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Treatment with Alternate Unequal Fixed Dose Dexamethasone in Sheehan's Syndrome: A Case Study

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Abstract

An old lady, 69 years of age, has been on levothyroxine therapy for the last 35 years, since she had been diagnosed as a case of sheehan's syndrome. On presentation, she was suffering from extreme weakness, inability to perform daily routine tasks, poor memory as well as lack of concentration. There was history of corticosteroid (prednisolone) therapy also, which was withdrawn gradually due to development of undesired side effects. On examination, she was found to have pallor, hypotension, low weight for height, poor memory and lack of concentration. Interestingly, there was gait disturbance too, with difficulty to put the heel of left leg on the ground during walking. Dexamethasone in fixed, unequal dose of 1 mg and 0.5 mg, orally on alternate day was found to be very effective along with levothyroxine. The case has been monitored for the next 12 months with marked improvement of symptoms and almost no side effects due to corticosteroids as noted previously with prednisolone.

Key words: Sheehan's syndrome, Corticosteroids, Unequal Dose Therapy

Introduction

Sheehan's syndrome is a rare clinical presentation of pituitary infarction due to hypovolumic shock. The usual presentation is a case of excessive postpartum bleeding and the resultant shock, leading to pituitary infarction and necrosis.

The basic treatment of Sheehan's syndrome is, replacement with thyroid hormone and corticosteroid. Most patients tolerate the thyroid hormone well but, corticosteroid therapy poses many undesired side effects on long term basis. In a normal individual, cortisol inhibits growth hormone and TSH secretion. Cortisol is also concerned with diabetic like state in Cushing's syndrome due to its anti-insulin like action. Catecholamines require presence of cortisol to produce their calorigenic and vasoconstrictor effects (permissive action). Similarly calorigenic effect of glucagon also requires the presence of cortisol. None the less, both deficiency and excess of cortisol is dangerous. The anti-inflammatory and anti-allergic actions of the same require high doses of exogenous glucocoticoids, which always tend to manifest with the signs of glucocorticoid excess.

Corisol therapy with dexamethasone on alternate unequal fixed dose in sheehan's syndrome has demonstrated excellent prognosis and improvement of symptoms.

Background and History

A female developed Sheehan's syndrome after the labour for her third issue, due to excessive postpartum hemorrhage and the resultant shock. She was put on levothyroxine and prednisolone. Due to development of the signs of cortisol excess, prednisolone was withdrawn gradually in tapering doses. Later on, she again presented to her doctor with hypotension and weakness. Low dose prednisolone was started again, but she failed to tolerate the titrated dose also. She was on levothyroxine alone, for 6 months. She developed all the signs of cotisol deficiency along with poor concentration and memory. She was unable to perform the daily routine tasks and for most of the time remain seated or on bed due to extreme weakness. Her blood pressure and pulse rate was was 88/60 mm of Hg and 78/min, respectively. Furthermore, her skin turgor was low and pigmentation in pressure areas were evident.

Materials and Method

Dexamethasone in a dose of 1 mg orally daily, at early morning was started. Within a week facial swelling and other signs of cortisol excess became evident. Interestingly, blood pressure improved to 110/72 mm of Hg. She also admitted that, weakness and tierdness were less bothersome. She started taking interest in few household works too. Observing these sign of improvement and considering the previous failure of cortisol therapy, she was put on 1 mg and 0.5 mg, alternate day oral dexamehasone for the next one week. Examination after one week revealed as the disappearance of the facial swelling as well as further improvement of weakness and mental condition. She was more active in household activities apart from her daily routine works.

The case had been regularly examined for the next 12 months, with oral dexamethasone continued. No undesired side-effects of cortisol appeared during this phase.

Results

On presentation, the hormone assay revealed:
Plasma cotisol (Fasting, AM) - 2.1 micro gram / dL (5 - 25 µg/dL)
Free T3 - 0.3 nano gram / dL (0.23 - 0.42 ng/dL)
Free T4 - 1.6 nano gram / dL (0.8 - 1.8 ng/dL)

After starting dexamehasone, the hormone assay after 6 weeks demonstrated:
Plasma cortisol (Dexamethasone equivalent) - 6.3 micro gram / dL
Free T3 - 0.29 nano gram / dL
Free T4 - 1.2 nano gram / dL

Free T3 and Free T4 levels are also affected by cortisol, since cortisol inhibits the residual TSH release, as usually present in the cases of partially functional pituitary.

Discussion

Lactotroph, somatotroph and thyrotroph failure are common in patients with Sheehan's syndrome. In addition to known preservation of gonadotroph axis, corticotroph axis may be preserved in some of these patients arguing against the universal treatment of these patients with glucocorticoids. Patients with sheehan's syndrome have varying degree of anterior pituitary hormone deficiency.

The present case of sheehan's syndrome was having partial functioning pituitary, with low ACTH secretion. Initially, there were signs of cortisol deficiency which responded well to oral dexamethasone along with continuation of levothyroxine.

Cortisol and levothyroxine are the mainstay of treatment for Sheehan's syndrome. While levothyroxine is well tolerated on long term basis, cortisol on long term generally poses problems. The diverse effects of cortisol, including those on other hormones like growth hormone, glucagon and catecholamines, make the picture very complicated.

Apart from these, cortisol also acts on the HPA axis, and inhibits ACTH. Fixed and equal dose cotisol exerts a tonic inhibitory effect on HPA axis. Furthermore, the circadian rhythm of the hypothalamus permits maximum cortisol release only in the early morning hours (4 AM to 10 AM). These facts necessitate the dose modulation of exogenous cortisol when given on long term basis. The present case provides an excellent example of the same.

Dexamethasone has 100% glucocorticoid activity (relative potency 25:0, compared to cortisol), whereas prednisolone has both glucocorticoid as well as mineralocorticoid activity (relative potency 4:0.8, compared to cotisol). Since the mineralocorticoid secretion primarily depends on Angiotensin ll level, and not on ACTH, it will be wise to avoid a corticosteroid with both glucocorticoid and mineralocorticoid activity, for the treatment of sheehan's syndrome. Many of the undesired side-effects of such corticosteroids are due its mineralocorticoid content.

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Prevalence and Drug Resistance Pattern of Organisms in Nosocomial Urinary Tract Infections in a Tertiary Care Hospital

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Abstract

Fifty patients, with urinary tract intervention, admitted to different wards of Medical College, Kolkata from October to December 2013 were taken in the study. Samples of urine were collected; cultured, isolated organisms were identified biochemically. The antibiogram is prepared to account for resistance pattern. 46% cases out of 50 developed nosocomial urinary tract infections. Ward wise distribution of cases were 46% in Obstetrics & Gynaecology wards, 56% in Medicine, 37.5% in Surgery, 38% in Urology. Organisms isolated were Pseudomonas sp., Escherichia coli, Enterococcus sp., Klebsiella sp., Staphylococcus saprophyticus, Staphylococcus aureus, Acinetobacter sp., and Proteus vulgaris. All the isolates from different w s were un iformly and totally resistant to Penicillin, Amoxycillin, and Cotrimoxazole. Resistance to Amoxycillin Clavulenic acid combination was found in Staphylococci, Proteus sp. and Acinatobacter sp. On the other hand, all isolates were sensitive to Imipenem and resistant to Doxycycline. Gram-negative bacilli were very sensitive to Nitrofurantion.

Key words: *Nosocomial*, Urinary Tract Infections (U.T.I.), Antibiotic Resistant

Introduction

Nosocomial infections have been known to cause serious problems these days as it leads to loss of man-days, emergence of multi drug resistant hospital strains of bacteria and less bed occupancy in hospitals[1,2].Serious nosocomial urinary tract infections are common after any urinary tract interventions like catheterization, cystoscopy, and genito-urinary surgery, caesarian section delivery, due to the use of instruments in the perineal region that may be contaminated if extra care is not taken by the surgeon or due to prevalence of resistant bacteria present in the hospital environment[3].

Death rate in India from nosocomial infections is approximately 20,000 per year[1] and as such it requires a thorough investigation to know the extent to which it is prevalent in a particular hospital. The micro organisms involved in such cases along with their antibiotic sensitivity pattern and at the end to advocate the necessary measures to be taken for the prevention of any hospital acquired infections.

The study was mainly aimed at obtaining the number of cases who are acquiring urinary tact infections from hospital and the cause of such infections if any, to ascertain the nature of pathogens in different wards, to obtain necessary data to find out the antibiotic sensitivity / resistance pattern of each isolate in each war

Materials and Methods

The patients admitted to the different wards of Medical College, Kolkata requiring any urinary tract intervention in the hospital has been chosen for the study.Patients admitted in Medicine, Surgery, Obstetrics & Gynaecology and Urology wards who have the chance of undergoing surgical intervention of urinary tract or admitted with such condition which would require at least urinary catheterization have been initially selected for the study. If the first sample of urine from these prospective cases were found to be infected on culture, they were not included in the study.

When the first sample was sterile, these patients were included in the study. A second sample was collected from them to find out whether they there was any hospital acquired infections or not. A total of 50 patients were not included in the study. If the first sample was sterile then after 7 days or after the surgery urine was collected from the same patient to find out any hospital acquired infection. Either a mid stream sample of urine or a catheter specimen was used for the study[4]. Samples were processed on immediate transfer to the Microbiology Laboratory which included culture of the sample on Blood agar, Mac Conkey agar and nutrient agar and incubated at 37^0C overnight. Micro organisms, if any, come on culture, its identification and antibiotic sensitivity testing was done[4]

Observations

Table I: Prevalence of Hospital Acquired Urinary Tract Infections in patients of different wards

Serial no.	Ward		Total no. of Patients	Community acquired infections		Hospital acquired infections	
				Total no.	Percent.	Total no.	Percent.
1.	G &O	1-14	14	1	7.14%	6	46%
2.	Medicine	15-30	16	0	0%	9	56%
3.	Surgery	31-41	11	3	27.27%.	3	37.5%
4.	Urology	42-54	13	0	0%	5	38%
TOTAL :			54 (100%)	4	7.4%	23	46%

The discs[4] used for antibiotic sensitivity tests were as follows:

Penicillin 10 I.U., Gatifloxacin 10 mcg, Amoxy-Clav 30 (20/10) mcg, Chlorumphenicol 30 mcg, Amoxicillin10 mcg, Doxycycline 30 mcg, Cefotaxime 30 mcg, Gentamicin 10 mcg, Ceftriaxone

30 mcg, Amikacin 30 mcg, Imipenem 10 mcg, Nitrofurantoin 300 mcg, Norfloxacin 10 mcg, Erythromycin 15 mcg Ciprofloxacin 5 mcg, Cotrimoxazole 25 mcg.

Table II: Microorganism types obtained from different wards in nosocomial UTI

Organisms	Gynecology & Obstetrics (n=13)	Medicine (n=16)	Surgery (n=8)	Urology (n=13)	Number (Percent.)
Staphylococcus aureus	1	0	0	0	1 (4.3%)
CoN Staphylococcus	1	0	1	0	2 (8.9%)
Enterococcus sp.	0	1	1	1	3 (13%)
Escherichia coli	1	1	1	1	4 (17.4%)
Klebsiella sp	1	1	0	1	3 (13%)
Proteus vulgaris	1	0	0	0	1 (4.3%)
Acinatobacter sp.	1	0	0	0	1 (4.3%)
Pseudomonas sp.	0	6	0	2	8 (34.8%)
Total:	6	9	3	5	23 (100%)

Out of 50 patients included in this study, 23 (46%) developed hospital acquired Urinary Tract Infections (Table I). It's ward-wise distribution was 6 (46%) in Obstetrics & Gynaecology wards, 9 (56%) in Medicine, 3 (37.5%) in Surgery and 5 (38%) in Urology.

A total of 4 patients selected from the Obstetrics and Gynaecology ward (1) and Surgery ward (3) developed community acquired urinary tract infection as was evident from the first sample showing significant bacteruria. So these patients were excluded from the study and rest 50 patients were included in the study showing no bacterial growth in the 1st urine sample.

Table II shows the distribution pattern of isolated organisms in different hospital wards. All the isolates from different wards were uniformly and totally resistant to Penicillin, Amoxycillin, and Cotrimoxazole (Table III). Resistance to Amoxycillin Clavulenic acid combination varied in different isolates although in *Staphylococci, Proteus sp.* and *Acinatobacter sp*. it was found to be 100% resistant. On the other hand, all isolates were 100% sensitive to Imipenem. All isolates were resistant to Doxycycline except *Proteus vulgaris* which was sensitive to this drug. All Gram-negative bacilli were totally sensitive to Nitrofurantion.

Pseudomonas aeruginosa strains as also *E.coli* & *Klebsiella sp.* showed variable resistance to Aminoglycosides, Quinolones and Cephalosporins but when all isolates were sensitive to Amikacin, pseudomonas aeruginosa strains were only 37.5% sensitive and *Proteus vulgaris* strain was resistant it.

Table III: Percentage of organisms resistant to a particular antibiotic

Antibiotic Discs used	*E. coli*	*Klebsiella sp.*	*Proteus vulg.*	*Acinatobacter sp.*	*Pseudomonas*	*Enterococcus*	*Staph. aureus*	*CONS*
Penicillin	100	100	100	100	100	100	100	100
Amoxycillin	100	100	100	100	100	100	100	100
Amoxy-Clav	75	66.7	100	100	50	66.7	100	100
Imipenem	0	0	0	0	0	0	0	0
Norfloxacin	75	100	100	0	100	100	100	100
Ciproflox.	75	100	100	100	75	66.7	100	50
Gatifloxacin	25	66.7	100	100	62.5	66.7	100	100
Gentamicin	75	66.7	100	0	100	100	100	50
Amikacin	0	0	100	0	62.5	0	0	0
Erythromy.	-	-	0	0	-	0	0	100
Ceftriaxone	75	66.7	100	100	100	100	100	50
Cefotaxime	50	100	100	100	100	100	100	50
Chlorumph.	25	66.7	0	0	62.5	33.3	-	-
Doxycycline	100	100	0	100	100	100	100	100
Nitrofuran.	0	100	100	100	100	-	-	-
Cotrimoxazo.	100	100	100	100	100	100	100	100

Discussions

A total of 46% incidence of hospital acquired Urinary Tract Infections is much higher than the incidences observed by other workers[5]. It has been observed that the Medicine ward of The Medical College, Kolkata is most notoriously implicated in the incidence (56%) of hospital acquired Urinary Tract Infections amongst of hospital acquired Urinary Tract Infections is more or less same as the other studies[6]. It might be due to the fact that in Medicine ward there had been rampant use of different antibiotics and there had been selection of resistant organisms under antibiotic pressure[7].

The next common incidence of nosocomial urinary tract infection was from Obstetrics & Gynaecology ward (46%). As all patients there were females that might have increased the incidence of nosocomial urinary tract infection[6].

The commonest bacteria isolated was *Pseudomonas aeruginosa* (34.8%), although it was prevalent in only Medicine and Urology wards *E.coli* (17.4%) and *Klebsiella sp.* (13%) were the next two common organisms incidences, when *E.coli* was prevalent in all the four wards and

Klebsiella sp. was not found in surgery. Rests of the organisms were isolated from one or two wards. Other studies had shown that *E.coli* was most common organisms in causing nosocomial urinary tract infections[8] but in this study *Pseudomonas aeruginosa* was found to be the commonest organism. It may be assumed that although E.coli was the most prevalent organism in all the wards, Pseudomonas aeruginosa was found mostly in medicine ward. Other studies have also indicated that *Pseudomonas aeruginosa* was prevalent in nosocomial Urinary Tract Infection in about 16-24% cases[9].

As the *Proteus vulgaris* strain isolated from Obstetrics and Gynaecology Ward had a variable drug resistance pattern, hence it may be assumed that this particular organism isolated in this study was not a hospital flora but might be patients own flora from the perineum. Most of the organisms isolated were sensitive to less used drugs like Imipenens, Chloramphenical and Nitrofurantion and those hospital strains, under pressure from the commonly used antibiotics showed multi-drug resistance.

It has also been found in this study that the organisms isolated from Urology ward was more sensitive to antibiotics than the organisms from other wards, which might be due to more judicious use of antibacterials in Urology. The study conducted in a tertiary care hospital setting that the hospital environment is the store house of several multi drug resistant pathogens. The different wards showed high incidence of hospital acquired urinary tract infections indicating that enough measures have not been taken to check this incidences. This has occurred, might be due to indiscriminate use of recent antibiotics as also less stringent sterilization procedures[10]. As such incidences might lead to long hospital stay and morbidity of the patient including less bed occupancy, immediate measures need to be taken to prevent it. The Government too has to bear the extra burden of taxation in order to provide remedy for these unwanted nosocomial infections[11].

In this scenario, it is necessary to take immediate measures so as to curb the overwhelming preponderance of hospital acquired urinary tract infections in the admitted patients[12].

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Designing of Some Potential New 4-aminoquinoline Analogs: Identification of Some Potential New Leads against PfLDH using Structure Based Drug Design (SBDD) Approach

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Abstract

Malaria is one of the lethal parasitic diseases caused by different strains of Plasmodium sp. *Plasmodium falciparum* lactate dehydrogenase (PfLDH) has been considered as one of the potential target for malaria due to its critical role in glycolysis. The lactate dehydrogenase found in different strains of *Plasmodium* such as *P. vivax*, *P. malariae* etc. exhibiting around 90% identity. Antimalarial agent chloroquine interacts specifically with PfLDH in the NADH binding pocket and acts as a competitive inhibitor of NADH which is critical a co-factor of PfLDH. In this study some analogs of chloroquine has been designed from the 4-aminoquinoline scaffold and docked in the NADH binding pocket using an integrated structure based drug design approach (SBDD) approach to identify some potential new leads for the treatment of malaria.

Keywords: Malaria; *Plasmodium falciparum* lactate dehydrogenase (PfLDH); Chloroquine; Structure Based Drug Design (SBDD); Docking

Introduction

Malaria is one of the prominent mosquito borne infectious disease caused by different species of protozoa with the genus Plasmodium. It is one of the most lethal parasitic diseases in the world especially in Sub-Saharan Africa, Asia and Americas. Transmission of malaria mainly occurs through the bite of Anopheles mosquitoes infected with the parasite, which introduces the parasite into human circulatory system. As per the estimation of WHO in the year 2010 over 500 million cases of malaria resulting more than half a million death worldwide. The sub-Saharan Africa is one of the most affected areas where 85-90% malaria fatalities occur. Among many species of Plasmodium, Plasmodium falciparum is the most pathogenic species, which creates a greater threat towards humanity[(1)].

Over the time many antimalarial agents have been discovered to treat malaria. The most effective treatment against *P. falciparum* is the currently available artemisinin combination therapy (ACT). Besides artemisinin some very prominent agents in ACT are quinoline compounds, which act mainly by inhibiting hematin polymerization[(2)]. For many years chloroquine has been widely used to treat *P. vivax*, which is the most prevalent human parasite. But recent emergence of chloroquine resistant strains across different part of the world posses a great challenge to

discover new antimalarial agents[3].

Recently *Plasmodium falciparum* lactate dehydrogenase considered as potential molecular drug target for malaria. Hematin one of the product of hemoglobin degradation by malaria parasites intoxicates the parasite by competiting with NADH for the active site of PfLDH. The survival of malaria parasite depends on polymerization of hematin to hemozoin. Chloroquine acts as a competitive inhibitor of NADH for the active site of PfLDH thus formed complexes with hematin, which ultimately prevents the formation of hemozoin [4, 5]. Because of this reason compounds having structural similarity with NADH have been researched as potential new inhibitor targeting PfLDH. In this study some chloroquine analogs have been designed and analyzed using structure based drug design (SBDD) approach to identify some potential new inhibitors, which predicted to act as a better competitive inhibitor for PfLDH.

Over the time structure based virtual screening approach have been used widely to identify potential drug like candidates[6]. In this study a number of chloroquine analogs have been designed and virtual screening has been done against the NADH binding pocket of PfLDH to identify best ligands, which can act as better competitive inhibitor than chloroquine (CHL). With this approach we come up with some potential new analogs having better in-silico profile as future lead candidate for treatment of *P. falciparum*.

Computational Methods

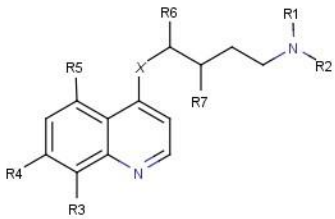
Ligand library generation:

As chloroquine acts a competitive inhibitor for PfLDH hence the 4-aminoquinoline scaffold of chloroquine has been taken as the core scaffold to design some structural analogs (Table 1; Figure 1). Over the time 4-aminoquinoline scaffold has been used to develop many potential drug candidates for the treatment of malaria[7]. The molecular property of these designed molecules has been predicted using internet based 1-click molecular property prediction tool developed by Mcule.Inc[8]. Molecular properties are essential for every stages of drug development from design to synthesis hence predictions of these parameters are important for drug development point of view[6].

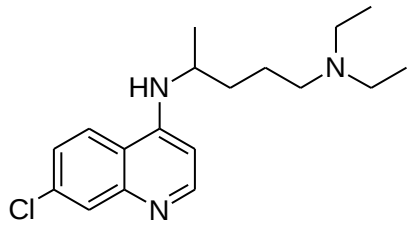
Protein Preparation:

The 3D structures of the PfLDH complex with NADH and oxamate was obtained from protein data bank (PDB: 1LDG)[9]. The protein has been prepared using Chimera by deleting existing oxamate, water and processed accordingly [10,11]. The centre of co-crystallized NADH was considered as centre of receptor grid as it covers all reported interacting amino acid residues. The binding affinity of chloroquine and the designed analogs at this pocket will be critical because of the competitive binding mechanism which ultimately inhibits the formation of hemozoin.

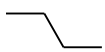
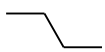
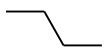
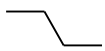
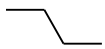
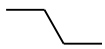
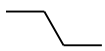
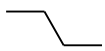
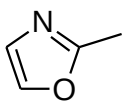
Table 1 An overview of structure modifications adapted in this study. A) Showing the core scaffold used for structure modification at different positions B) Structure of the chloroquine used as a reference molecule



(a)



(b)

Molecule Id	R1	R2	R3	R4	R5	R6	R7	X
Lig1			H	Cl	H	H	H	NH
Lig2			H	Cl	H	CH_3	H	NH
Lig3			H	F	H	H	H	NH
Lig4			H	F	H	CH_3	H	NH
Lig5			OH	H	F	CH_3	NH_2	CH_2
Lig8			H	SO_2CH_3	H	CH_3	H	NH
Lig9			H	F	H	CH_3	H	NH

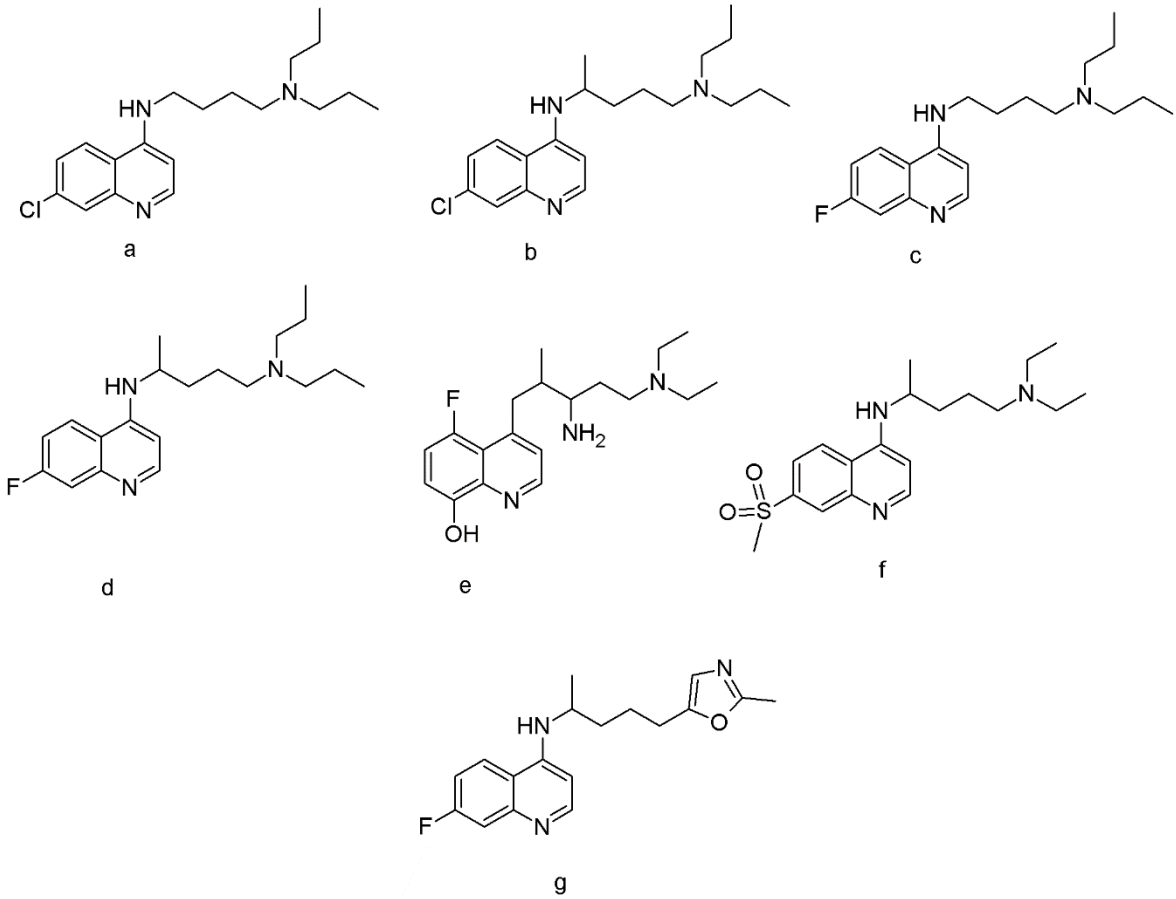


Figure 1 Structure of all designed ligands used for virtual screening. a,b,c,d,e,f and g represents Lig1, Lig2, Lig3, Lig3, Lig4, Lig5, Lig8 and Lig9 consecutively.

Virtual Screening of the Ligands & Validation of Docking Protocol:

The known inhibitor chloroquine and the designed compound library have been combinedly screened against the binding pocket of PfLDH. The ligands have been energy minimized and geometry optimized using Avogadro with MMFF94 force field and saved as MOL2 format for subsequent docking analysis[12]. The files are then processed using Raccoon software for generating pdbqt files required for docking analysis[11]. Virtual screening was conducted using AutoDock Vina[13]. The screening procedure has been run using Vina's default settings with exhaustiveness of the screening have been set to 8.0. The grid box has been defined to accommodate Asn140, His195, Val138, Phe100, Gly99, Thr97, Gly32, Ile31, Met30, Gly29, Asp53, Ile54 and Tyr85, as this corresponded crucial interaction at NADH binding pocket in the PfLDH (Figure 2)[1]. The X, Y and Z centres were defined as 31, 27 and 31, respectively. The X, Y and Z size dimensions were defined as 32, 24 and 26, respectively. AutoDock Vina generated screening results in the pdbqt format. All the compounds with corresponding binding energy have been

visualized in Chimera using the ViewDock plugin and the ligands have been ranked accordingly on the basis of binding energy. The best pose of the prototype and the top ranked ligand on the basis of binding energy have been visualized using Maestro's LigInteraction option to understand the crucial amino acid residues interacting with the ligands and the nature of interactions[14].

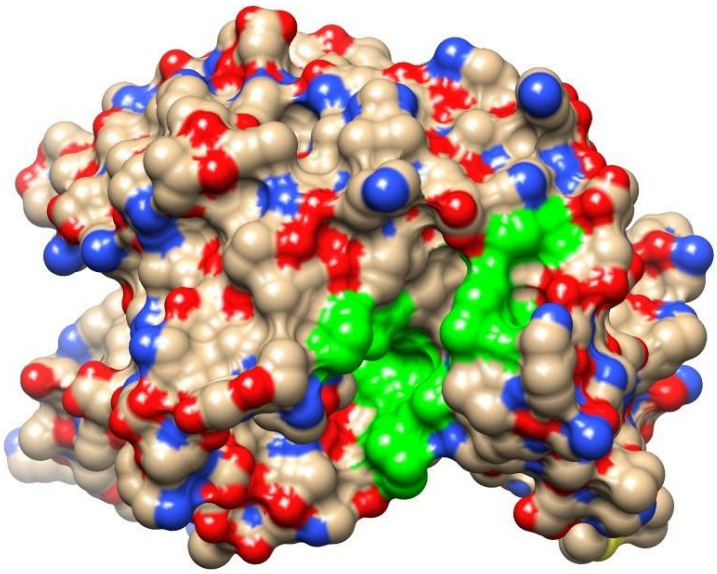


Figure 2 The surface of PfLDH in green showing crucial interacting residues at NADH binding pocket of 1LDG used for GRID generation.

To validate our docking protocol we adapt a similar approach that of our previous reported approach. Validation of docking is one of the prominent steps to narrow down the selection of false hits. We extracted the NADH from the co-crystallized structure and redocked using our docking protocol at exactly same binding pocket. It has been noticed that the NADH binds at exactly same place with a single docking pose having binding affinity of -11.0 kcal/mol and RMSD zero. This certified that efficiency of our docking engine and protocol. To further validate our protocol we docked three previously researched compounds which has been docked at the same NADH binding pocket using different docking software and tested for anti-PfLDH activity[1]. It has been observed that all the three ligands ranked exactly in the same order in our docking protocol compared to the previous docking programme (ranked according to most negative binding energy) (**Figure 3**). If this trend holds true, this could indicate that our designed compounds may have excellent activity against PfLDH.

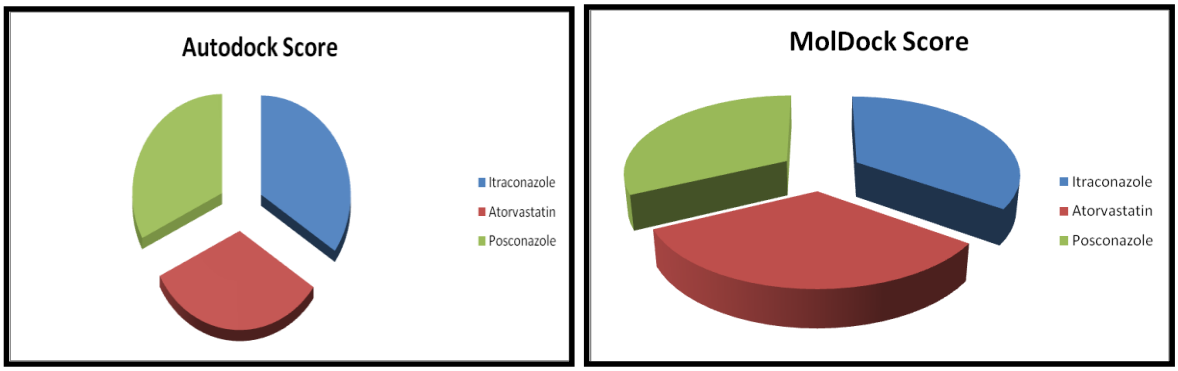


Figure 3 The comparative docking scores of Autodock and MolDock of previously reported ligands docked at the same binding site within PfLDH. MolDock scores have been taken from Penna-Coutinho et al., 2011. In both cases Itraconazole, Atorvastatin and Posconazole contributed 35%, 33% and 32% of the total score which validates our docking protocol using Autodock Vina.

Results and Discussion

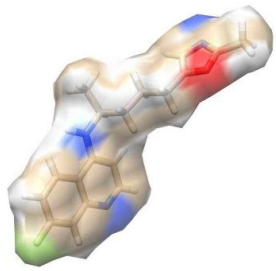
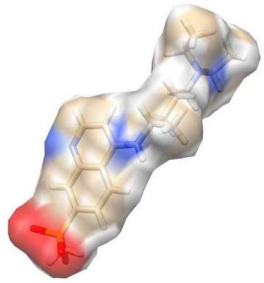
The designed ligands and the reference chloroquine has been subjected to molecular docking using Autodock Vina at the NADH binding pocket to identify best competitive inhibitor of NADH. After validation of our docking protocol to narrow down the false hit count we ranked all the ligands based on their binding energy. It has been observed that Lig9 posses a significantly higher binding energy (more negative) of -8.1 kcal/mol while the reference chloroquine which acts as a competitive inhibitor of NADH in PfLDH has a significantly lower binding energy of -6.9. Moreover Lig8, Lig5, Lig4 and Lig2 have got better binding energy than the reference ligand (Table 3). It is quite interesting to notice from superimposition of all the docking posses of the ligands that all ligands binds around the same binding cavity of NADH. More importantly Lig8 and chloroquine binds almost at the same region of the binding pocket (Figure 5). The prediction of molecular properties of all the ligands confirmed their drug-like small molecule behavior as no violation from Lipski Rule of 5 has been observed (Table 2)[(15)]. From the interaction map it has been observed that chloroquine has got a hydrogen bond interaction with Asp53 whereas Lig9 has a hydrogen bond interaction with Asp34. Moreover chloroquine posses hydrophobic interactions with Ile54, Ile123, Phe52, Val26, Tyr85, Ala98, Met30, Ile31, Val55; polar interactions with Ser28, Thr101, Thr97; Glycine interactions with Gly99, Gly29, Gly32, Gly27 and charged interaction with Glu122 whereas Lig9 posses hydrophobic interactions with Phe81, Ile104, Tyr66, Val9, Ile108, Phe33, Val124, Ile14, Met13, Ala79, Val36, Ile35; polar interactions with Ser11, Thr82, Thr78 and glycine interactions with Gly80, Gly15, Gly12, Gly10 (Figure 4). The overall interaction map and the binding energy confirms a better binding affinity of Lig9 which emerged as the top ligand from this structure based screening approach.

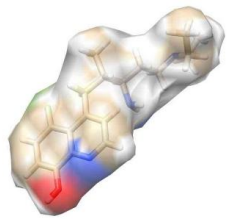
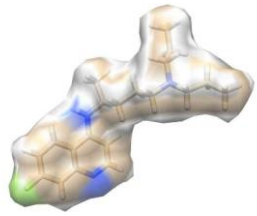
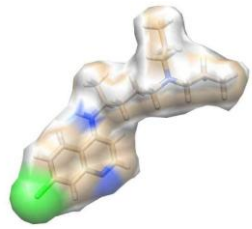
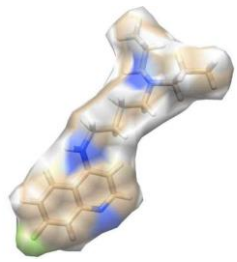
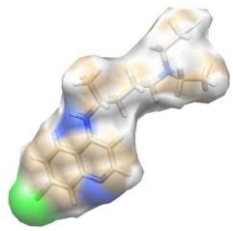
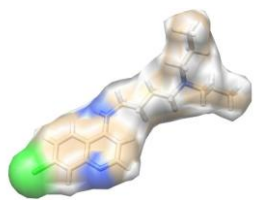
Table 2 The predicted molecular property of all the designed ligands using 1-click molecular property prediction application from Mcule. Inc

Molecule Id	Mass	LogP	No. of HBA	No. of HBD	No. of Rotatable Bonds	PSA	RO5 violation
Lig9	313.37	4.57	4	1	6	50.95	None
Lig8	363.52	4.71	5	1	9	70.68	None
Lig5	333.44	4.02	4	2	8	62.38	None
Lig4	331.47	5.15	3	1	10	28.16	None
Lig2	347.92	5.66	3	1	10	28.16	None
Lig3	317.44	4.76	3	1	10	28.16	None
Lig1	333.89	5.27	3	1	10	28.16	None

HBA= Hydrogen Bond Acceptors; HBD= Hydrogen Bond Donors; PSA= Polar Surface Area; RO5= Lipnski's Rule of 5

Table 3 List of ligands with their binding energy presented in this study. Ligands are ranked from lower to higher binding energy.

Rank	Molecule Id	Structure	Binding Energy (kcal/mol)
1	Lig9		-8.1
2	Lig8		-7.2

3	Lig5		-7.1
4	Lig4		-7.1
5	Lig2		-7.0
6	Lig3		-6.9
7	Chloroquine		-6.9
8	Lig1		-6.7

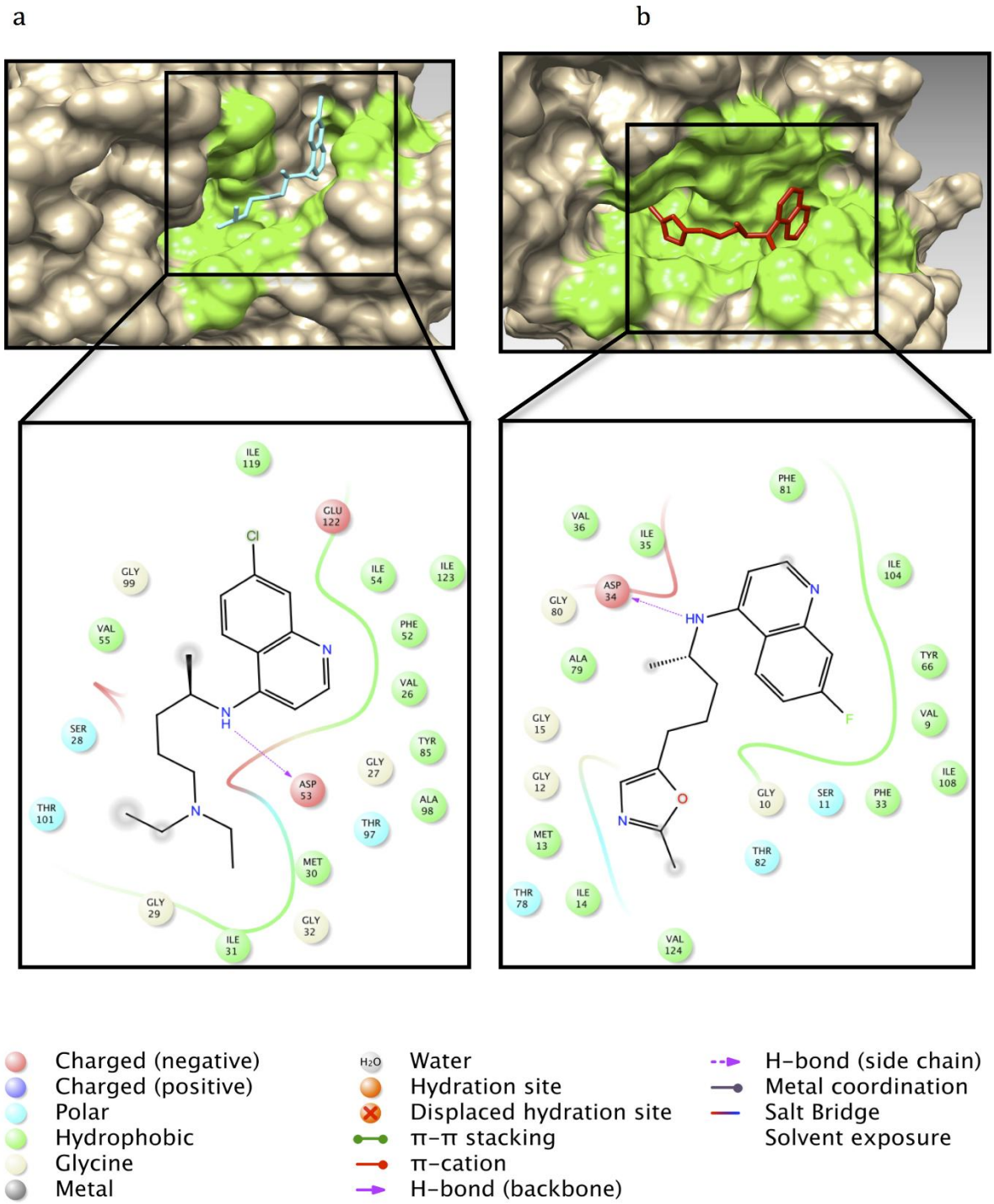


Figure 4 Maestro ligand interaction diagram showing a) key amino acid residues interacting with the reference ligand Chloroquine and b) key amino acid residues interacting with the top ranked ligand Lig8.

A B

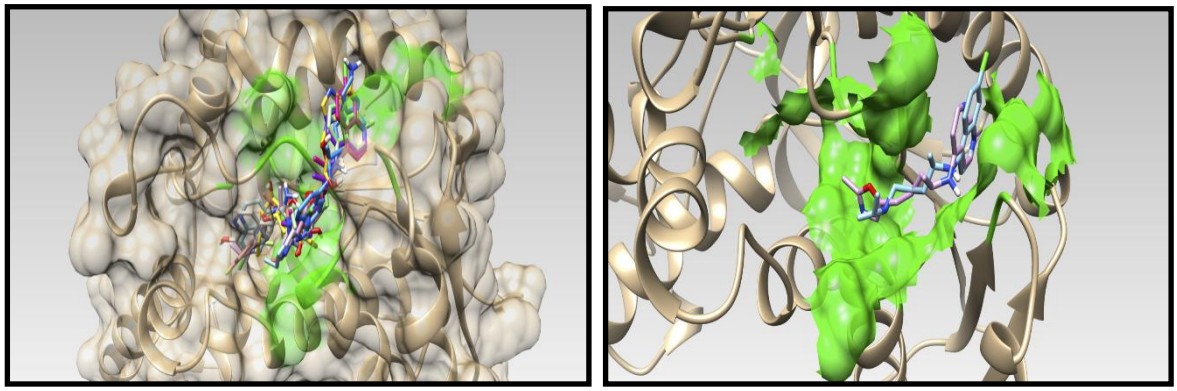


Figure 5 A) Showing superimposition of best docking conformations in NADH binding cavity used for GRID generation, B) superimposed best conformers of Lig9 and chloroquine at the binding cavity.

Conclusion

With the validated docking protocol and structure based drug design approach a number of ligands have been subjected to virtual screening to identify a better competitive inhibitor of NADH in PfLDH. Chloroquine, a potent antimalarial agent has been considered as a competitive inhibitor of NADH but herein we emerged with five potential new ligands which can bind more efficiently at the NADH binding pocket within PfLDH. Among these five ligands Lig9 showed a better binding than chloroquine at the critical NADH binding pocket. All molecular property prediction confirmed potential drug-likeness as the designed molecules have not found to violate the Lipnski's rule of five. Moreover all designed molecules including the best ligand Lig9 posses the core 4-aminoquinoline scaffold which is considered as the essential scaffold to have antimalarial activity. It is quite interesting to notice in molecules where the –Cl group has been replaced with –F or $–SO_2CH_3$ group at the 7 position the binding affinity increases which is evident from the binding energies of Lig9, Lig8, Lig5, Lig4 and Lig2. It has also been noticed that the addition of an oxazole group increase the binding affinity of the ligand which is evident from the binding energy of Lig9. Moreover it has been found that two ethyl substitution in the -N terminal is more favourable over propyl substitution as well as – $NHC(CH_3)C$- chain is crucial for better binding. Over the time structure based drug design approach has been adapted to identify potential drug like molecules which can bind more efficiently to act as an inhibitor. Our approach leads us to identify five potential new ligands which can act as a better competitive inhibitor than chloroquine at the NADH binding pocket especially Lig9 promises to be the best ligand out of this virtual screening campaign which can potentially be a future lead molecule to develop better drug-like candidates for P. falciparum. This method could possibly lead drug discoverers to identify potential new compounds having 4-aminoquinoline scaffold as an antimalarial agent and in future could

also be beneficial to identify best ligands for further synthesis and development stages without wasting important time and resources.

Acknowledgement

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Haematinic activity of *Tridax procumbens* Linn.

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Abstract

The haematinic activity of an orally administered aqueous extract of *Tridax procumbens* L. was studied on haemolytic anaemic rats. Anaemia was induced by an oral administration of phenylhydrazine (PHZ: 10 mg/kg p.o.) for a period of 8 days. The red blood cell count (RBC), haemoglobin concentration (Hb), white blood cell count (WBC) and haemotocrit (PCV) were analyzed as indices of anaemia. The mean cell haemoglobin, mean cell volume and mean cell haemoglobin concentration were calculated accordingly. Phenylhydrazine induced a significant decrease ($P<0.05$) in the blood parameters indicating anaemia and also resulted to significant increase ($P<0.05$) in the mean cell haemoglobin, mean cell volume values, which are indicators of macrocytosis. *Tridax procumbens* L. induced a significant ($P<0.05$) increase in the red blood cell count, haemoglobin concentration, and pack cell volume which had been originally decreased by phenylhydrazine administration within one week of treatment. The presence of macrocytosis turn towards normal as the animals recovered from anaemic condition. The results obtained suggested that. *Tridax procumbens* L. may have haematinic properties.

Key words: Haematinic activity, *Tridax procumbens* L. Haematinic anaemia, phenyhydrazine

Introduction

Tridax procumbens L. (Compositae) is a hispid, procumbent herb, found as a weed throughout India. *Tridax procumbens* L. is a small herb having short, hairy blade like leaves. Corolla is yellow in color. *Tridax procumbens* L. has been claiming to promote wound healing in folk medicine. The wound healing study includes excision, incision and dead space wound. It is common weed in open places In traditional medicine, the leaves are reported to be used in bronchial catarrh, dysentery and diarrhoea and for restoring hair. The poultice of whole plant is used as an anti-inflammatory remedy by the tribal healers [1]. The leaves of the plant are used as a vegetable and as cattle feed [2].

Anaemia constitutes a serious health problem in many tropical countries because of the prevalence of malaria and other parasitic infections [3]. In anaemia there is decreased level of circulating haemoglobin, less than 13 g/dl in male and 12 g/dl in females [4]. In the tropics, due to endemicity of malaria, between 10 to 20% of the population presents less than 10 g/dl of Hb [5].

Materials and Method

Plant material

Sample of *Tridax procumbens* L. whole plant was collected from Greater Noida in the month of November 2014 Identification of the plant was confirmed by the Professor of Birla Institute of Technology Dr. S. Jha. The specimen copy of the plant was submitted in the herbarium for reference purpose. The whole plant was air-dried. The extract was prepared by cold maceration method using 2.5 litre of methanol for 1 kg of plant material. The extract was freeze dried to obtain the methanol extract.

Animals

Male albino rats (150-180g) were used for the study. The rats were housed in wire-mesh cages with a 12 h light/dark cycle. They had continuous access to food and water during the entire period of experimentation.

Experimental design

Six rats were kept as normal control group (Group 1 below), while 30 rats were made anaemic by oral intubations of phenylhydrazine (10 mg/kg body weight) daily for 8 days [6]. Rats that developed anaemia with haemoglobin concentration lower than 13 g/dl were recruited for the study [7]. Anaemic rats were randomly divided

4 groups (2 to 5 below, 6 rats per group) and treated as follows:

Group 1: received distilled water (1 ml) daily (normal control),

Group 2: received distilled water (1 ml) daily (anaemic control),

Group 3: received oral single dose (1 ml) of the extract 200 mg/kg body weight/day

Group 4: received oral single dose (1 ml) of the extract 400 mg/kg body weight/day,

Group 5: received oral single dose (1 ml) of the extract 800 mg/kg body weight/day.

The experiment lasted for 3 weeks.

Haematological investigation

Blood was collected by ocular puncture after overnight fast. The blood was collected before induction of anaemia, after induction of anaemia with PHZ and during 1, 2, and 3 weeks of treatments. The volume of blood collected (0.25 to 0.45 ml) did not affect blood parameters as earlier reported [5]. The red blood cell count (RBC), white blood cell counts (WBC), haemoglobin concentration (Hb) pack cell volume (PCV) [3, 8] were calculated.

Statistical analysis

Experimental data were analyzed using one way analysis of variance (ANOVA) and LSD multiple range test to determine significant differences between means. Difference between means were regarded as significant at $p<0.05$.

Results

The changes in the haematological parameters of the rats during the study are presented in Tables 1, 2, 3 and 4. The RBC, Hb, and PCV of rats administered phenylhydrazine (PHZ) decreased significantly ($P<0.05$) while (Table 1) giving rise to macrocytic anaemia. One week of treatment of anaemic rats (Groups 3, 4, and 5) with *Tridax procumbens* L. extract reversed the effect of PHZ resulting to a significant ($P<0.05$) increase in RBC, Hb, and PVC (Table 2). During the experimental period, the Hb, RBC, and PCV of the untreated anaemic rats (anaemic control, Group 2) also increased but at a slow rate. The Hb and PCV only reached the normal range at the second week of the experiment (Table 3) while the RBC reached normal range at the 3 rd week of experiment (Table 4). The Hb, RBC and PCV of group 3, 4 and 5 reached normal values after one week of treatment (Table 2) with maximum level of increase in the second week (Table 3). At this point, the Hb and PCV were significantly ($P<0.05$) higher in group 5 rats than in the normal control rats while no significant difference ($P>0.05$) was observed between the normal control rats and group 3 and 4 rats (Table 3). This explains that the response to treatment was dose related. After the 3 rd week of experiment, the Hb, RBC and PCV return to normal with no further increases Table 4) Figures 1-3 presents the changes in Hb, PCV and RBC per group during the experimental period. The Hb of anaemic rats increased sharply within the first week of the experiment, though the increase was higher for the groups treated with *Tridax procumbens* L. than the anaemic control. This increase slowed down at week 2 and stabilises in week 3 (Figure 1). Similar results were obtained for PCV (Figure 2) and RBC (Figure 3).

Table 1. Effect of phenyl hydrazine (10 mg/kg, o.p daily for 8 days) on some haematological parameters (T=0) (RBC, Hb, PCV and WBC).

Parameter	Group 1 (control)	Group 2 (anaemic)	Group 3 (anaemic)	Group 4 (anaemic)	Group 5 (anaemic)
Hg (g/dl)	17.08 ± 0.32	12.98 ± 0.68	13.03 ± 0.65	13.37 ± 0.98	13.01 ± 0.11
RBC (x10 6 /µl)	6.10 ± 0.19	3.82 ± 0.27	3.09 ± 0.23	2.88 ± 0.49	2.66 ± 0.28
PCV (%)	49.20 ± 1.80	40.27 ± 0.82	42.53 ± 0.99	43.98 ± 0.82	41.69 ± 2.10

Table 2. Haematological parameter of rats after one week treatment with extract of *Tridax procumbens* L.

Parameter	Group 1 (control)	Group 2 (anaemic control)	Group 3 (200 mg/kg)	Group 4 (400 mg/kg)	Group 5 (800 mg/kg)
Hg (g/dl)	17.33 ± 1.38	15.54 ± 0.91	15.69 ± 1.12	17.13 ± 0.97	17.99 ± 0.10
RBC (x10 6 /µl)	6.23 ± 0.32	3.98 ± 0.32	4.52 ± 0.98	4.98 ± 1.23	5.89 ± 0.56
PCV (%)	49.33 ± 0.22	42.56 ± 0.28	48.92 ± 0.89	50.86 ± 1.23	52.69 ± 0.32

Table 3. Haematological parameters of rats after 2 weeks treatment with extract of *Tridax procumbens* L.

Parameter	Group 1 (control)	Group 2 (anaemic control)	Group 3 (200 mg/kg)	Group 4 (400 mg/kg)	Group 5 (800 mg/kg)
Hg (g/dl)	17.39 ± 0.23	15.98 ± 1.21	16.06 ± 0.02	18.58 ± 0.01	19.09 ± 0.65
RBC (x10 6 /µl)	6.98 ± 0.67	4.99 ± 0.02	5.68 ± 0.32	6.99 ± 0.11	7.11 ± 0.11
PCV (%)	50.55 ± 0.49	46.78 ± 0.11	49.98 ± 0.08	51.99 ± 0.02	53.09 ± 0.23

Table 4. Haematological parameters of rats after 3 weeks treatment with extract of *Tridax procumbens* L.

Parameter	Group 1 (control)	Group 2 (anaemic control)	Group 3 (200 mg/kg)	Group 4 (400 mg/kg)	Group 5 (800 mg/kg)
Hg (g/dl)	17.02 ± 1.23	16.55 ± 0.36	16.96 ± 0.89	17.0 ± 0.01	17.21 ± 0.10
RBC (x10 6 /µl)	7.15 ± 0.76	6.02 ± 0.16	6.79 ± 0.46	7.87 ± 0.43	7.98 ± 0.09
PCV (%)	50.55 ± 0.49	46.78 ± 0.11	49.98 ± 0.08	51.99 ± 0.02	53.09 ± 0.23

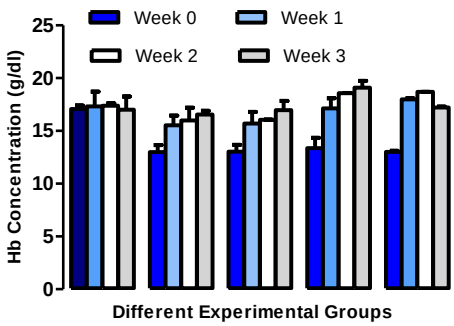

Figure 1. Changes in Hb concentration per group during experimental period.

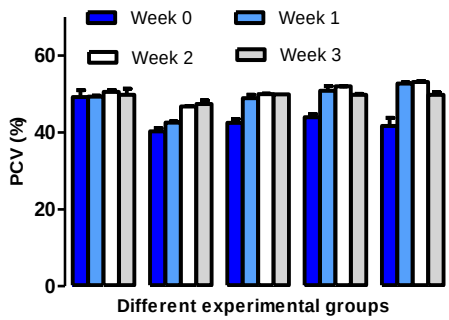

Figure 2. Changes in PCV per group during experimental period.

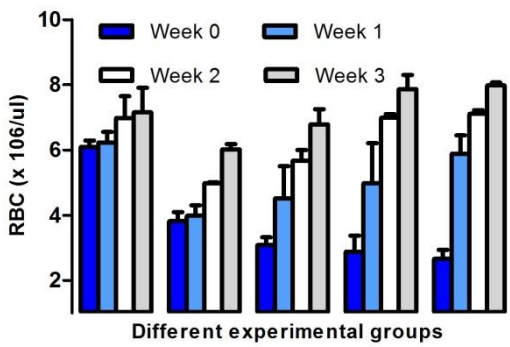

Figure 3. Changes in RBC per group during experimental period.

Discussion

The anaemia was induced by using PHZ in albino rats. It is already proved that intraperitoneal administration of PHZ decreased haemoglobin concentration, red blood cells number and haematocrit [9, 10]. The results of this study indicated that the whole methanol extract of *Tridax Procumbens* L. markedly increase the concentration of haemoglobin, red blood cell count, white blood cell count and the packed cell volume mainly one week after the treatment. *Tridax procumbens* L. is the well known source of minerals, Sterols, Proteins and other vitamins as In conclusion the Chemical constituents of the plant may be responsible for the haematinic activity [11]. Anaemia is the disease condition characterised by a reduction in the concentration of haemoglobin, circulating red blood cell and pack cell volume per unit of the peripheral blood below the normal for the age and sex of the patient [12].

The Recovery of the anaemic rats indicates that the *Tridax procumbens* L. is helpful in increasing the erythropoisis. The results from the current study are indication that this plant can be used for the treatment of anaemia. However the mechanism of action is by which the *Tridax procumbens* L. is helpful in increasing the RBCs, Hb, and PCV in experimental animals needs to be analysed.

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Evaluation of Physical Stability of Azithromycin dihydrate Suspensions Formulated by using *Plantago ovata* Mucilage and HPMC as Suspending Agents Employing Step-Technology

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Abstract

The aim of the present study was to evaluate the sedimentation stability of azithromycin suspensions formulated with different concentrations of two suspending agents in a rapid way by employing near infrared transmission measurements. The mucilage extracted from *Plantago ovata* seeds was used as a suspending agent and was compared with the traditional suspending agent HPMC. Stability studies of suspensions are very important in formulation of suspensions for accuracy of doses administerd. Physical stability of azithromycin suspensions was studied in terms of sedimentation stability in a rapid way employing infrared extinction profiles by using the instrument separation analyzer (LUMiReader®) in the present work. The LUMiReader® instantaneously measures the extinction profiles of the transmitted light across the entire length of a suspension sample employing STEP-Technology (Space- and Time-resolved Extinction Profiles Technology). Instability indices determined on different suspension formulations indicated that *Plantago ovata* mucilage (POM) and HPMCK4M can be used as suspending agents for the preparation of stable suspensions of azithromycin and POM was found to be a better suspending agent.

Key words: suspension stability, azithromycin, *Plantago ovata,* LUMiReader®, STEP-Technology, instability index.

Introduction

Suspensions are biphasic heterogeneous coarse dispersions containing essentially the insoluble particulate matter or drug suspended with the help of suspending agent(s) in a liquid medium. The continuous or external phase is generally a liquid or semisolid. The liquid phase may be aqueous or in some instances may be organic or oily liquid for non oral use. The dispersed or internal phase is the insoluble particulate matter dispersed throughout the continuous or external phase. These are thermodynamically unstable; almost all suspension systems separate on standing. Because some products occasionally are prepared in a dry form to be placed in suspension at the time of dispensing by the addition of an appropriate liquid vehicle, this definition is extended to include these products[1, 2].

Stability study of suspensions is a very important aspect to enable the patient to receive the

intended amount of the drug(s) in the dose administered. Physico-chemical stability of suspensions is important for maintaining the quality of the product.

Azithromycin is (2R, 3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R)-13-[(2, 6-dideoxy-3-C-methyl3-O-methyl-α-L-ribohexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10 ,12,14-heptamethyl-11 [[3,4,6trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]-1-oxa-6 azacyclopentadecan-15-one. Azithromycin is a new alternative macrolide to erytrhromycin that has a similar spectrum of activity. Azithromycin is acid-stable antibiotic, so it can be taken orally with no need of protection from gastric acids. It is readily absorbed, but absorption is greater on an empty stomach[3-5].

Physical stability testing of azithromycin suspensions

Different procedures have been suggested in the past for evaluating the physical stability of suspensions[6-9]. Some of these are experiential in the sense that they have no mathematical base. The evaluation methods used may well be classified into:

i. Sedimentation methods
ii. Rheological methods
iii. Electro-kinetic methods and
iv. Micromeritic methods

Normally there is a need to carry out a quick assessment of particle size change since no formulator can afford to wait for the normal shelf storage periods to study such changes. Hence, suspensions are subjected to artificial stress conditions in the form of freezing and thawing. Such a treatment is known to promote particle growth and can be used to predict future behaviours. However, an important point to remember is that sometimes hydrocolloids which are usual additives in suspensions can themselves get affected by freezing and thawing leading to caking of suspensions. Hence, observations may not be quite correlated to shelf-life of the products.

STEP-Technology (Space- and Time-resolved Extinction Profiles Technology)

STEP-Technology stands as acronym for Space and Time Resolved Extinction Profiles Technology. It can be used to measure the infrared extinction profiles of the transmitted light across the entire length of a suspension sample from top to bottom instantaneously[10]. By using an instrument called LUMiReader® which operates on STEP-Technology, it is possible to observe and understand different stability/instability phenomena of a disperse system like emulsion or suspension concurrently; e.g., creaming, sedimentation, coalescence, aggregation and flocculation at original product concentration. Basing on these phenomena the instability index is generated by the software SEPview installed in the instrument. Depending on the instability index measured for different suspension samples prepared with various suspending agents and other

excipients, an ideal suspending agent and its concentration required to get a stable suspension can be selected.

Different program components are provided in a LUMiReader® for the qualitative and quantitative analysis of the samples, e.g.,

- The Front Tracking for settling, creaming and consolidation (separation velocity)
- The Integral Transmission for the clarification speed
- The PSA-Module for the calculation of the particle size distribution.
- Stability analysis for determination of instability index and for comparison of stability of different samples.

Separation analyser LUMiReader®

The Separation analyser LUMiReader® PSA-453 manufactured by LUM GmbH, Germany was used in the present work to carry out physical stability studies on azithromycin suspensions formulated with different concentrations of suspending agents. The sample cell in a LUMiReader® is illuminated by a multi-colour light source I_0, including one near infrared wavelength (870 nm). Behind the sample cell the transmitted light I is detected using a CCD-line detector. The detector contains about 6434 elements, with a detector resolution of 9 µm and detection length of 45 mm. Transmission is converted into extinction by ln (I_0/I).

Frequently optical particle size measurement techniques are used to determine the volume weighted particle size distribution. For this purpose the size and material dependent extinction coefficient is needed which can be calculated with Mie-theory using the complex refractive index of the particles. In this case strong assumptions have to be made like spherical homogeneous particles. However, the determination of the refractive index can be very difficult especially in the submicron range and for heterogeneous particles. No standard measurement methods are available up to now. The way out could be the evaluation of space and time resolved extinction profiles at different wavelengths for sedimenting or creaming particles in gravitational or centrifugal field[11, 12].

Illuminating the dispersion across its entire sample height, and by having many thousand detectors LUMiReader® can measure the light source extinction profile instantaneously, even the smallest changes in concentration can be detected. The instrument measures the extinction profiles over the whole sample length during physically accelerated separation. Changes in the extinction profile are representative for the changes in particle concentration and allow to determine the velocity of individual particle classes with no assumptions regarding particle properties. Particle size distribution is obtained based on Stokes' law[10]. Mostly 2 mm cells made of polycarbonate are used for measurements (sample volume 0.4 cm^3). For samples with very low turbidity 10 mm cells (sample volume 2 cm^3) are used alternatively.

Materials and Methods

Materials

Azithromycin IP was obtained as gift sample from VerGo Pharma Research Laboratories Pvt. Ltd, Goa, India. Hydroxypropyl methylcellulose (HPMC) - Methocel K4M was procured as a gift sample from Colorcon Asia Pvt. Ltd., Verna, Goa. India.

Plantago ovata mucilage (POM) was extracted from the seeds of *Plantago ovata* purchased from local market following the procedure described by Kulkarni et al.[13]. The seeds were soaked in distilled water for 48 hours and boiled for 10 minutes thereafter. The resulting viscous gel mass was pressed through a muslin cloth. The filtrate so isolated was treated with equal volume of acetone which resulted in precipitation of the mucilage. The isolated precipitate was dried at 40 °C for 2 hours. The dried mass was subjected to size reduction which yielded a powder mass. The powder was finally passed through sieve number 80 and stored in desiccators for further analysis and use. The yield was around 30 % w/w. All chemicals, solvents and reagents used in the study were of analytical grade.

Methods

Compatibility studies: Compatibility studies were carried out to investigate the incompatibilities between azithromycin and the suspending agents by using differential scanning calorimetry (DSC) and Fourier transform infrared (FT-IR) spectroscopy.

Sample preparation: Drug to excipient ratio of 1:1 provides maximum possibilities of interaction between the drug and various suspending agents thus enabling easy detection of any incompatibility. Therefore, homogeneous 1:1 physical mixtures of azithromycin and suspending agents were prepared by trituration in a clean and dry glass mortar and pestle[14]. These mixtures were stored in glass vials in a stability chamber at 25±2 °C for four weeks after which they were subjected to DSC and FT-IR studies using differential scanning calorimeter, DSC-4000 and FT-IR, model IR Affinity-1, Shimadzu Corporation, Japan.

Preparation of azithromycin suspensions: Azithromycin suspensions were prepared with four different concentrations of each suspending agent HPMC K4M and POM as described hereunder. Each suspending agent was used in four concentrations at 0.25 %, 0.5 %, 0.75 % and 1.0 % as shown in Table 1. Thus, eight formulations were prepared with four suspending agents.

Table 1. Formulation of azithromycin suspensions

Formulation code	Amount of azithromycin (g)	Suspending agent used	Amount of suspending agent (g)
F1S1	4	HPMCK4M	0.25
F2S1	4	HPMCK4M	0.50
F3S1	4	HPMCK4M	0.75
F4S1	4	HPMCK4M	1.00

Formulation code	Amount of azithromycin (g)	Suspending agent used	Amount of suspending agent (g)
F1S2	4	PO mucilage	0.25
F2S2	4	PO mucilage	0.50
F3S2	4	PO mucilage	0.75
F4S2	4	PO mucilage	1.00

HPMC K4M – Hydroxypropyl methylcellulose K4M; POM- Plantango ovata mucilage

Procedure

The suspending agent was kept in contact with about 90 ml of water containing 100 mg of sodium benzoate for 12 hours to allow the swelling of suspending agent. The dispersion was thoroughly mixed with a laboratory stirrer (REMI) for 30 minutes at an average speed of 200 rpm to get a uniform dispersion. Azithromycin was then added to the dispersion under stirring and stirring continued for another 30 minutes and made up to volume. The prepared suspensions were stored at room temperature until further studies.

Physical stability determination

Separation analyser LUMiReader® PSA 453 manufactured by LUM GmbH, Germany was employed for stability determinations.

Sample cells: LUM 10 mm, PC, synthetic cells were used for separation studies basing on the sample properties like freedom from organic solvents, viscosity of the suspensions etc., as recommended by the manufacturer of the instrument.

Selection of tilt angle and temperature

The instrument has a provision for measurements from 0 to 30˚ tilt allowing the sample to remain in upright or inclined position depending on the angle of tilt selected. Tilting the sample from its normal upright position allows an increase in the separation rate at gravity without any additional external forces. The magnitude of acceleration (upto 10 times) depends on geometric factors, such as tilt angle, vial dimensions, and sample type. The LUMiReader® has a provision to maintain the temperature between ambient temperature to 60 °C i.e., 25 °C to 60 °C. Measurements were carried out at 0˚ and 30˚ tilt at 30°C and 60°C for the suspension samples. A sample volume of about 2 ml was used in the determinations.

Procedure

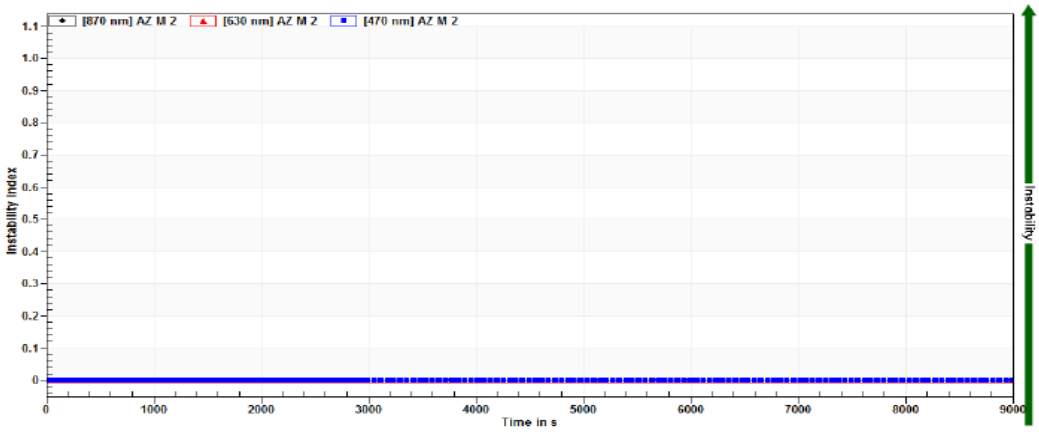
The suspension sample of about 2 ml was filled in the sample cell. The instrument was switched on and the SOP was programmed by selecting various parameters like the tilt angle, temperature, number of profiles, interval, number of cycles etc. Once, the instrument was ready with normalization and base line correction applied, a message appeared to insert the sample.

The sample tube was gently shaken to disperse the sample and inserted into the sample holder. The instrument started recording the profiles as per the set SOP once the sample holder lid is closed by sliding in the direction shown on the instrument. The extinction profiles were recorded at three wavelengths i.e., 470 nm (blue), 630 nm (red) and 870 nm (NIR) with the help of the software SEPView® installed in the instrument. The profiles were automatically saved in the instrument[10]. The extinction profiles of 870 nm (near infrared) wavelength were taken into account for determination of sedimentation stability in the present work. Different suspension samples were analyzed as per the set parameters discussed above.

Some of the representative profiles recorded are shown in Figure 1 and Figure 2. The relevant data is shown in Table 2, Table 3 and Table 4.

Data Range Analysed:

Sample Name	Range from in mm	Range to in mm	Range in mm	Time in s	Channel/ Wavelength
[870 nm] AZ M 2	24.59	38.59	14.00	8,997.49	870 nm
[630 nm] AZ M 2	24.81	38.81	14.00	8,997.49	630 nm
[470 nm] AZ M 2	24.39	38.39	14.00	8,997.49	470 nm



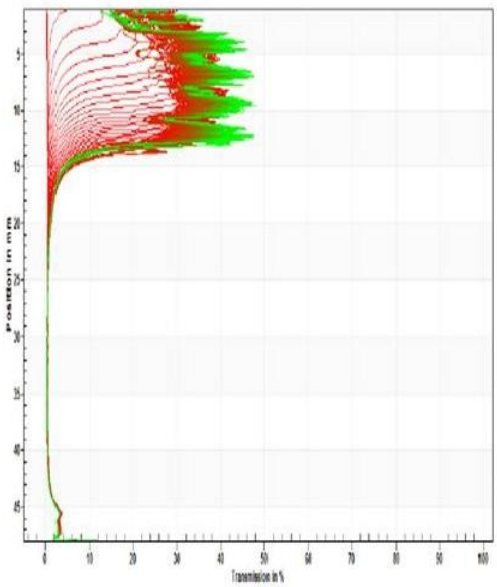
Results:

Sample Name	Time in s	Instability Index	Mean RCA in g	Mean	StdDev
[870 nm] AZ M 2	8,997	< 0.0001	-x-	< 0.0001	< 0.0001
[630 nm] AZ M 2	8,938	< 0.0001	-x-	< 0.0001	< 0.0001
[470 nm] AZ M 2	8,938	< 0.0001	-x-	< 0.0001	< 0.0001

Figure 1. Extinction profiles for azithromycin suspension containing 0.5% of Plantango *ovata* mucilage (POM) as suspending agent measured at 30º tilt and 60 ˚C.

Data Range Analysed:

Sample Name	Range from in mm	Range to in mm	Threshold in %	Start in s	End in s	Channel/ Wavelength
[870 nm] AZ M 2	24.59	38.59	11.00	0.00	8,997.49	870 nm
[630 nm] AZ M 2	24.81	38.81	11.00	0.00	8,997.49	630 nm
[470 nm] AZ M 2	24.39	38.39	11.00	0.00	8,997.49	470 nm



Results:

Sample Name	Start in s	End in s	Value at End in µm	Mean RCA in g	Velocity in µm/s	StdDev in µm/s
[870 nm] AZ-POM	-x-	-x-	-x-	-x-	-x-	-x-

Figure 2. Extinction profiles for azithromycin suspension containing 0.5% of Plantago *ovata* mucilage as suspending agent measured at 30º tilt and 60 ºC temperature: Front tracking – sedimentation velocity

Determination of resuspendability of suspension samples

Resuspendability is the ability to resuspend the settled particles with a minimum amount of shaking after a suspension has sedimented on standing for some time.

Procedure

The resuspendability of the suspensions was evaluated qualitatively. The suspensions were allowed to sediment in stoppered glass jars for 3 months. The test was performed on samples in triplicate by shaking the sedimented suspensions manually at 180° movement, after sedimentation was completed[15]. Based on the number of shakings required to disperse the sediment uniformly into a suspension, the formulations were evaluated. Cake formation was also evaluated qualitatively. Formulations requiring more than 10 shakings were considered positive for cake formation.

Results and discussion

Compatibility studies

DSC Studies

Results of DSC studies shown in Figure 3, describe the thermal behavior of azithromycin and its physical mixtures with HPMC K4M and POM. The endothermic peak of pure drug seen at 132.49˚C indicates the melting point of drug. The thermal peaks were observed at, 93.57 ˚C, and 104.15 ˚C for HPMC K4M and POM respectively. The thermal peaks of physical mixtures of drug with excipients were seen at 132.49 ˚C and 90.89 ˚C for drug with HPMC K4M, and 130.5 ˚C and 97.08 ˚C for drug with POM. There was no significant change in the peak positions and peak shapes suggesting that there was no interaction between the drug and the excipient.

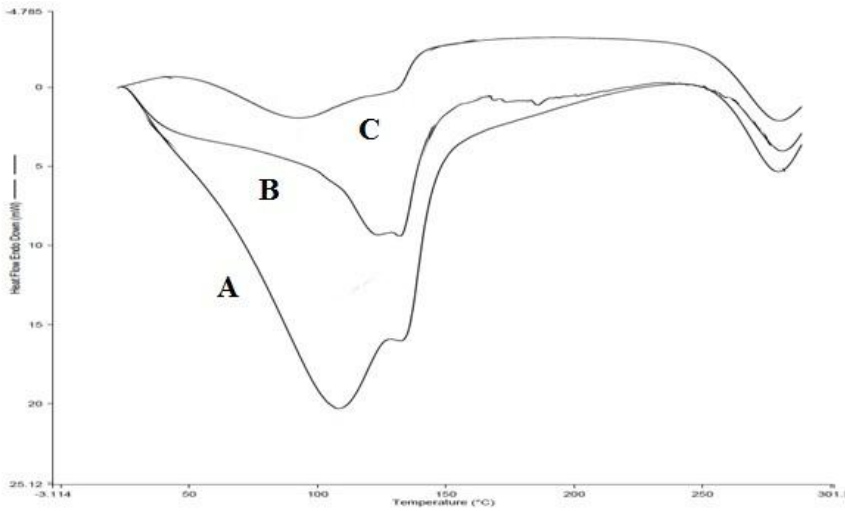


Figure 3. DSC thermograms of azithromycin (AZ) and mixtures of AZ and suspending agents; A- Azithromycin (AZ); B- (AZ + HPMC K4M); C- (AZ+ POM)

FT-IR Studies

Compatibility of azithromycin with different suspending agents was studied using FT-IR spectroscopy. Interactions in the sample are derived or deduced by FT-IR studies from changes in the characteristic peaks. However, some broadening of peaks due to hydrogen bonding was expected while using the excipients from natural origin and also due to moisture without indicating any significant interaction. If all the characteristic peaks are retained and there is no significant change in the peak position, compatibility can be expected.

The broad peak around 3500 cm^{-1} explains the presence of OH stretching due to intermolecular bonding while 2998.47 cm^{-1} explains about CH aliphatic stretching vibration. A prominent peak at 1759.16 cm^{-1} represents C=O carbonyl stretching. The characteristic peak at 1453.43 cm^{-1} defines about CH_3-O alkyl ether group while 1302.01 cm^{-1} describes presence of CH_2O of alkyl ether. COC functionalities are represented by peaks at 1190.13 cm^{-1} and 1014.6

cm^{-1}. The FT-IR spectra of azithromycin dihydrate showed characteristic peaks of pure azithromycin. The presence of characteristic peaks for OH stretching, CH aliphatic stretching vibration, C=O carbonyl stretching, CH_3-O alkyl ether group, CH_2O of alkyl ether, COC functionalities and no new bands or shifts in characteristic peak appeared in the physical mixtures of azithromycin with suspending agents HPMC K4M and POM as shown in Figure 4, indicated that there was no significant interaction between the drug and the selected suspending agents.

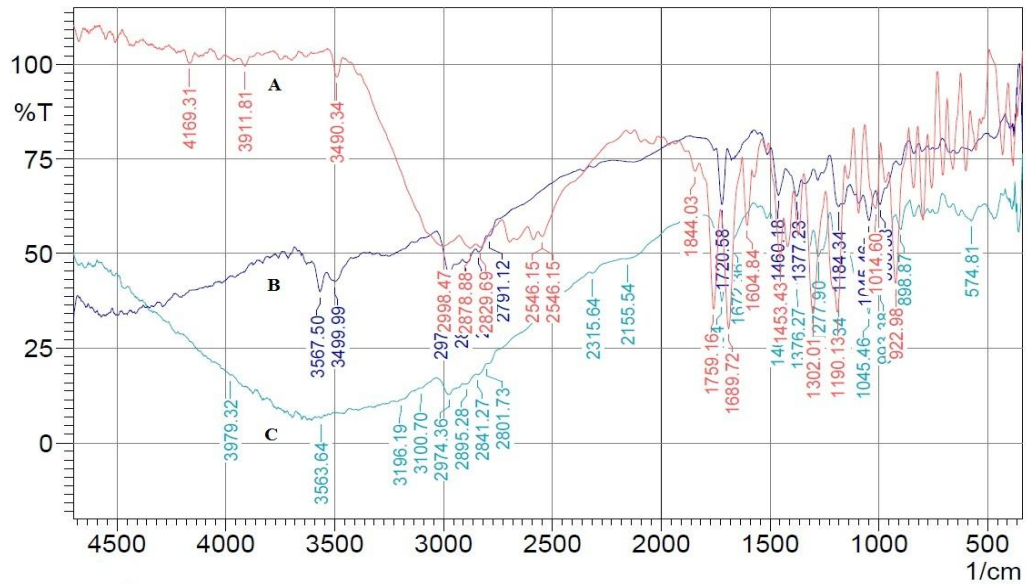


Figure 4: FTIR spectra of azithromycin (AZ) and mixtures of AZ with suspending agents

A- Azithromycin (AZ); B- AZ+ HPMC K4M); C- (AZ + POM)

Preparation of azithromycin suspensions

Eight suspension formulations were prepared using four concentrations (0.25 %, 0.5 %, 0.75 % and 1.0 %) of each of the two suspending agents i.e., HPMC K4 M and POM. Sodium benzoate was included as a preservative. The prepared suspensions were studied for sedimentation stability with the help of near infrared extinction profiles.

Determination of instability index and sedimentation velocity of suspensions

Separation analyser LUMiReader® PSA 453 was employed for stability determinations. LUM 10 mm, PC, synthetic sample cells were used for separation studies. Measurements were carried out at 0° tilt and 30 °C, 30° tilt and 30 °C and 30° tilt and 60 °C for the suspension samples. Tilting the sample from its normal upright position allows an increase in the separation rate at gravity without any additional external forces. The inclined position of the sample tube due to a tilt of 30° resulted in accelerated sedimentation velocity compared to the upright position of the tube at 0° tilt as evident from the results shown in Table 2, Table 3 and Table 4.

Table 2. LUMiReader® stability data for azithromycin suspensions at 0º tilt and 30 °C

Suspending agent	Instability index at suspending agent concentration of				Front tracking Sedimentation velocity (µm/s) at suspending agent concentration of				Extinction ratio (470 nm/870 nm) at suspending agent concentration of			
	0.25 %	0.5 %	0.75 %	1.0 %	0.25 %	0.5 %	0.75 %	1.0 %	0.25 %	0.5 %	0.75 %	1.0 %
HPMC K4M	0.0594	<0.0001	0.0039	0.4397	x	x	x	x	1.275	1.108	1.167	1.244
POM	0.0072	0.0046	0.0067	0.0002	x	x	x	x	1.105	1.113	1.181	1.166

Table 3. LUMiReader® stability data for azithromycin suspensions at 30º tilt and 30 °C

Suspending agent	Instability index at suspending agent concentration of				Front tracking Sedimentation velocity (µm/s) at suspending agent concentration of				Extinction ratio (470 nm/870 nm) at suspending agent concentration of			
	0.25 %	0.5 %	0.75 %	1.0 %	0.25 %	0.5 %	0.75 %	1.0 %	0.25 %	0.5 %	0.75 %	1.0 %
HPMC K4M	0.0275	0.0002	0.0130	0.2629	x	x	x	0.0749	1.195	1.103	1.198	1.191
POM	0.0033	0.0016	0.0010	0.0001	x	x	x	X	1.112	1.142	1.177	1.210

Table 4. LUMIReader stability data for azithromycin suspensions at 30 tilt and 60 ˚C

Suspending agent	Instability index at suspending agent concentration of				Front tracking Sedimentation velocity (µm/s) at suspending agent concentration of				Extinction ratio (470 nm/870 nm) at suspending agent concentration of			
	0.25 %	0.5 %	0.75 %	1.0 %	0.25 %	0.5 %	0.75 %	1.0 %	0.25 %	0.5 %	0.75 %	1.0 %
HPMC K4M	0.0657	0.1869	0.0190	0.1215	0.5806	9.325	x	131.4	1.352	1.204	1.207	1.185
POM	0.0072	<0.0001	0.0001	0.0005	x	x	24.56	x	1.105	1.075	1.112	1.183

A sample volume of 2 ml was used in the determinations. Measurements at 30° tilt resulted in accelerated measurements. Further, the measurements at higher temperatire of 60°C resulted in further acceleration as seen from the enhanced sedimentation rate of suspensions.

The extinction profiles were recorded at three wavelengths i.e., 470 nm (blue), 630 nm (red) and 870 nm (NIR) with the help of the software SEPView® installed in the instrument. The profiles were automatically saved in the instrument. The extinction profiles of 870 nm (near infrared) wavelength were taken into account for determination of sedimentation stability in the present work as the near infrared light region is sensitive for measurement of data of coarse particles. Blue light (470 nm) is sensitive for nano range particles.

The results shown in Table 3 indicate that the instability index ranged between 0.0001 to 0.0033 for suspensions with 0.25 % to 1.0 % POM as suspending agent. It was highest (0.2629) with formulation containing 0.5 % HPMCK4M. Similar observations were made with measurements made at 30° tilt and 60 °C as shown in Table 4. The instability index ranged between 0.0001 to 0.0072 for suspensions with 0.25 % to 1.0 % POM as suspending agent. It was the highest (0.1869) with formulation containing 0.5 % HPMCK4M. Instability index generally ranges between 0 to 1 and the higher this value, more unstable the suspension is. Therefore, Instability index is a very useful tool for comparison of different suspending agents and selection of suitable suspending agents during suspension formulation development. Basing on the results of instability index it is presumed that 0.5 to 1.0 % of POM is a better suspending agent for preparation of stable suspensions of azithromycin when compared to HPMCK4M.

Comparison of multi wavelength extinction ratios

Multi wavelength extinction ratio (like MWL Extinction ratio 470nm/870nm) measurement gives an idea about changes in particle size. Extinction ratios (470 nm/870 nm) were smaller in general for formulations containing POM, compared to those containing HPMC K4M as shown in Tables 3 and 4. This suggests that the differences in extinctions at 470 nm and 870 nm are small for the formulations containing POM resulting in lower extinction ratios for these formulations, indicating minimum changes in particle sizes in these suspensions. Thus, POM is considered a better suspending agent compared to HPMC K4M for azithromycin suspensions.

Determination of resuspendability of suspension samples

Resuspendability is the ability to resuspend settled particles with a minimum amount of shaking after a suspension has sedimented on standing for some time. The suspension should redisperse with minimum effort on shaking for ease of administration. It is an important prerequisite for a good and stable suspension. Results of resuspendability are given under Table 5.

Table 5. Results of resuspendability evaluation on azithromycin suspensions (n=3)

Sample	Suspending agent	% of suspending agent	Number of shakings required to get a uniform dispersion n=3	Remarks
F1S2	HPMCK4M	0.25	9.33	
F2S2	HPMCK4M	0.50	17.33	Caking +
F3S2	HPMCK4M	0.75	5.00	
F4S2	HPMCK4M	1.00	1.33	
F1S4	POM	0.25	1.33	
F2S4	POM	0.50	1.00	
F3S4	POM	0.75	1.00	
F4S4	POM	1.00	3.33	

Results shown in Table 5 indicate that the formulations containing POM were easily resuspendable compared to suspensions containing HPMCK4M as suspending agents, as they required less number of shakings for obtaining uniform dispersion.

Considering the results of instability index, sedimentation velocity, multi wavelength extinction ratio (470nm/870nm) and resuspendability of the suspensions it can be further inferred that 0.5 to 1.0 % of POM can be considered suitable as a suspending agent for preparation of stable suspensions of azithromycin. Further the FT-IR and DSC studies showed that the selected suspending agents were compatible with azithromycin. The experimental data indicates that POM is a better suspending agent than HPMCK4M for azithromycin suspensions.

Conclusions

From the results obtained from separation analysis of suspensions employing LUMiReader®, it can be concluded that the suspension formulations can be easily compared by the parameter “Instability index” because this parameter takes into account of all the properties of the suspension like sedimentation velocity, clarifying velocity, particle size distribution changes etc. Instability index ranges between 0 to 1 and the higher this value, more unstable the suspension is. Therefore, Instability index is a very useful tool for comparison of different suspending agents and selection of suitable suspending agents during suspension formulation development. *Plantago ovata* mucilage at a concentration of about 0. 5 to 1.0 % w/v can be considered as a suitable suspending agent in formulation of azithromycin suspensions.

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