference product (Zithromax) containing 600 mg AZI was also completely dispersed in 100 ml distilled water to obtain a homogenous dispersion for 10 minutes before administering to the rabbits. A volume of the liquid suspension equivalent to 10 mg/kg was carefully withdrawn and fed to the rabbit by a syringe. The drug content uniformity among rabbits was assured by gently shaking the flask bottle containing liquid suspensions upside down and up in a vertical direction by hand 10 times before each feed. Blood samples (2 ml/sample) were withdrawn from the ear artery after 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24 hours (hrs.) and supplemented with equal amounts of saline containing heparin 50UI. Plasma was collected by centrifugation of the above samples at 3500 rpm within 10 minutes and stored in a deep freezer at -40°C until the day of analysis.

2.8. LC-MS/MS analysis of azithromycin in rabbit plasma

An AB Sciex 5500 QQQ mass spectrometer (AB Sciex, USA) coupled with LC-20AD high-pressure pumps, column compartment and autosampler (Shimadzu, Japan) was used for the determination of azithromycin in plasma. LC separation was obtained by using an Eclipse plus C18 column (10 cm x 2.1 mm; 5 µm particle size) and a pre-column (Agilent, USA) with a mobile phase composition of 10 mM ammonium acetate and acetonitrile. The gradient program was initially set at 5% acetonitrile for 0.5 min then increased linearly from 5% to 90% acetonitrile over 3 min. After that, the eluent composition was maintained at 90% acetonitrile from 3 to 4 min then returned to 5% acetonitrile in 1 minute, and re-equilibrated until the end of the analysis process. The flow rate was kept constant at 0.5 mL min⁻¹. The injection volume was 5 µL. Total run time was 6 min.

The mass spectrometer was operated in positive ESI mode with the capillary voltage and temperature set at 5000 V and 400°C, respectively. MS experiments were carried out in multiple reaction monitoring modes with the transition m/z 749.5 to 591 for quantification and m/z 749.5 to 116 for confirmation. The transition for internal standard roxithromycin was m/z 837.5 to 158.

One milliliters aliquot of the plasma sample was transferred into a 15 mL centrifuge tube. Twenty microliters of an internal standard solution of 1 µg mL⁻¹ (roxithromycin in methanol) and 0.5 ml solution of 0.10 M ammonium hydroxide were added to the tube. Diethyl ether (2
mL) was then added, and the tube was mixed by a vortex mixer for 5 minutes. The supernatant (0.8 mL) was transferred to a 10 mL glass tube and evaporated under the flow of nitrogen gas and 40°C. The residue was reconstituted with 0.8 mL methanol, mixed by a vortex mixer for 60 seconds and filtered through 0.45 µm. Finally, the filtrate (5 µL) was injected into the LC-MS/MS system.

The method was fully validated according to guidelines for bioanalytical method validation from the U.S. Food and Drug Administration (FDA).

2.9. Data analysis

The experiment was run triplicates. The data were expressed as a mean ± standard deviation and analyzed for statistical significance by one-way ANOVA and Student’s t-test using Excel (Microsoft, U.S.A.). Pharmacokinetics parameters were determined by the non-compartment model using Phoenix® software (A Certara™ Company, U.S.A, WinNonlin® 6.5).

3. Results and discussion

3.1. Preparation of bitter taste masking microparticles containing azithromycin

Based on guidelines from the British Pharmacopoeia 2013, the bitter taste threshold of AZI was determined by an experiment with six volunteers. Data in Table 1 showed that the bitter taste threshold of azithromycin was 32.43 ± 6.81 µg/ml which was around 6 times higher than that of the standard, i.e. quinine hydrochloride (roughly 5 µg/ml). When compared to USP solubility criteria, this threshold (32.43 µg/ml) lied in the practically insoluble range and the bitter taste of AZI might result from the trace of AZI in solution. This indicated that the taste of AZI was extremely bitter and development of taste masking microparticles was essential.

Previous studies have attempted to mask the bitter taste of AZI by preparing microparticles containing AZI using different carriers such as ethylcellulose or glyceryl behenate. Most notably, Hu et al. [32] developed microspheres of azithromycin with ethyl cellulose by the modified solvent diffusion method. The obtained results demonstrated that the bitter taste of azithromycin was significantly masked by microparticles and the relative bioavailability in human of microspheres was 102.7% compared to a commercial product. Similarly, Lo et al. [30] used a melt-congealing process to prepare matrix microparticles
containing AZI. The microparticles had small size (~200 μm) with a narrow particle size distribution and good tolerability. The present study employed a roughly similar solution to that by Hu et al. [32] to mask the bitter taste of AZI that was to maintain the released amount of drug in water medium under the bitter threshold (32.43 μg/ml). However, a novel bitter taste masking microparticles containing AZI and Eudragit L100, whose taste masking mechanism was different from that of ethyl cellulose and glyceryl behenate, was developed by spray drying. After spray drying of free drug, the dissolved amount of spray dried AZI (262.63 ± 0.85 μg/ml) was 8.1 times higher than the bitter threshold. It was explained by the formation of porosity on the surface of spray dried AZI, which accelerated the dissolution rate of the drug. To inhibit the bitter taste of AZI by modification of AZI solubility, several polymers with pH-dependent solubility (Eudragit E, L, S100) were used to prepare the microparticles. The experiment to evaluate the bitter taste of microparticles was conducted with 10 ml which mimicked the usage guideline of the reference product. To make it easier for the bitter taste evaluation of AZI, the polymers were considered as intact excipients which would not have any specific tastes. Plus, these polymers were mainly used to reduce the drug solubility in the saliva, thereby masking the bitter taste of AZI. The impact of these polymers on the drug solubility was also seen in Table 1. Specifically, the released amount of AZI from microparticles using Eudragit E100, Eudragit EPO, Eudragit S100 and Eudragit L100 were 229.77 ± 4.12, 190.41 ± 3.46, 76.16 ± 2.53 and 63.47 ± 3.24 μg/ml, respectively. This meant anionic polymers (Eudragit L100 and Eudragit S100) were more effective than cationic polymers (Eudragit E100 and Eudragit EPO) regarding bitter taste masking ability.

To find out the molecular mechanism for the taste masking ability of microparticles, FTIR and NMR spectroscopies were used to study the interactions between AZI and Eudragit L100. As shown in FTIR spectroscopy (Figure 1), AZI, Eudragit L100 and physical mixture of AZI and Eudragit L100 had a carbonyl peak in wavenumber of 1722 cm⁻¹. Because of hydrogen bonds of intermolecular interactions between carbonyl groups (-C=O) and hydroxyl groups (-OH) in Eudragit L100, the carbonyl peaks (-C=O) shifted to the lower wavenumber (1705 cm⁻¹) and formed a shoulder-type peak in FTIR spectra of Eudragit L100 and physical mixture. This shoulder-type peak, however, disappeared in FTIR spectra of microparticles (AZI: Eud L100 = 1:4) and the carbonyl peak (-C=O) in microparticles moved from 1722 to 1724 cm⁻¹. These changes in FTIR spectra of microparticles were caused by the interaction between the carboxyl group (-COOH) in Eudragit L100 and an amine group in azithromycin. Besides, the fact that there was an appearance of a new peak at 2300 cm⁻¹ in microsphere
spectra also proved a formation of a new interaction. This new peak was formed from N\(^+\)-H after interaction of the acid group (Eudragit L100) and the amine group (AZI). Besides, the \(^1\)H-NMR spectra (Figure 2a) showed the shift of -CH\(_3\)-N- to the upfield in the range of 2-3 ppm, especially the CH\(_3\)-N- group. This movement of CH\(_3\)-N- group proved that the interaction of the amine group (AZI) and the acid group (Eudragit L100) turned the nitrogen into a positively charged center which would attract electron and moved to upfield in NMR spectra. Based on these results, it proved that acid-base interaction of drug and polymer was the first main reason for the taste masking effect of microparticles. The molecular interaction between functional groups of Eudragit L100 and AZI was described in Figure 2b. Besides, Eudragit L100, known as a polymer soluble in intestinal fluid from pH 6.0, partly inhibited the drug release in gastric fluid which may even have an impact on bitter receptors in the stomach and elicit responses such as an intense lingering aftertaste. The characterization of a large family of putative mammalian taste receptors (T2Rs) was first reported by a research group of Zuker et al. [33, 34]. Later, Depoortere et al. [35] proved that these bitter taste receptors (T2Rs) presented in smooth muscle tissue along the mouse gut and human gastric smooth muscle cells (hGSMC). In the present study, the advantage of Eudragit L100 in the bitter taste masking might be seen in the lower release of AZI in upper GI tract, thereby preventing the contact of dissolved drug from T2Rs in extra-oral tissues, including gut endocrine cells. Consequently, the endings of the vagus nerve that lie underneath the lining of the GI contain bitter receptors was inactivated to signal the bitter information to the brain [36]. The obtained results also proved that water soluble polymer (hypromellose) or gastric-soluble polymer (Eudragit E100) might not be effective in masking the bitter taste of AZI because they can not inhibit the contact of the dissolved drug with bitter receptors in saliva and stomach. The kind of interaction between drug and a polymer soluble in intestinal fluid from pH 6.0 was also used to mask the bitter taste of other drugs. For example, Gao et al. [37] prepared taste-masking microspheres containing roxithromycin by the emulsion solvent diffusion method. The effect of different polymers on the characteristics of the microspheres was investigated by DSC, X-ray diffraction, and IR. The authors concluded that Eudragit S100 was the best effective polymer among the six kinds of polymers for masking the unpleasant taste of roxithromycin. Similar results obtained from Lu et al. [38] and Yi et al. [39]. Lu et al. [38] also proved that the ionic bonding of the amine group in clarithromycin to the high molecular weight polyacrylic acid (carbomer) was the mechanism to remove the free drug from the solution phase, thus effectively masking the bitter taste of this drug.
Even though Eudragit L100 was effective in inhibiting the bitter taste of AZI, the release amount of AZI (63.47 µg/ml) still doubled the threshold (32.43 µg/ml), causing the bitter taste of microparticles. Table 2 demonstrated the effect of different preparation methods, particle sizes and ratios of drug to polymer on the amount of AZI released in water. Microparticles of similar size (≤ 45 µm) were prepared by the two methods of spray drying and solvent evaporation. As shown in Table 2, the released amount of AZI from these two kinds of microsphere was almost the same (63.47 ± 3.24 vs. 67.51 ± 1.41 µg/ml), which indicated that different preparation methods did not change the bitter taste of AZI.

Because of the easy preparation process, solvent evaporation method was chosen for further study. The four-size distribution range (≤ 45, 45 – 75, 75 – 125 and 125 – 212 µm) was selected to investigate the effect of microsphere size on the bitter taste of AZI. Due to the smaller surface area, the bigger microparticles had a lower release rate of AZI and less bitter taste compared to the smaller ones (Figure 3a). For microspheres sized over 45 µm, the released amount of AZI in water was under the bitter threshold (32.43 µg/ml), resulting in almost non-bitter microparticles (Table 2). Consequently, the size of microparticles was identified as the second main reason for their taste masking effect. Guo et al. [40] also studied the effect of structure, morphology, and size of the microparticles on the taste-masking performance of microparticles. Based on results obtaining from synchrotron radiation X-ray computed microtomography (SR-mCT), the authors managed to figure out the changes of fine structure for particle formulations during the dissolution test. Accordingly, particles with regular shape and a smooth surface having a smaller surface area as well as slower dissolution rate were likely to be less bitter than the rough and smaller ones.

Lastly, the impact of different ratios of AZI to Eudragit L100 (1:1, 1: 2 and 1:4) on the bitter taste of microparticles was determined. The size distribution of these microparticles was fixed from 75 to 125 µm. The results in Table 2 proved that the amount of Eudragit L100 was inversely proportional to the concentration of AZI in water. The higher amount of Eudragit L100 the lower the drug release and bitter taste of AZI were inhibited. Consequently, the microparticles in which the ratio of AZI to Eudragit L100 was 1:4 and whose size distribution was 45 – 212 µm were considered optimal because the released amount of AZI in water was under the threshold. These optimal microparticles were used to determine some physiochemical properties before incorporating into tablets.
Regarding the dissolution patterns of AZI from microparticles (Figure 3), those from microparticles having sizes ≤ 75 µm displayed an initial burst followed by a very slow and incomplete release (Figure 3a). The burst release phenomenon was caused by the high dissolution rate of microparticles which were in the lower limit of size distribution range. The existence of these microparticles was shown in SEM and optimal microscopy image (Figure 5). According to images displaying in Figure 5, there were microparticles or clusters of microparticles having sizes of around 2 µm on the surface of bigger microparticles. Following the fast dissolution of AZI from these small-sized microparticles, AZI from the bigger-sized microparticles which mainly existed in a matrix form with regular cube shape and many layers was gradually released. The dissolution of AZI from this matrix was mainly managed by the solubility of Eudragit L100 in the medium. Since Eudragit L100 was insoluble in pH 5.0 and water, the drug was kept mainly inside the matrix then released slowly and incompletely out of the microparticles. Meanwhile, as shown in Figure 3b, the dissolution rate of AZI in phosphate buffer medium pH 6.8 was significantly higher than those in phosphate buffer medium pH 5.0 and distilled water. The reason was the phosphate buffer medium pH 6.8 facilitated the solubility of microparticles using anionic polymer (Eudragit L100). Furthermore, the high dissolution rate of AZI in pH 6.8 also resulted from the amorphous state of AZI in microparticles. The amorphous state of AZI was confirmed by DSC analysis (Figure 4). Accordingly, DSC spectra of AZI and the physical mixture had one endotherm peak at 126°C. However, the fact that this endotherm peak (126°C) disappeared in DSC spectra of microparticles proved the existence of AZI in an amorphous state in microparticles.

3.2. Preparation of dispersible tablets containing bitter taste masking microparticles

As an antibiotic, AZI has a high potential to be decomposed in acidic medium. Stability kinetics of AZI in several pH media (pH 1.0, 2.0, 3.0, 4.0 and 5.0) was studied to screen stabilizers for the dispersible tablets. As shown in Figure 6a, the concentration of AZI strongly depended on pH of media and the reduction of AZI concentration followed the first-order degradation kinetics. When pH increased from 1.0 to 2.0, the degradation constant reduced about 10 times (from 2.07.10^{-2} down to 1.87.10^{-3}). However, AZI was only quickly decomposed in pH ≤ 2.0. When pH increased from 3.0 to 5.0, the concentration of AZI did not change after 4 hours. This was explained by the fact that the chemical connection between glycoside and cladinose (2-desoxy) in AZI structure was easily cut out by very low pH acidic media.
To further clarify the degradation process of AZI, the samples with immediate neutralization by 0.2 M phosphate buffer before analyzing by HPLC were compared with samples without neutralization step. Samples which were not immediately neutralized by 0.2 M phosphate buffer had two degradation products (Figure 6b). Meanwhile, those that neutralized had only one degradation product, i.e. the 2nd degradation product in the chromatogram. The two phases in degradation process of AZI in pH 1.0 included: (1) quick degradation for the first 60 minutes and (2) very slow degradation or level off for the remaining time. This proved a reversible reaction between AZI and the first degradation product. Besides, reduction in the concentration of the 1st degradation product indicated that it was an intermediate product of degradation process from AZI to the 2nd degradation product. Based on this assumption, the degradation process of AZI could be predicted as follows:

\[
\begin{align*}
&AZI 
\quad k_1 
\quad \text{Quick} 
\quad k_2 
\quad \text{1st degradation product} 
\quad k_3 
\quad \text{2nd degradation product} 
\end{align*}
\]

This result showed that neutralization step was quite essential to stop the degradation process and turned the first degradation product into AZI. Consequently, the screening of pH modulators to the composition of tablets, especially basic agents, was crucial to maintain the stability of AZI.

The aim of the screening step was to find out the suitable alkaline agents which kept the pH of the gastric medium around 5.0 – 6.0. At this pH range, the released amount of AZI in the gastric medium was minimized (Figure 3b), thus maintaining the stability of AZI in this medium. Furthermore, Curatolo [41] reported that GI side effects of AZI were caused by the binding of AZI to the human gastric antrum motilin receptor which existed primarily in the upper part of GI wall. Thus, minimization of exposure of free drug in the upper GI tract reduced the undesirable effects of AZI [30] [41] like intense lingering aftertaste, abdominal pain, diarrhea, nausea, and vomiting.

The effect of the three groups of alkaline agents including soluble alkaline agents (Na₃PO₄, Na₂HPO₄, and NaH₂PO₄), insoluble alkaline agents (Al(OH)₃, Mg(OH)₂ and CaCO₃) and combination of soluble and insoluble alkaline agents (CaCO₃ : NaH₂PO₄ = 2:1) on modulating gastric medium was investigated (Figure 7). Insoluble alkaline agents displaying
a broad plateau of the titration curve indicated their effectiveness in enhancing and maintaining the pH of the gastric medium at stable levels for a longer time compared to soluble alkaline agents. Especially, only CaCO₃ could create a pH range of 5.0 – 6.0 with an additional amount of hydrochloric acid from 40 to 120 ml. However, right after the dispersion of CaCO₃ in gastric medium, the pH was 9.0. In contrast, only one soluble alkaline agent, NaH₂PO₄, created pH of 5.0 right after being dispersed in the gastric medium. Nonetheless, the pH of gastric medium added by NaH₂PO₄ quickly decreased to a risk level (pH 2.0) for the stability of azithromycin.

To create and maintain a pH of 5.0 – 6.0 right after addition of alkaline agents to gastric medium, a mixture of insoluble and soluble alkaline agents (CaCO₃ and NaH₂PO₄ = 2:1) was developed. As shown in Figure 7, when the ratio of CaCO₃:NaH₂PO₄ was 2:1, the pH of the gastric medium was maintained around 5.0 – 6.0 with the additional amount of hydrochloric acid up to 120 mL. The ability to maintain a pH range of 5.0 – 6.0 of this mixture depended on the amount of CaCO₃ and NaH₂PO₄. When using this couple of alkaline agents, AZI would have a higher chance to prolong the lifetime in a harsh environment, gastric medium, where hydrochloric acid was continuously secreted from parietal cells in the stomach. Consequently, these two excipients (CaCO₃ and NaH₂PO₄ = 2:1) were used as stabilizers for dispersible tablets.

Dissolution profiles of AZI and 2nd degradation product from microparticles with pH modulators and the reference product (Zithromax oral suspension) in pH 1.2 and 6.8 were displayed in Figure 8. As shown in Figure 8a, the concentration of the 2nd degradation product was ignorable, and AZI virtually did not release in the gastric medium. AZI displayed a burst release phase right after changing the dissolution medium from gastric medium (pH 1.2) to intestinal medium (pH 6.8). The addition of a couple of pH modulators (CaCO₃ and NaH₂PO₄) enhanced the pH of the gastric medium (hydrochloric acid 0.1 N) to a new pH level (5.0 – 6.0) thus avoiding the drug contact to the very acidic medium (pH 1 – 2) and improving the drug stability. Besides, at this new pH level of gastric medium, the matrix using Eudragit L100 as the carrier was insoluble and kept the drug inside. AZI only released from the microparticles when the pH of dissolution medium increased to 6.8 at which Eudragit L100 was completely soluble.

Meanwhile, Figure 8b showed that Zithromax was quickly hydrolyzed to the 2nd degradation product in pH 1.2. AZI quickly dissolved in pH 1.2 and had the maximum
concentration after 5 minutes because this drug was a weak base drug (pKa = 8.74). However, the drug was immediately decomposed right after exposing to the gastric medium. If the dissolution test of Zithromax in pH 1.2 lasted to 60 or 120 minutes, AZI would be completely decomposed. Consequently, the dissolution test of Zithromax was only carried out in 30 minutes in pH 1.2 before changing to pH 6.8. The remaining AZI concentration in pH 6.8 was only around 13.07%. This result proved the pivotal role of the incorporation of the enteric coating polymer (Eudragit L100) as a carrier of microparticles and pH modulators (CaCO$_3$ and NaH$_2$PO$_4$) in maintaining the stability of AZI in the gastric medium.

Together with pH modulators, other additives were also screened to prepare the tablets. Based on the preliminary results (disintegration time of tablets and dissolution rate of AZI) obtained from screening steps, optimal microparticles, diluents (Avicel PH 101, lactose or mannitol), lubricants (magnesium stearate, Aerosil, or Talc), stabilizer (NaLS) and pH modulators (CaCO$_3$ and NaH$_2$PO$_4$ = 2:1) were chosen as components of the DT-MP. The effect of some main factors including diluents and lubricants on disintegration time of DT-MP and dissolution rate of AZI was shown in Figure 9. Regarding the effect of kinds of diluent, the disintegration time of DT-MP decreased following the order: mannitol > lactose SD ~ Avicel PH101. Due to the crystallized state of mannitol, the dissolution rate of this excipient was longer than that of lactose and Avicel. Consequently, the formation of porosity in tablets using mannitol was slower than that using lactose and Avicel. Meanwhile, both lactose and Avicel accelerated the disintegration process of DT by microcapillaries mechanism after the dissolution of lactose and swelling mechanism of Avicel. Despite using different kinds of lubricants, these two diluents (lactose SD and Avicel PH 101) always had the smallest disintegration time compared to mannitol.

Regarding the impact of lubricants, DT-MP using a combination of different lubricants had the shorter disintegration time than that using single lubricant (magnesium stearate). The increasing order of disintegration time of DT-MP using different lubricants was magnesium stearate + aerosil + sodium lauryl sulfate < magnesium stearate + aerosil < magnesium stearate. Consequently, tablets only using magnesium stearate had the longest disintegration time. Microparticles using Eudragit L100 as a polymeric carrier were highly sticky. Meanwhile, Aerosil, for its very high surface area, when adhered to the surface of microparticles would inhibit the sticky tendency of microparticles and reduce the disintegration time of DT-MP. On the other hand, the introduction of a small amount of sodium lauryl sulfate (NaLS), a surfactant, would promote the wettability of dissolution.
medium in tablets, thus activating the disintegration process of DT-MP (Figure 9b). Due to the fast disintegration of tablets to liberate the small microparticles in pH 6.8, the initial burst phenomenon was once again observed in the dissolution profiles of DT-MP. However, the addition of hydrophobic lubricants including Talc, Aerosil, and magnesium stearate inhibited the drug release in the later phase of the dissolution process. One more reason for the slow release was the formation of bigger granules containing the bigger microparticles after compression process in a tableting machine. The adsorption of hydrophobic lubricants on the surface of these bigger granules inhibited the water uptake inside the granules thus delaying the drug release in the later phase of the dissolution process. Accordingly, the ratio of main components in the DT-MP included 78% of optimal microparticles (equivalent to 100 mg AZI), 4% Avicel, 1% Disocel, 0.5% Aerosil, 0.5% magnesium stearate, 1% sodium lauryl sulphate and 15% pH modulators (CaCO₃: NaH₂PO₄ = 2:1). These tablets were used to investigate the impact of crushing strength on physical properties of tablets. When the crushing strength increased from 30-40 N to 90-95 N, the disintegration time of the tablets increased from 53 to 89 seconds while the friability of these tablets decreased from 1.03 % to 0.79%, respectively. It was explained by the fact that the higher compression force and crushing strength, the lower porosity of the tablets and the harder it was for water uptake inside the tablets. The tablets undergoing 30 – 40 N of breaking force were used for further study because of their lower disintegration time compared to those experiencing 90 - 95 N and their friability which almost met the general requirement for tablets in pharmacopeia (1%). Consequently, these tablets were used to compare the relative bioavailability with the reference product (Zithromax®, Pfizer) in the rabbit model.

3.3. Pharmacokinetics study in rabbits

The pharmacokinetics study of AZI in both the optimal DT-MP and reference product was conducted in the rabbit model. The drug concentration in rabbit plasma was analyzed by LC-MS/MS. The pharmacokinetics profiles and pharmacokinetics parameters of AZI were shown in Figure 10 and Table 3, where similar patterns could be observed in both the optimal DT-MP and reference product. Specifically, AZI was quickly absorbed after oral administration, and the drug concentration peaked after 0.5 hours. Later, AZI had a very fast elimination phase from 0.5 to 4 hour. Finally, due to the long half-life of AZI, the drug concentration gradually reduced in the last phase. The pharmacokinetics profile looked like a plateau in the last phase. Besides similar PK patterns, as it was shown in Figure 10, both DT-MP and Zithromax had the same tₘₐₓ (0.5 h). The relationship between this in-vivo value
(t_{max}) and the in-vitro dissolution profile was explained by the following assumption. Regarding the t_{max} of the reference product (Zithromax), this value was almost in agreement with the dissolution profile in Figure 8b. For its quick solubility in the gastric medium, t_{max} of Zithromax was observed after 0.5h in PK profile. According to Merchant et al. [42], the pH of rabbit stomach medium was around 1.6, which led to Zithromax being quickly soluble and decomposed. Therefore, the C_{max} of Zithromax was lower than that of DT-MP. One more reason for the quick absorption of AZI in both formulations (Zithromax and DT-MP) was the rapid gastric emptying phenomenon. Pilot et al. [43] reported that gastric emptying was accelerated by macrolide antibiotics such as erythromycin and azithromycin because they were also motilin receptor agonists [44]. Moreover, Larson et al. [45] proved that the mean gastric emptying half life (t_{1/2}) in human for azithromycin (10.4 ± 7.2 minutes) and erythromycin (11.9 ± 8.4 minutes) were almost similar. Due to the rapid gastric emptying phenomenon, AZI rapidly passed to the intestinal medium where the reported pH was higher than 6.4 [42]. Because of the fast dissolution rate of DT-MP and Zithromax in the intestinal medium, the t_{max} of the two formulations (DT-MP, Zithromax) were around 0.5 h.

Even though the drug concentration in plasma of DT-MP was higher than that of the reference product, Figure 10 displayed the overlapping error bars of the two formulations for the initial phase of pharmacokinetics profiles. One of the main reason for these unwanted results was the small number of experimental animals (n=3) which increased the standard deviations of the means. Furthermore, azithromycin and its microparticles were pH-dependent soluble drug and dosage form, thus the pharmacokinetics response would be more likely to be influenced by inter-subject and intra-subject variability. In this case, as the pharmacokinetics test was of parallel type, the risk of inter-subject variability would be one considered reason for the overlapping error bars. The difference in pH and motility of stomach and intestine among experimental rabbits strongly influenced the dissolution and absorption process of AZI in GI tract. One of the feasible solutions was to enhance the number of experimental animals. The recommended number for bioequivalence study was from 12 to 18 volunteers, and the type of experimental design was crossover study.

The results in Table 3 proved that the optimal DT-MP offered higher bioavailability than the reference product. The mean area under the curve (AUC) of DT-MP was 2.19 times higher than that of the reference product. And the 90% confidence interval (CI_{90%}) of the ratio (AUC_{DT-MP}/AUC_{reference}) transformed by the logarithm was from 130.83 to 345.92%. The obtained results of CI_{90%ln(AUC)} proved that the bioavailability of DT-MP was
significantly higher than that of the reference product. Besides, the mean $C_{\text{max}}$ of DT-MP (81.77 ng/ml) also doubled that of the reference product (40.50 ng/ml). However, the fact that the CI$_{90\%}$ _lower$_{\text{ln}(C_{\text{max}})}$ (87.61%) was lower than the upper limit of bioequivalence (125%) indicated the non-significant difference between $C_{\text{max}}$ of DT-MP and $C_{\text{max}}$ of the reference product. This result was explained by the inter-subject variability in the initial phase of pharmacokinetics profile which was discussed earlier. Assumingly, the higher bioavailability of DT-MP was caused by some of the following factors.

First, since AZI is a weakly basic drug, it is very likely to precipitate in the intestinal medium right after leaving the gastric medium, causing a reduction in drug absorption. Zithromax was easily dissolved in the stomach and also decomposed in this medium. The remaining part of dissolved AZI passed to the intestinal medium where the drug solubility declined. Consequently, this part of AZI would have a high potential of precipitation. In the dissolution profile of AZI, a slight reduction of Zithromax concentration (from 13.07% to around 11.15%) was observed after the remaining drug passed to the intestine. This reduction might be caused by the precipitation phenomenon of AZI. However, the remaining concentration of AZI was very small, which suggested that it might be under or around the saturated solubility of AZI. As a result, it was hard to see a marked reduction of Zithromax concentration in the intestinal medium. In contrast, the bitter taste masking microparticles behaved differently from Zithromax regarding dissolution profile in the two mediums (pH 1.2 and 6.8). After passing to the intestine, the dissolution rate of AZI from microparticles increased dramatically to more than 80% after 180 minutes. Because of the amorphous state of AZI in microparticles, its solubility in the intestinal medium was improved, and its precipitation was limited. AZI might easily re-dissolve after supersaturation state in intestinal medium, and its absorption accelerated.

In recent years, several research groups [46] [47] [48] have looked into the mechanism of enhancing oral bioavailability of weakly basic drugs using amorphous solid dispersion. One approach was based on the supersaturation, precipitation, and re-dissolution process of weakly basic drugs in the intestinal tract. Tanaka et al. [48] used a hydrochloric acid solution containing fluorescein isothiocyanate dextran (FD-4), a non-absorbable marker, to investigate how supersaturation, precipitation, and re-dissolution processes influenced the intestinal absorption of cinnarizine (CNZ), a lipophilic weak base. From the luminal concentration-time profiles of FD-4 and CNZ, the team concluded that the key process for the absorption of CNZ was re-dissolution, not supersaturation. Similarly, Rubbens et al. [47] employed a weak base,
indinavir, as a model drug to investigate its intraluminal dissolution, supersaturation and precipitation behavior in GI tract. The experiment was conducted among five healthy volunteers in a cross-over study in fasted state, fed state and fasted state with concomitant proton pump inhibitor (PPI) use. Their conclusion was the presence of duodenal supersaturation in all three testing conditions.

In another approach, Indulkar [46] used liquid−liquid phase separation (LLPS) theory to explain the oral bioavailability enhancement of the three weakly basic drugs (clotrimazole, nicardipine, and atazanavir). Right after exiting the acidic stomach environment, these model drugs could form liquid−liquid phase separation prior to crystallization. Accordingly, if liquid−liquid phase separation happened, a turbid solution consisting of a continuous solution phase and a colloidal phase would be formed. The high drug concentration in the continuous solution phase corresponding to the amorphous solubility of the drug was a driving force for the enhancement of drug absorption. Besides, the colloidal phase composing of a disordered drug-rich phase was a drug reservoir to maintain the supersaturation at a constant value during absorption.

Second, the combination of two stabilizers (CaCO$_3$ and NaH$_2$PO$_4$ = 2:1) protected AZI from degradation in gastric medium and maintained the high level of parent drug in the upper part of gastrointestinal tract. The result in Figure 10 also indicated that the drug concentration of AZI from DT-MP within the first hour after oral administration was always higher than that from reference product. In this case, both CaCO$_3$ and NaH$_2$PO$_4$ were alkalizes for modulation of microenvironmental pH in microparticles. The potential of these agents in the enhancement of drug solubility and stability was also proved in other studies [49] [50] [51]. Tung, et al. [51] used l-lysine as a pH modulator to increase the solubility of rebamipide, an anti-ulcer drug. The solid dispersion using rebamipide, l-lysine, PVP-VA 64 and poloxamer 407 increased the relative oral bioavailability of the drug about 1.74-fold compared with the reference product in a rat model. Similarly, Park et al. [50] used various alkalizes (MgO, Na$_2$CO$_3$, Na$_2$HPO$_4$, and NaHCO$_3$) to modulate the microenvironmental pH of clarithromycin in a crystalline solid dispersion system. The authors concluded that alkalizes in crystalline-solid dispersion maintained the microenvironmental pH of the tablet above pH 5 under acidic conditions thus providing an useful method to improve the dissolution rate and stability of clarithromycin without changing drug crystallinity. Even though the pharmacokinetics study was conducted with a small number of experimental animals, the improved bioavailability of AZI primarily proved the high potential of the newer dosage form, DTs-MP. When the
relative bioavailability of the DTs-MP doubled that of the commercial product, other therapeutic advantages of the DTs-MP can be considered such as reduction of dose, total weight of tablets, and minimization of GI side effects.

4. Conclusion

The study was successful in developing bitter taste masking microparticles containing azithromycin loaded in dispersible tablets. The kind of polymeric carrier and microsphere size played an important role in masking the bitter taste of AZI. An incorporation of soluble and insoluble alkalizes into DTs-MP for the maintenance of the microenvironmental pH of tablets around 5.0 was useful for the improvement of the drug stability and minimization of side effects of AZI on the GI. The two times enhancement of the relative bioavailability of DTs-MP (2.19 times) compared to that of the commercial powder for oral suspension (Zithromax, Pfizer) resulted from the high solubility of the amorphous drug in the intestinal medium and the improved stability of AZI in the gastric medium.
TABLE LISTS

Table 1. The bitter threshold of azithromycin and effect of polymers on the bitter taste masking of microparticles (n=6, Mean ± SD).

Table 2. Effect of preparation method, the ratio of AZI: Eud L100 and particle size to bitter taste masking of microparticles.

Table 3. Pharmacokinetics parameters of azithromycin from reference product and DT containing bitter taste masking microparticles (n = 3, Mean ± S.E).
FIGURE LEGENDS

**Figure 1.** FTIR diagram of A) Azithromycin, B) Eudragit L100, C) physical mixture, D) microparticles (AZI: Eud L100 = 1:4).

**Figure 2.** a) NMR diagram of A) Eudragit L100, B) Azithromycin, C) microparticles; b) structures of azithromycin and Eudragit L100.

**Figure 3.** a) Effect of microparticles size to drug release from microparticles prepared by a solvent evaporation method in pH 5.0; b) Effect of different mediums to drug release from microparticles (45 – 212 µm) prepared by a solvent evaporation method (n = 3, Mean ± S.D).

**Figure 4.** DSC diagram of A) Azithromycin, B) Eudragit L100, C) physical mixture, D) microparticles.

**Figure 5.** Images of microparticles prepared by solvent evaporation method taken by A) optical microscopy and B, C, D) scanning electron microscope.

**Figure 6.** Stability kinetics of AZI a) AZI in pH 1.0 and 2.0 with neutralization step; b) AZI and degradation products in pH 1.0 without neutralization step.

**Figure 7.** Ability to neutralize the gastric acid of soluble pH modulators (Na₃PO₄, Na₂HPO₄, NaH₂PO₄), insoluble pH modulators (Al(OH)₃, Mg(OH)₂, CaCO₃) and the combination of soluble and insoluble pH modulators (CaCO₃: NaH₂PO₄).

**Figure 8.** Dissolution profiles of AZI in pH 1.2 and 6.8 from; a) Bitter taste masking microparticles with pH modulators (CaCO₃: NaH₂PO₄ = 2:1); b) Zithromax.

**Figure 9.** Effect of lubricants to a) disintegration time of tablets; b) dissolution profiles of azithromycin in pH 6.8 (n = 3, Mean ± S.D).

**Figure 10.** Pharmacokinetics profile of azithromycin in rabbit model from reference product and a dispersible tablet containing bitter taste masking microparticles (n = 3, Mean ± S.E).
Table 1. The bitter threshold of azithromycin and effect of polymers on the bitter taste masking of microparticles (n=6, Mean ± SD).

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>Released amount of AZI in water (µg/ml)</th>
<th>Bitter threshold (µg/ml) of azithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZI: Eudragit L100 (1:4)</td>
<td>63.47±3.24</td>
<td></td>
</tr>
<tr>
<td>AZI: Eudragit S100 (1:4)</td>
<td>76.16±2.53</td>
<td></td>
</tr>
<tr>
<td>AZI: Eudragit E100 (1:4)</td>
<td>190.41±3.46</td>
<td>32.43 ± 6.81 (µg/ml)</td>
</tr>
<tr>
<td>AZI: Eudragit EPO (1:4)</td>
<td>229.77±4.12</td>
<td></td>
</tr>
<tr>
<td>Spray dried AZI</td>
<td>262.63±0.85</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Effect of preparation method, the ratio of AZI: Eud L100 and particle size on bitter taste masking of microparticles.

<table>
<thead>
<tr>
<th>Method</th>
<th>Ratio of AZI: Eud L100</th>
<th>Size distribution (µm)</th>
<th>Mean of particle size (µm)</th>
<th>Released amount of AZI in water (µg/ml)</th>
<th>Bitter threshold (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray drying AZI: Eud L100 = 1:4</td>
<td>≤ 45</td>
<td>17.4</td>
<td>63.47±3.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent evaporation AZI: Eud L100 = 1:4</td>
<td>≤ 45</td>
<td>17.6</td>
<td>67.51±1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZI: Eud L100 = 1:4</td>
<td>45-75</td>
<td>58.1</td>
<td>15.28±0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZI: Eud L100 = 1:4</td>
<td>75-125</td>
<td>117</td>
<td>4.79±1.22</td>
<td>32.43</td>
<td></td>
</tr>
<tr>
<td>AZI: Eud L100 = 1:4</td>
<td>125-212</td>
<td>205</td>
<td>3.22±0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZI: Eud L100 = 1:2</td>
<td>75-125</td>
<td>n/a</td>
<td>47.53±2.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZI: Eud L100 = 1:1</td>
<td>75-125</td>
<td>n/a</td>
<td>196.78±1.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Pharmacokinetics parameters of azithromycin from reference product and DT containing bitter taste masking microparticles (n = 3, Mean ± S.E).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reference product</th>
<th>DT-MP</th>
<th>90% CI (Lower; Upper) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (ng.h/ml)</td>
<td>250.83 ± 25.18</td>
<td>548.29 ± 98.17</td>
<td>(130.83; 345.92)</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>40.50 ± 16.47</td>
<td>81.77 ± 17.35</td>
<td>(87.61; 577.05)</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.50</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

*90% Confidence Intervals of the ratio (AUC_{DT-MP}/AUC_{reference} or C_{max_DT-MP}/C_{max_reference}) were transformed by the logarithm
Figure 1. FTIR diagram of A) Azithromycin, B) Eudragit L100, C) physical mixture, D) microparticles (AZI: Eud L100 = 1:4).
Figure 2. a) NMR diagram of A) Eudragit L100, B) Azithromycin, C) microparticles; b) structures of azithromycin and Eudragit L100.
Figure 3. a) Effect of microparticles size to drug release from microparticles prepared by a solvent evaporation method in pH 5.0; b) Effect of different mediums to drug release from microparticles (45 – 212 µm) prepared by a solvent evaporation method (n = 3, Mean ± S.D).
Figure 4. DSC diagram of A) Azithromycin, B) Eudragit L100, C) physical mixture, D) microparticles
Figure 5. Images of microparticles prepared by solvent evaporation method taken by A) optical microscopy and B, C, D) scanning electron microscope.
**Figure 6.** Stability kinetics of AZI a) AZI in pH 1.0 and 2.0 with neutralization step; b) AZI and degradation products in pH 1.0 without neutralization step.
Figure 7. Ability to neutralize the gastric acid of soluble pH modulators (Na₃PO₄, Na₂HPO₄, NaH₂PO₄), insoluble pH modulators (Al(OH)₃, Mg(OH)₂, CaCO₃) and combination of soluble and insoluble pH modulators (CaCO₃: NaH₂PO₄).
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Figure 10. Pharmacokinetics profile of azithromycin in rabbit model from reference product and a dispersible tablet containing bitter taste masking microparticles (n = 3, Mean ± S.E).
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Taste masking

Improve Stability

Improve Bioavailability