RESEARCH ARTICLE

Azithromycin Nanosuspension Preparation using Evaporative Precipitation into the Aqueous Solution (EPAS) Method and its Comparative Dissolution Study

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Abstract: Aim: EPAS (evaporative precipitation into aqueous solution) was used in the current studies to prepare azithromycin nanosuspensions and investigate the physicochemical characteristics for the nanosuspension batches with the aim of enhancing the dissolution rate of the nanopreparation to improve bioavailability.

Methods: EPAS method used in this study for preparing azithromycin nanosuspension was achieved through developing an in-house instrumentation method. Particle size distribution was measured using Zetasizer Nano S without sample dilution. Dissolved azithromycin nanosuspensions were also compared with raw azithromycin powder and commercially available products. The total drug content of nanosuspension batches was measured using an Ultra-Performance Liquid Chromatography (UPLC) system with Photodiode Array (PDA) detector while residual solvent was measured using Gas Chromatography (GC).

Results: The average particle size of azithromycin nanosuspension was 447.2 nm and total drug content was measured to be 97.81% upon recovery. Dissolution study data showed a significant increase in the dissolution rate for nanosuspension batch when compared to raw azithromycin and commercial version (microsuspension). The residual solvent found for azithromycin nanosuspension is 0.000098023 mg/mL or 98.023 ppb.

Conclusion: EPAS was successfully used to prepare azithromycin nanoparticles that exhibited a significantly enhanced dissolution rate. Further studies are required to scale up the process and determine long term stability of the nanoparticles.

Keywords: Azithromycin nanoparticles, EPAS, Particle size, dissolution enhancement, residual solvent, poorly water soluble drugs.

1. INTRODUCTION

Most of the drugs that are developed have poor solubility or are insoluble in water. More than 85% of the drugs sold in the USA and Europe are administered orally, which is a largely aqueous route. As such, improving the solubility of an otherwise hydrophobic drug is one of the major challenges for pharmaceutical scientists. Solid dispersion [1] and nanoparticle preparation [2] are some of the techniques that have been used to improve the solubility of hydrophobic drugs. Nanoparticles are 100 to 10,000 times smaller than human cells but are similar in dimensions to big bio-molecules such as enzymes and receptors. Nanoparticles are particles that are less than 100 nm in size in at least one of its dimensions and this small size confers the particles with specific properties that have favoured their application as a novel drug delivery system [3]. The reactive surface chemistry of nanoparticles in addition to their sizes has also been employed to boost medication efficiency via targeted delivery of drugs, improving the bioavailability either sustaining the release of the drugs or solubilizing them for systemic delivery. Nanoparticles are suitable to serve as personalized, targeted medication delivery vehicles to carry large doses of...
chemotherapeutic agents or therapeutic genes [4]. The significant technical advantages of nanoparticles as drug carriers when compared to conventional drugs are their high stability, large carrier capacity, the feasibility of formation of both hydrophilic and hydrophobic compounds and the possibility of varying routes of administration, most notably oral application and inhalation. An excellent example is in the use of liposomes made up of phospholipids that mimic the plasma membrane lipid bilayer. This affords the liposome easy entry into the cells delivering payload of drugs that will otherwise take longer to traverse the cell membrane and also in small concentration. In addition, both hydrophobic and hydrophilic drugs can be loaded into a liposome due to the possibility of the hydrophobic drug being entrapped within the lipid bilayer and the aqueous cavity within the liposome holding hydrophilic drugs. Depending on the method of preparation, nanoparticles can be constructed to contain various physico-chemical attributes and release characteristics [5-8]. As such, nanoparticles can be tailored to promote sustained drug release out of the matrix [9]. For instance, nanoparticles with a surface chemistry that is modulated based on pH, has been developed to deliver drug payload at acidic pH, which is often found within the tumour microenvironment, preventing normal cells with basic pH from the toxic effect of such drug [10].

Azithromycin (AZI) is a new macrolide antibiotic that exhibits a much wider gram-negative anti-bacterial activity. It is a class II drug, according to Biopharmaceutics Classification Systems. The mechanism of action of the drug is through its binding to the 50s ribosomal subunit of sensitive microorganism and disrupting microbial protein synthesis. Azithromycin is widely used in the treatment of infections caused by both gram-positive and negative bacteria [11, 12], but its oral bioavailability is limited due to its poor water solubility [13]. Nanotechnology is gaining much attention from formulation scientists for developing a dosage form for poorly soluble compounds. As mentioned above, hydrophobic drugs can be entrapped in liposome lipid bilayer and the polar head of the phospholipid allows the liposome to stay within an aqueous environment such as the physiological environment. Nanotechnology can reduce particle size from 8 micron to 200 nm, which can increase a 40-fold surface area to volume ratio [14-19]. This increase in surface area increases the dissolution rate leading to improved bioavailability. Evaporative precipitation into aqueous solution (EPAS) is a technique that is used in producing submicron particles of a drug coated with stabilizers. EPAS was first developed by Sarkari et al. [18] to improve the dissolution of carbamazepine, a highly hydrophobic drug. Guangpu et al. [20] found that nanocrystal prepared by EPAS process showed better particle size distribution with enhanced dissolution rate. EPAS process was also used to control the size distribution of precipitated particles and the enhancement of the dissolution rate of drugs [21-23]. Therefore, the aim of this study was to develop azithromycin nanosuspensions using EPAS method and compare the dissolution rate with raw azithromycin powder and commercial version of microsuspension. Azithromycin drug content and the residual solvent in the prepared nanosuspensions batches were also analyzed using a chromatographic approach.

2. MATERIALS AND METHODS

2.1. Materials

Azithromycin dihydrate (purity 95.9%), HPMC (Hydroxypropyl Methylcellulose; HLB values in the range of 10 -12) were obtained from Incepta Pharmaceuticals Ltd, Dhaka, Bangladesh. Tween 80 (from Aldrich, St. Louis, MO, USA; HLB value of 15), Ultrapure Type I water (from Millipore water purification system, Merck KGaA, Darmstadt, Germany), all reagents used dichloromethane, acetone and isopropyl alcohol were of analytical grade. The chemical structures of azithromycin dihydrate and the surfactants are shown in Fig. (1).

![Molecular structure of azithromycin dihydrate and surfactants](image1)

**Fig. (1).** Molecular structure of azithromycin dihydrate and surfactants used in the current study.

2.2. Evaporative Precipitation into Aqueous Solution (EPAS)

The EPAS apparatus is a slight modification of the design made by Xiaoxia Chen [18, 22] Bonnecaze and Lloyd in 2004 [24]. The drug solution or pure solvent is fed using an HPLC pump. The organic azithromycin solution (8 mg/ml in dichloromethane; Boiling Point 39.6°C) was fed through a SS tube having 0.18 mm i.d. and 350 mm length with a heating plate and cramped nozzle to produce an atomizing effect (Fig. 2A). Organic azithromycin solution was pre-heated using a heating plate which was submerged in the water bath. Aqueous solution (200 ml) containing stabilizer (3% HPMC, 1% Tween 80) was submerged in a temperature-controlled water bath. The nozzle was submerged approximately 3 cm under the surface of the aqueous solution. HPLC system was washed using Isopropyl alcohol, dichloromethane and finally using organic azithromycin solution. The aqueous stabilizing solution (3% HPMC, 1% Tween 80) was constantly stirred with a glass rod to mini-
mize foam production. Azithromycin solution was sprayed into aqueous solution and stirred for a few minutes (Fig. 2B). The resulted suspension was then sonicated at 50°C for 5 minutes to evaporate the remaining organic solvent. It was then cooled down to room temperature and the volume was made up to 350 ml with an aqueous stabilizing solution in 500 ml measuring cylinder.

2.3. Ultra-performance Liquid Chromatography (UPLC)

Azithromycin nanosuspensions were analysed by Ultra Performance Liquid Chromatography (UPLC) method developed by our group [25]. The study employed an UPLC system (ACQUITY UPLC System with binary solvent manager) equipped with auto sampler and a Photodiode Array (PDA) detector (from Waters Corporation, USA). The separation was obtained by reverse phase isocratic elution using a mobile phase consisting of filtered and degassed mixture of buffer solution (potassium phosphate buffer adjusted to the pH 6.5±0.05, acetonitrile and water at a ratio of 10:35:55) at a flow rate of 0.3 ml/min through an ACQUITY UPLC C18 Column (130Å, 1.7 μm, 2.1 mm X 100 mm). Standard and equivalent quantity of samples was diluted using diluent (water with Acetonitrile at a volume ratio 60:40) and were injected.

2.4. Gas Chromatography (GC)

The amount of residual dichloromethane in the prepared suspension was determined using Shimadzu GC 2010 plus with HS-20 Headspace Autosampler [26]. 5 ml of suspension was transferred in a dry HS-20 vials and sealed properly and heated up to 90°C and injection time was 0.10 min. The temperature program was set at 60°C as initial temperature for 1 min with a temperature ramp of 5°C/min up to 90°C and final temperature ramp of 15°C/min up to 120°C for 1min. The separation was achieved by fused silica DB-624 length 30-meter column.

2.5. Particle Size and Particle Size Distribution

Particle size distribution analysis of the API (Azithromycin Dehydrate) was done using Laser diffraction (Mastersizer 2000, hydro-unit, with 12.20% obscuration, water as a solvent) particle size analyzer. Particle size distribution and polydispersity index (PDI) of nanosuspension batches were measured by Zetasizer Nano S (Malvern Instruments) at 25°C using disposable cuvettes without any dilution. The analysis was performed in triplicate and the average value was used from the data.

2.6. In Vitro Dissolution Study

The dissolution experiment of azithromycin nanosuspension formulation was determined using the USP Apparatus II (paddle) with a paddle speed of 100 rpm and 37°C ± 0.5°C temperature. Phosphate buffer (pH= 6.0) was used as a dissolution medium and the release of the nanosuspension was compared to raw azithromycin powder and leading azithromycin suspension brand. The amount of drug used was equivalent to 5 mg. Samples (5 ml) were collected using a cannula 5 min after the addition of samples to the dissolution media. The samples withdrawn were filtered through 0.2μm microfilter prior to analysis using UPLC system. All experiments were carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1. Particle Size Measurements

Malvern zetasizer data (Table 1 and Fig. 3) showed that azithromycin nanosuspensions batches prepared using Tween 80 exhibited low average particle size (447.2 nm) compared to batch prepared using HPMC (1267.0 nm). Based on the DLS values, the highest intensity values for the Tween 80 prepared suspension was 447 nm making up 69% of the nanoparticle size values (Fig. 3A). However, the HPMC prepared nanosuspension had only 14% of the nanoparticles in size range of 459 nm, which were the smallest obtained, while the remaining 86% had sizes above 1.2 micron (Fig. 3B). Tween-80 has been documented to reduce due to Tween-80 adherence to the drug particles surface, forming a protective layer that reduces the solid–liquid interfacial tension, preventing agglomeration of the particles. However, narrow particle size distribution was observed for the batch prepared using HPMC and this may have accounted for the lower polydispersity index (PDI) of the HPMC derived nanosuspension (0.555) compared to the Tween 80 ver-
sion (0.865). Laser diffraction particle size distribution data indicate that average particle size of raw azithromycin powder was 9.16 μm. Azithromycin nanosuspension prepared using EPAS process decreases particle size down to 447 nm, which is more than a 20-fold reduction in particle size.

3.2. Assay of Nanosuspension Encapsulation Efficiency by UPLC

Theoretically, the azithromycin nanosuspensions that were prepared were estimated to contain 0.75 mg/ml. UPLC data (Fig. 4A and 4B) showed that drug content found for nanosuspensions batches of azithromycin is 0.73 mg/ml. This indicated a 97.33% yield for the expected drug content in nanosuspensions batch.

3.3. Residual Solvent Analysis

The standard solution for residual solvent analysis was prepared by weighing dichloromethane into a volumetric flask containing 1% Tween 80 and diluted up to the mark, making a final concentration of 0.73 mg/ml. Dichloromethane is a class 2 solvent (solvents with less toxicity but requires specific exposure limits) with permitted daily exposure of 6 mg/day according to US Pharmacopeia United States Pharmacopeia (USP) chapter 467. Gas chromatography data (Fig. 4C and D) suggest that the content of dichloromethane found in the final preparation of the azithromycin nanosuspensions is 0.000098023 mg/mL or 98.023 ppb. This concentration is way below than the daily allowed concentration of 6 mg/ml, which suggest that azithromycin nanosuspension is safe to use.

![Graph A](image1.png)

**Fig. (3).** Malvern zetasizer particle size distribution data for azithromycin nanosuspension prepared by (A) Tween 80 and (B) 3% HPMC. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

**Table 1.** Particle size distribution and zeta potential data for the batches prepared using EPAS process.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Z-Average (d.nm)</th>
<th>±SD</th>
<th>Polydispersity Index (PDI)</th>
<th>SD</th>
<th>Zeta Potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>HPMC</td>
<td>1267.0</td>
<td>112.0</td>
<td>0.555</td>
<td>0.17</td>
<td>-19.6</td>
</tr>
<tr>
<td>Tween 80</td>
<td>447.2</td>
<td>26.0</td>
<td>0.865</td>
<td>0.11</td>
<td>-14.2</td>
</tr>
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</table>
3.4. In Vitro Dissolution Study

The bioavailability of hydrophobic drugs like azithromycin is dependent on dissolution rate; hence reducing particle size can significantly improve the performance of the drug. Formulation of poorly water soluble drugs as nanosized drug particles has a significant effect on drug solubility, dissolution and consequently, bioavailability. The dissolution rate of the nanosuspension was significantly faster (≥90% after 5 minutes) compared to raw azithromycin powder and leading azithromycin microsuspension brand, which showed a dissolution rate of 37% and 74%, respectively (Fig. 5A-C). In agreement with the previous studies, nanosuspensions can increase the dissolution rate significantly. Previously, Cerdeira et al. in 2013 [28] prepared nanosuspensions of antifungal drugs, miconazole and itraconazole, both of which are highly hydrophobic drugs, which limits their bioavailability and antifungal effects. The nanosuspensions were prepared by the media milling method and the mean particle size of the suspensions was 210 nm with sodium dodecyl sulfate and cellulose ethers or poloxamers used as stabilizer. When compared with the coarse drug suspension, the nanosuspensions were found to have two-fold higher dissolution rates in 10 and 20 min.

This is because smaller particles have a greater surface area to volume ratio. In fact, nanosizing of drugs is known to increase the total effective surface area that is in contact with the solvent, increasing the dissolution rate [29]. Reducing particle size results in a reduction of the diffusion layer thickness surrounding drug particles. This subsequently results in increased concentration gradient all of which leads to improved bioavailability of the drugs.

3.5. Future Recommendations on Azithromycin Nanosuspension as a Combined Therapy with Antiviral Drugs in the Treatment of Viral Diseases

A recent outbreak of the severe acute respiratory syndrome-related coronavirus, SARS-CoV-2, has just been officially declared a pandemic threat to global public health. Novel zoonotic coronavirus disease, also termed COVID-19, was first identified during an outbreak of pneumonia in Wuhan City, Hubei Province, China, in November 2019 [30]. This new strain of coronavirus was announced by
the World Health organization (WHO) to have not been previously identified in humans. With a high infectious rate and no vaccine in sight, the virus is now rapidly spreading in Europe, Asia and North America, with cases in many African countries and the Middle East and Latin America also on the rise.

Currently, there are no specific treatments available for COVID-19. The mainstay of the disease management is therefore optimized supportive care to relieve symptoms and support organ function in more severe cases. Interestingly, China is currently running more than 80 clinical trials on potential treatments for COVID-19 [31].

Chloroquine and hydroxychloroquine have been approved by the U.S. Food and Drug Administration for the treatment of malaria, lupus and rheumatoid arthritis, but preliminary research in human and primate cells suggests that the drugs could effectively treat COVID-19. A study conducted in France in a small number of patients with COVID-19 received either hydroxychloroquine alone or hydroxychloroquine in combination with an antibiotic called azithromycin. The authors reported that a significant drop in the viral load of SARS-CoV-2 at a faster rate in the study subjects when compared with coronavirus patients in other French hospitals who did not receive either of the drugs. This promising effect appeared to be significant in a group...
of six other patients [32], Azithromycin efficacy in the treat-
ment of COVID-19 may be due to the antibacterial activity
on secondary infections common in viral disease such as
those caused by Streptococcus pneumonia. Azithromycin is
known to be effective in treating community-acquired pneu-
monia even those that are insensitive to macrolide antibi-
otics [33]. Thus, improving azithromycin dissolution rate
through nanoencapsulation or in nanosuspension can thus im-
prove its efficacy in different viral diseases such as
COVID-19, especially in combination with other antiviral
drugs currently being tested. In addition, azithromycin nano-
suspension may also be effective in combination with antivi-
ral drugs in different diseases such as in Human Immunodefi-
ciency Virus (HIV) Syndrome. Azithromycin has been wide-
ly used as a treatment modality in HIV patients in the past
two decades, either alone or in combination with other drugs
such as zidovudine, an antiviral drug [34, 35]. As such, with
a higher dissolution rate, further studies testing the effective-
ness azithromycin nanosuspension in combating viral diseas-
es in combination with antiviral drugs may open up a more
promising future for patients suffering from untreatable viral
diseases such as HIV/AIDS.

CONCLUSION

Azithromycin (AZI) nanoparticles were successfully pre-
pared by the EPAS method and this nanosuspension showed
better dissolution property compared to raw azithromycin
powder and the commercially available microsuspension.
In addition, the EPAS process showed better drug yield of
the nanosuspension batches, and the residual solvent was
within the accepted limit of compendia. Since the dose 0.73
mg/ml is much lower and tween 80 (a non-irritant to the eye-
s) has been used as a stabilizer, the nanosuspension prepara-
tion has a potential application as an ophthalmic drug deliv-
ery system. AZI nanoparticulate preparation by EPAS can be
thus scaled up for commercial production. However, further
studies need to be conducted for formulation and process op-
timization.

AUTHORS’ CONTRIBUTIONS

Mohammad Hossain Shariare and Tonmoy Kumar Mond-
al contributed to the concept and design of the study. Tonmoy
Kumar Mondal, MD Wadud & Md. Didaruzzaman So-
hel did the literature search and wrote part of the paper.
Hani’s authorship contribution consisted of data curation &
analysis, recourses, and help in drafting & editing the
manuscript. Mohammad Hossain Shariare, Md Abdur
Rashid, Mohsin Kazi and Mohammed Aldughaim contribut-
ed to the critical revision of the paper and approved the final
version before submission. All authors discussed the results
and contributed equally to the final manuscript.

LIST OF ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>FID</td>
<td>Flame Ionization Detector</td>
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<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<td>LD</td>
<td>Laser Diffraction</td>
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<td>AZI</td>
<td>Azithromycin</td>
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<td>PFS</td>
<td>Powder for Suspension</td>
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<td>Susp</td>
<td>Suspension</td>
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ETHICS APPROVAL AND CONSENT TO PARTICI-
PATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the ba-
sis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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search).

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or
otherwise.

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Declared none.

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