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www.mcmed.us/journal/abs **Research Article**

DESIGN AND EVALUATION OF AZATHIOPRINE NANOPARTICLES

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ABSTRACT

Studies show nanoparticles containing a coat of PEG not only have a prolonged half-life in the blood compartment but also be able to selectively extravagates in pathological sites such as tumors or inflamed regions with a leaky vasculature. As a result, such long-circulating nanoparticles have increased the potential to directly target tumors located outside MPS-rich regions. The size of the colloidal carriers as well as their surface characteristics are the critical to the biological fate of nanoparticles. A size less than 100 nm and a hydrophilic surface are essential in achieving the reduction of opsonisation reactions and subsequent clearance by macrophages. Coating conventional nanoparticles with surfactants or PEG to obtain a long-circulating carrier has now been used as a standard strategy for drug targeting *in vivo*. Significant advances in biotechnology and biochemistry have led to the discovery of a large number of bioactive molecules and vaccines based on peptides and proteins. Development of suitable carriers remains a challenge due to the fact that bioavailability of these molecules is limited by the epithelial barriers of the gastrointestinal tract and their susceptibility to gastrointestinal degradation by digestive enzymes. Polymeric nanoparticles allow encapsulation of bioactive molecules and protect them against enzymatic and hydrolytic degradation. For instance, it has been found that insulin-loaded nanoparticles have preserved insulin activity and produced blood glucose reduction in diabetic rats for up to 14 days following the oral administration.

Keywords :- Evaluation, Azathioprine, Nanoparticles, Design.

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INTRODUCTION

Nanotechnology is the science that deals with matter at the scale of 1 billionth of a meter (i.e., 10^{-9}m = 1 nm), and is also the study of manipulating matter at the atomic and molecular scale [1]. A nanoparticle is the most fundamental component in the fabrication of a nanostructure, and is far smaller than the world of everyday objects that are described by Newton' s laws of motion, but bigger than an atom or a simple molecule that are governed by quantum mechanics. The United States instituted the National Nanotechnology Initiative (NNI) back in 2000, which was soon followed by a plethora of projects in nanotechnology in nearly most of the U.S. Departments and Agencies. About 20 Research Centers were subsequently funded by the Nationa1 Science Foundation (NSF), an agency responsible solely to the President of the United States and whose mandate is to fund the best of fundamental science and technology projects. NSF was the lead U.S. agency to carry forward the NNI. The word "nanotechnology" soon caught the attention of various media (TV networks, the internet, etc.) and the imagination and fascination of the community at large [2].

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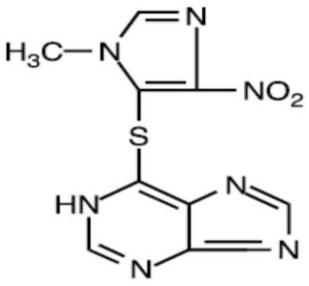
Azathioprine

Azathioprine belongs to the class of medicines called Immunosuppressive agents to suppress Immunity.

Molecular formula: $C_9H_7N_7O_2S$

Molecular weight: 277.263

Fig. 1. Structure of Azathioprine



Category: Immunosuppressive agents.

Description: It is a pale yellow color powder which has a purine anti-metabolite, 6-mercapto purine. 6-MP is further metabolized by hypoxanthine-guanine phosphorribosyl transferase (HGPRT) into 6-thioguanosine-5^1-phosphate (6-thio-GMP) and 6-thioinosine monophosphate(6-thio-IMP),both inhibit nucleotide conversions and de novo purine synthesis. This leads to inhibition of DNA, RNA and protein synthesis. As a result, cell proliferation may be inhibited, particularly in lymphocytes and leukocytes.

M.P: 238-245^{0}c

Solubility: Practically insoluble in water and in ethanol (96%). It is soluble in dilute solutions of alkali hydroxides and sparingly soluble in dilute mineral acids.

Indication: Used in combination with anticancer chemotherapy to maintain the immunity balance during the therapy

Pharmacodynamics

Azathioprine is a chemotherapy drug, now rarely used for chemotherapy but more for immunosuppression in organ transplantation and autoimmune disease such as rheumatoid arthritis or inflammatory bowel disease or crohn's disease. It is a pro drug converted in the body to the active metabolite 6-MP.

Pharmacokinetics

Absorption:

Well absorbed following oral administration.

Distribution: Azathioprine and its major metabolite mercaptopurine distribute throughout the body and appear to cross the placenta

Metabolism: Primarily converted into the active metabolites 6-mercaptopurine and 6-thioinosinic acid via a non-enzymatic process and glutathione transferases. Activation of 6-mercapto purine occurs via hypoxanthine-guanine phosphoribosyltransferase and a series of multi enzymatic processes involving kinases to form 6-thio guanine nucleotides (6-TGNs) as major metabolites [3].

Volume of distribution: Apparent volume of distributions of azathioprine is 1.54.

Protein binding:

Azathioprine and the metabolite mercaptopurine are moderately bound to serum proteins 20-30%.

Route of elimination:

Both compounds are rapidly eliminated from blood and are oxidized or methylated in erythrocytes and liver; no azathioprine or mercaptopurine is detectable in urine after 8 hours.

Half-life:

26-80 min (Azathioprine)

3-5 hours (Drug and its metabolites).

Toxicity:

The oral LD_{50} for single doses of azathioprine in mice and rats are 250 mg\kg and 400 mg/kg respectively. Very large doses of this antimetabolite may lead to marrow hypoplacia, bleeding, infection and death.

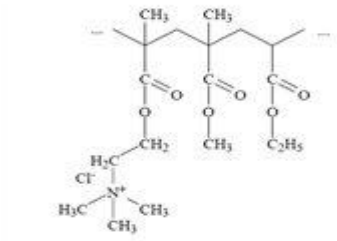
Polymer Profile

Eudragit RL 100

EUDRAGIT RL 100 is a copolymer of ethyl acrylate, methyl methacrylate and a low content of methacrylic acid ester with quaternary ammonium groups. The ammonium groups are present as salts and make the polymers permeable.

Physical properties: It is a solid substance in form of colorless, clear to cloudy granules with a faint amine-like odor.

Fig. 2. Structure of Eudragit RL 100



Weight average molar mass approximately: 32,000 g/mol

Alkali Value: 28. 1 mg KOH/ g polymer

Glass Transition Temperature (Tg): 63°C (+/- 5°C)

Product Form: Granules

Dissolution:

- Insoluble
- High permeability
- pH independent swelling

Characteristics:

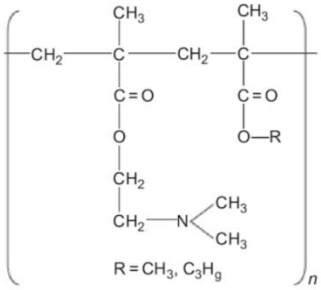
- Customized release profile by combination of RL and RS grades in different ratios
- Suitable for matrix structures

CAS number: 33434 – 24 – 1

Chemical/IUPAC name: Poly(ethyl acrylate-co-methyl methacrylate-co-trimethyl-ammonioethyl methacrylate chloride) 1:2:0.2
INCI name: Acrylates / Ammonium Methacrylate Copolymer
Monographs:
Ph. Eur.: Ammonio Methacrylate Copolymer, Type A
USP/NF: Ammonio Methacrylate Copolymer, Type A - NF
JPE: Aminoalkyl Methacrylate Copolymer RS

Eudragit E100
Eudragit E100 is cationic polymer based on dimethylaminoethyl methaacrylate, butyl methacrylate, and methyl methacrylate.
Physical properties: It consists of colorless to yellow tinged granules with characteristic amine-like odor.

Fig. 3. Structure of Eudragit E100



Weight average molar mass approximately: 399.528grams/mole
Alkali value: 180 mg KOH/g of polymer
Glass transition temperature [Tg]:48^0c
Product form: Powder
Dissolution:

- Particle size reduction
- Surface area enhancement

Characteristics: low viscosity, high pigment binding capacity, good adhesion.
Low polymer weight gain.
CAS number: 24938-16-7
Chemical\IUPAC name: Poly (butyl methacrylate-co-(2-dimethyl amino ethyl) methacrylate-co-methyl methacrylate) 1:2:1
INCI name: Acrylates/dimethylaminoethyl methacrylate copolymer

Excipient Profile
Tween 80

Chemical Name:	Tween 80
Synonyms:	Tween;crill11;crill10;monitan;sorlate;tween81;durfax80
Molecular Formula:	$C_{24}H_{44}O_6$
Formula Weight:	428.600006103516
Tween 80 Property:	
Boiling point:	$>100^0$c
Density :	1.08 g/mL at 20 °C
Storage temp. :	Store at RT.
Vapor pressure :	<1 mm Hg (20 °C)
Form :	viscous liquid
Water solubility:	5-10 g/100 mL at 23 ºC
Chemical properties:	yellow to amber liquid

General Description
Amber-colored viscous liquid. pH (5% aqueous solution) 5-7. Faint odor and bitter taste.
Air & Water Reactions: Water soluble.
Applications
Lonzest(R) SMO-20 is used as an emulsifier for textiles, personal care and industrial applications. Lonzest(R) SMO-5 is a general purpose mid-range HLB, ethoxylated, nonionic surfactant suggested for use in textile chemicals (emulsifier, lubricant), household products and cosmetic formulations (o/w emulsifier, viscosity modifier). Glycolube(R) AFA-1 is used as an antistat for PVC and as an antifog for PP, PE, PVC, PS.

MATERIALS AND METHODS

Table 1. List of Materials and their sources

S.No.	Materials	Sources
1	Azathioprine	John rakirs
2	Eudragit E 100	Degussa
3	Eudragit RL 100	Degussa
4	Ethanol	Honyon
5	Tween 80	SDFCL
6	Glutaraldehyde	MERCK

Table 2. List of equipments and their sources

S.No.	Equipments	Make/model
1	Magnetic stirrer	REMI
2	Balance	INFRA
3	U.V-Visible spectrophotometer	Ageielent-1200
4	FT-IR Spectrometer	Ageielent cary-680
5	Scanning electronic microscopy(SEM)	INFRA
6	Homogenizer	REMI

Preformulation Studies
Preformulation may be described as an initial stage of formulation development during which the physicochemical and biopharmaceutical properties of a drug substance are characterized [4]. It is an important part of the drug development process. The information relating to drug development acquired during this phase is used for making critical decisions in subsequent stages of development. A wide variety of information must be generated to develop formulations rationally.

Characterization of the drug is a very important step at the preformulation phase of product development followed by studying the properties of the excipients and their compatibility. Preformulation testing is the first step in the development of dosage forms of a drug substance. It can be defined as an investigation of physical and chemical properties of a drug substance alone and when combined with excipients [5].

The overall objective of Preformulation studies is to generate information useful to the formulator in developing stable and bioavailable dosage forms, which can be mass produce.

Preformulation study can divided into two subclasses

1) Preformulation studies with physicochemical parameters:-

Active pharmaceutical ingredient (API) characterization

a) Organoleptic Evaluation:

These are preliminary characteristics of any substance, which is useful in identification of specific material. Following physical properties of API were studied

- Color
- Taste
- Odor

Determination of Solubility

b) Solubility of API (Model Drug)

The solubility was determined using a method specified in the USP. The solubility profile of the investigational drug was important to determine a proper analytical method. There are two methods that are specified following the same dissolving procedures of the active substances [6].

Dissolving procedure

The substance is added to the solvent and shaken vigorously for 1 minute and placed in a constant temperature device. The temperature was maintained at 25±0.5ºC for 15 minutes [7]. If the substance is not completely dissolved, the stirring or shaking is done for 1 minute and the solution is placed in the constant temperature device for period of another 15 minutes, and changes in solubility are observed under the two methods.

Method 1:

For this test, a maximum of 1gram of substance for each solvent and solvent quantity of30ml was taken necessarily.

- **Very soluble** -100mg of finely divided powdered substance in a stopper tube (16mm in internal diameter and 160mm long). 0.1ml of solvent is added and preceded under dissolving procedure. The substance should be completely dissolved.
- **Freely Soluble** – Further 9ml of solvent is added and proceeded under dissolving procedure for complete solubility.
- **Soluble** – Further 2ml of solvent is added and proceeded as under dissolving procedure for complete solubility.
- **Sparingly soluble** – Additional 7.0ml is added and proceeded under dissolving procedure for complete solubility.
- **Slightly soluble** – 10mg of finely powdered substance is taken in a stopper tube, and10ml of solvent is added and preceded under dissolving procedure for complete solubility.
- **Poorly/Very slightly soluble** - 1mg of finely powdered substance in a stopper tube and 10ml of the solvent is added and proceeded under dissolving procedure. The Substance should be completely soluble.

Method 2:

For this test about 1 g of substance is taken and dissolved in approximate volume of solvent in milliliters following dissolving procedures. The volume of solvent taken ranging from less than 1ml to 10,000ml or 10 liters specifies the solubility range of the active drug by given specifications. In the present study, method 1 was followed for obtaining clear variable ranges of Solubility of the investigational drug.

C) FTIR Studies

It is one of the most powerful analytical techniques for chemical identification of drug.

Method: The pure drug and its formulation were subjected to FTIR studies. In the present study, the potassium bromide disc (pellet) method was employed.

2) Compatibility Study

The compatibility of drug and formulation components is important prerequisite before formulation. It is therefore necessary to confirm that the drug does not react with the polymers and excipients under experimental conditions and affect the shelf life of product or any other unwanted effects on the formulation.

Preparation of standard calibration curve

A) Determination of absorption maxima (λ_{max})

A solution of Azathioprine with concentration of 30µg/ml was prepared in 0.1N Hydrochloric acid. The solution was scanned in the range of 200-400 nm using shimadzu 1601-Double beam UV/Visible spectrophotometer.

B) Standard graph of Azathioprine in pH 6.8 phosphate buffer (SS-1)

100mg of pure Azathioprine was accurately weighed and dissolved in 50 ml of P^H 6.8 phosphate buffer in 100ml volumetric flask and the volume was made up to the mark using 6.8 phosphate buffer, to make (1000µg/ml) standard stock solution.

(SS-2): from the stock solution (SS-1), 5ml sample was withdrawn and transferred into 50ml volumetric flask and diluted with 6.8 phosphate buffer to make 100µg/ml stock solution2.

Standard: From the stock solution2 aliquots were taken and diluted to obtain different concentrations of 0, 5, 10, 15, 20,25, 30and 35µg/ml and absorbance was measured at λ_{max} of 281 nm against pH 6.8 phosphate buffer as blank.

Formulation of Azathioprine Nanoparticles

Nanoparticles (nanospheres) were prepared by nano-precipitation according to the method developed by Fessi and colleagues. Eudragit E 100 was dissolved in ethanol then Azathioprine was added and dissolved. The organic solution was injected at a rate of 48ml/min in distilled water containing Tween 80 and glutaraldehyde under homogenizer at room temperature. Ethanol and some proportion of water were eliminated under reduced pressure. The final nanosuspension was used for further characterization.. A placebo nanosupension (without drug) was prepared for comparison studies. Formulation optimization was pursued to obtain nanoparticles of desired physical properties. Effect of various drug polymer ratios from 1:1, 1:2, 1:3, 1:4 and 1:5 and the stabilizer concentration 1%w/v were assessed on drug encapsulation efficiency and particle Size.

By follow the above mentioned procedure five other batches of nanoparticles in the ratio of 1:1, 1:2, 1:3, 1:4 and 1:5 were prepared and named F1, F2, F3, F4 and F5 respectively. Similarly above mention procedure was applied to polymer of Eudragit E100for the preparations of Azathioprine nanoparticles.

Evaluation of Nanoparticles

Evaluation was performed to assess the physicochemical properties and release characteristics of the developed formulations [8].

1. Particle size

The surface morphology (roundness, smoothness, and formation of aggregates) and particle size were studied by scanning electron microscopy (SEM).

2. Drug recovery

3. Nanoparticle yield

$$\text{Percentage yield} = \frac{\text{Actual weight of product}}{\text{Total weight of excipient\&drug}} \times 100$$

4. Drug entrapment efficiency

$$\text{Drug entrapment \%} = \frac{\text{Mass of drug in Nanoparticles}}{\text{Mass of drug used in formulation}} \times 100$$

5. Drug content

Drug content was determined by centrifugation method. The redispersed nanoparticles suspension was centrifuged at 15,000 rpm for 40 min at 25°C to separate the free drug in the supernatant. Concentration of Azathioprine in the supernatant was determined by UV-Visible spectrophotometrically at 281 nm after suitable dilution.

6. *In vitro* release studies

In vitro release studies were carried out by using dialysis tubes with an artificial membrane [9]. The prepared Azathioprine nanoparticles and 10 ml of phosphate buffer pH 7.4 was added to the dialysis tube and subjected to dialysis by immersing the dialysis tube to the receptor compartment containing 250 ml of phosphate buffer pH 6.8.The medium in the receptor was agitated continuously using a magnetic stirrer a temperature was maintained at 37±1°C. 5ml of sample of receptor compartment were taken at various intervals of time over a period of 8 h and each time fresh buffer was replaced. The amount of drug released was determined spectrometrically at 281 nm.

7. Kinetic analysis of *in-vitro* release rates of Azathioprine nanoparticles

The results of *in-vitro* release profile obtained for all formulations were plotted in modes of data treatment as follows,

- Zero order kinetic model- cumulative % drugs released versus T.
- First order kinetic model – Log cumulative % drug retained versus T.
- Higuchi's model - cumulative % drug released versus square root of T.
- Korsemeyer equation or peppa's model - Log cumulative % drug released versus log T.

a. Zero order kinetics:

It describes the system in which the drug release rate in independent of its concentration.

$$\mathbf{C = C_0 - K_0\, t}$$

Where,

C = Concentration of drug to undergo reaction at time t

C_0= Initial amount of drug in the solution, which is often Zero and

K_0= Zero order release constant.

If the zero order drug release kinetic is obeyed, then a plot of C Vs t will give a straight line with a slope of K_0 and an intercept at zero.

b. First order kinetics

It describes the drug release from the systems in which the release rate is concentration dependent.

$$\mathbf{LogC = logC_0 - kt/2.303}$$

C= Amount of drug released in time t.

C_0= Initial amount of drug in the solution

K = First order release constant

If the first order drug release kinetic is obeyed, then a plot of log (C_0-C) vs t will give a straight line with a slope of k/2.303 and an intercept at t=0 of log Co.

c. Higuchi model

It describes the fraction of drug release from a matrix is proportional to square root of time.

$$M_t/M\infty = KHt^{1/2}$$

Where,

$M_t/M\infty$ is cumulative amounts of drug release at time t,

KH= Higuchi dissolution constant reflection formulation characteristics.

If the Higuchi model of drug release (i.e.Fickian diffusion) is obeyed, then plot of $M_t/M\infty$ versus$t^{1/2}$will be straight line with slope of KH.

d.Korsemeyer- peppas model (power Law)

The power law describes the drug release from the polymeric system in which release deviates from Fickian diffusion, as expressed in following equation.

$$M_t/M\infty = K\,t^n$$

$$Log[M_t/M\infty] = \log k + n \log t$$

Where,

M_t and $M\infty$ are cumulative amounts of drug release at time t and infinite time (i.e. fraction of drug release at time t),

K = Constant incorporating structural and geometrical characteristics of CR devices.

n = diffusional release exponent indicative of the mechanism of drug dissolution.

To characterize the release mechanism, the dissolution data{$M_t/M\infty$ <0.6} are evaluated. A plot of log {$M_t/M\infty$ } versus log t will be linear with slope of n and intercept gives the value of log k. Antilog of log K gives the value of K. the value of release exponent changes with change in the geometry of nanoparticles.

- In general if the exponent value n is 0.5, the release rate is termed "fickian"or square root of time dependent. Release is rapid at first,and then tailing off over time until 100% of the drug is released. In this type of release, the dominant mechanism for release is diffusion.
- If n is between 0.45<n=0.89, the release rate is described as "Non-fickian", or "anamolous". Release is rapid at first, although slower than the fickian release rate, again tails off over time.
- If n=0.89,"case II transport" has been achieved.

8. Stability studies

Stability of a drug can be defined as the time from the date of manufacture and the packaging of the formulation, until its chemical or biological activity is Not less than a predetermined level of labeled potency and its physical characteristics have Not change deleteriously [10]. The International conference on Harmonization (ICH) guidelines titled "Stability testing of New Drug substance and products"(QIA) describes the stability test requirements for drug registration applications in the European Union, Japan and the United States of America. ICH specifies the length of study and storage conditions. Long –Term Testing: 25^0 C±2^0C/60% RH ±5% for 12 months [11]. Accelerated testing: 40^0 C±2^0C/75% RH ±5% for 6 months. Stability studies were carried out at 25^0C, 30^0C and 45^0C for the selected formulation for three months.

Method

The stability study was carried out all formulations. Azathioprine containing nanoparticles were stored atelevated temperature (25±2^0C, 30±2^0C, 40±2^0C) over a period of time. Samples were kept for 21days for stability analysis and after 21days, drug release of nanoparticle was compared. The stability of drug loaded nanoparticles was evaluated in terms of its drug content. The stability of nanoparticles was evaluated in PBS (pH 6.8.) After specified time intervals, the suspension was centrifuged at 15,000 rpm for 40 min, supernatant was removed and nanoparticles were dissolved in dichloromethane. After adding of water and separation, the amount of drug was detected by UV-Visible spectrophoto metrically method at 281 nm.

Table 3. Formulation of Azathioprine nanoparticles with Eudragit RL 100 by using nano-precipitation method

Ingredients(mg)	F_1	F_2	F_3	F_4	F_5
Azathioprine	1	1	1	1	1
Eudragit RL 100	1	2	3	4	5
Gluturaldehyde	1	1	1	1	1
Ethanol(ml)	5	5	5	5	5
Tween 80(ml)	10	10	10	10	10

Table 4. Formulation of Azathioprine nanoparticles with Eudragit E 100 by using nano-precipitation method

Ingredients(mg)	F_1	F_2	F_3	F_4	F_5
Azathioprine	1	1	1	1	1
Eudragit E 100	1	2	3	4	5
Gluturaldehyde	1	1	1	1	1
Ethanol(ml)	5	5	5	5	5
Tween 80(ml)	10	10	10	10	10

RESULT AND DISCUSSION

Table 5. Standard calibration curve of Azathioprine at 281 nm in p^H6.8 phosphate buffer

S.No.	Concentration(µg/ml)	Absorbance(nm)
1	0	0
2	5	0.337
3	10	0.674
4	15	1.012
5	20	1.349
6	25	1.686
7	30	2.023
8	35	2.361

Table 6. Evaluation parameters of all formulations

Formulation code	Drug polymer ratio	Drug content (%)	Particle size(nm)	Entrapment efficiency (%)	Drug recovery (%)	Percentage yield (%)
AERL F1	1:1	84	543±6.4	62±0.82	85	76.12
AERL F2	1:2	81	510±3.2	65±0.51	80	79.23
AERL F3	1:3	76	584±1.09	68±0.24	77	81.49
AERL F4	1:4	73	624±8.12	73±0.19	74	84.64
AERL F5	1:5	70	651±1.32	82±0.42	70	86.28
AEE F1	1:1	83	432±5.04	70±0.23	84	72.12
AEE F2	1:2	80	473±4.2	69±0.56	82	73.07
AEE F3	1:3	78	502±8.9	73±0.58	79	75.31
AEE F4	1:4	74	540±10.5	76±0.42	75	80.42
AEE F5	1:5	71	568±10.7	81±0.36	72	81.08

Table 7. *In vitro* drug release profile for AERL

Time(hr)	% Drug Release				
	F1(1P:1D)	F2(2P:1D)	F3(3P:1D)	F4(4P:1D)	F5(5P:1D)
0	0	0	0	0	0
5	4.3	3.8	3.2	2.7	2.2
10	8.6	7.6	6.4	5.4	4.4
15	20.2	18	14	10	8
30	23.2	21.2	18.2	16.1	13.2
45	36	33	28	26	22
1:00	47	43	39	33	28
1:30	48	44	41	35	32
2:00	50	46	42	38	34
2:30	59	55	48	45	39
3:00	64	61	56	52	48
3:30	68	65	59	56	52
4:00	70	67	62	59	56
5:00	79	76	71	69	65
6:00	84	81	78	76	72
7:00	89	87	85	83	81
8:00	95	91	87	85	84

Table 8. *In vitro* drug release profile for AEE

Time(hr)	% Drug Release				
	F1(1P:1D)	F2(2P:1D)	F3(3P:1D)	F4(4P:1D)	F5(5P:1D)
0	0	0	0	0	0
5	2.5	2.2	1.9	1.6	1.5
10	5	4.4	3.8	3.2	2.0
15	12	8.9	8.5	8.0	6.1

30	24	17.8	17.4	16	12.2
45	32	26	25	22	18.3
1:00	39	34	31	26	24
1:30	42	37	34	29	28
2:00	45	40	37	35	32
2:30	51	46	44	40	37
3:00	57	52	49	46	43
3:30	60	57	53	49	46
4:00	63	60	58	55	52
5:00	75	72	69	66	61
6:00	82	79	76	73	69
7:00	86	84	81	78	74
8:00	90	87	85	83	79

***average of three values**

Table 9. Pharmacokinetic studies

Time in hours	√T	Log T	Cumulative % drug release	Log Cumulative % drug release	Cumulative % drug remain	Log cumulative % drug remain
0	0	0	0	0	100	2
1	1.0	0	28	1.4471	72	1.8573
2	1.414	0.301	34	1.5314	66	1.8195
3	1.732	0.477	48	1.6812	52	1.7160
4	2.0	0.602	56	1.7481	44	1.6434
5	2.236	0.698	65	1.8129	35	1.5440
6	2.449	0.778	72	1.8573	28	1.4471
7	2.645	0.845	81	1.9084	19	1.2787
8	2.828	0.903	84	1.9242	16	1.2041

Table 10. Kinetic values obtained from AERLF5 plot formulation

Formulation	Zero order r^2	First order r^2	Higuchi r^2	Korsemeyer peppas slope'n'
AERLF5	**0.985**	**0.982**	**0.984**	**0.814**

Table 11. Stability studies for AERL F5

Sl. No	25^{O}C	30^{O}C	45^{O}C
Initial	No change	No change	No change
Iweek	No change	No change	No change
II week	No change	No change	No change
III week	No change	No change	Slightly change

Fig. 4. Standard calibration curve of Azathioprine

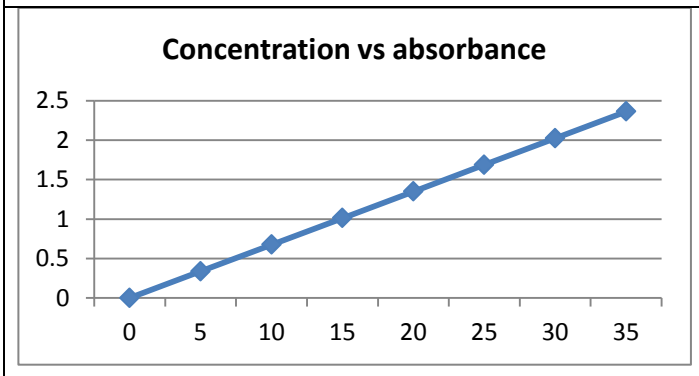


Fig 5. Typical SEM image of Optimized Formulation AERL F1

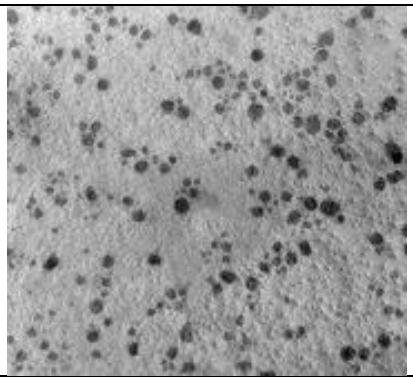


Fig. 6. Typical SEM image of Optimized Formulation AERL F2

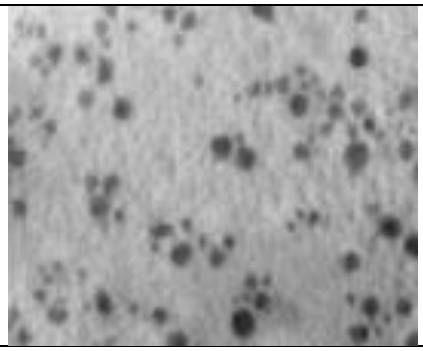


Fig. 7. Typical SEM image of Optimized Formulation AERL F3

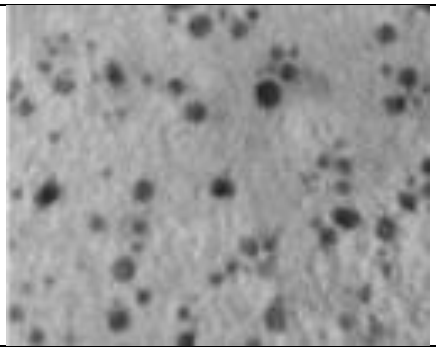


Fig. 8. Typical SEM image of Optimized Formulation AERL F4

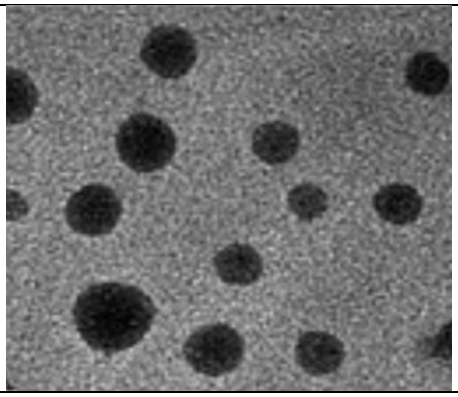


Fig. 9. Typical SEM image of Optimized Formulation AERL F5

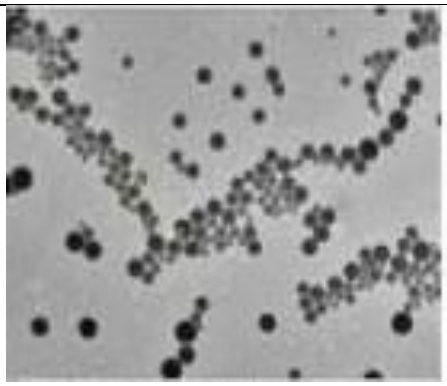


Fig. 10. Typical SEM image of Optimized Formulation AEE F4

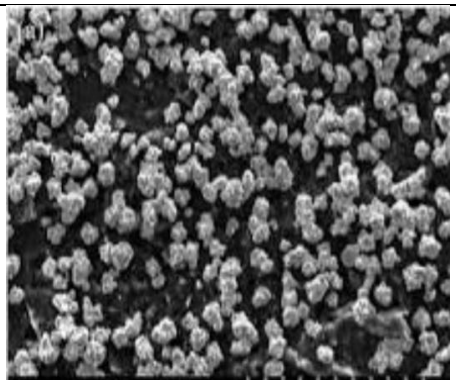


Fig. 11. Typical SEM image of Optimized Formulation AEE F5

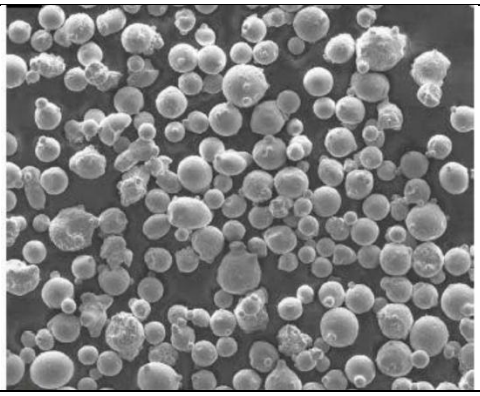


Fig. 12. Drug release profile of formulation AERL

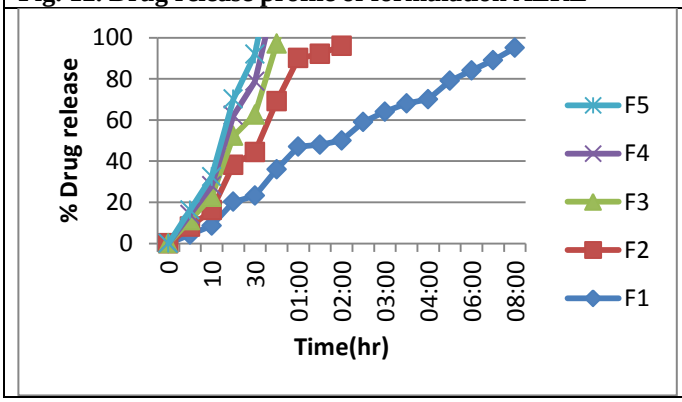


Fig. 13. First order plot

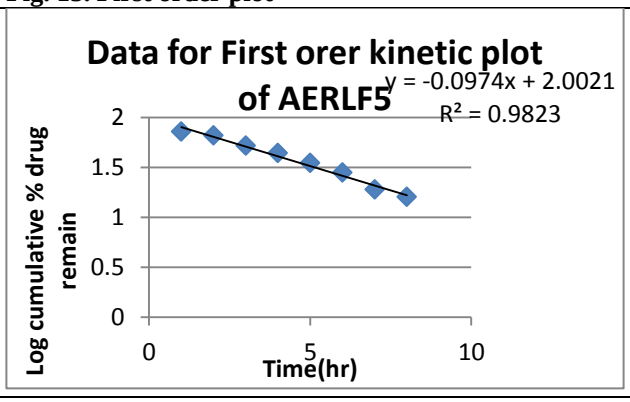


Fig. 14. Higuchi plot

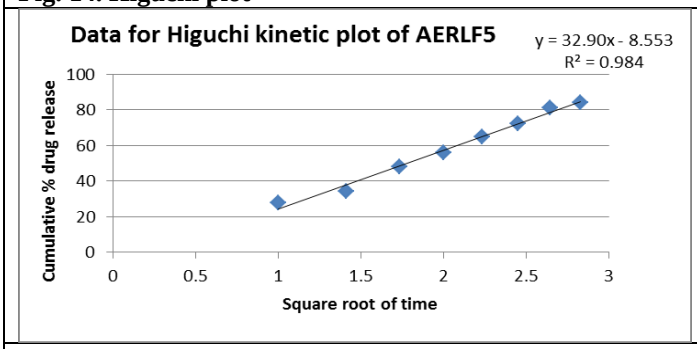


Fig. 15. Korsmeyer peppas plot

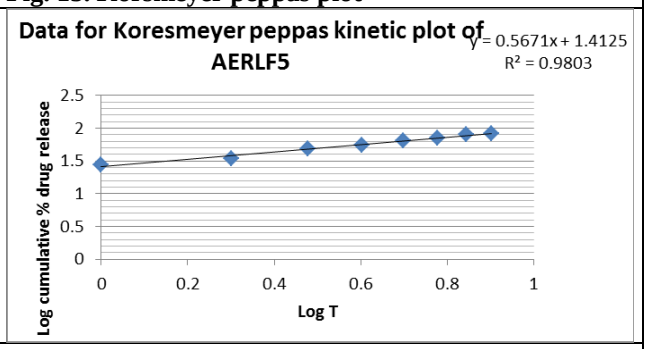


Fig. 16. FTIR spectrum of Azathioprine pure drug

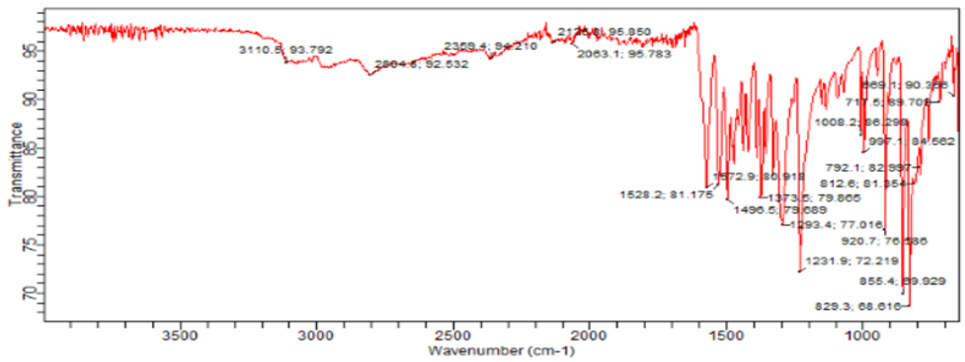


Fig. 17. FTIR spectrum of Eudragit RL 100

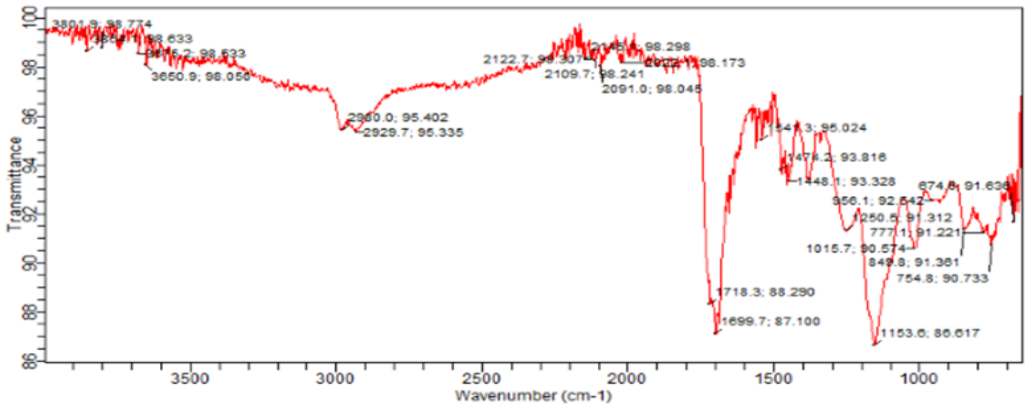


Fig. 18. FTIR spectrum of Eudragit E 100

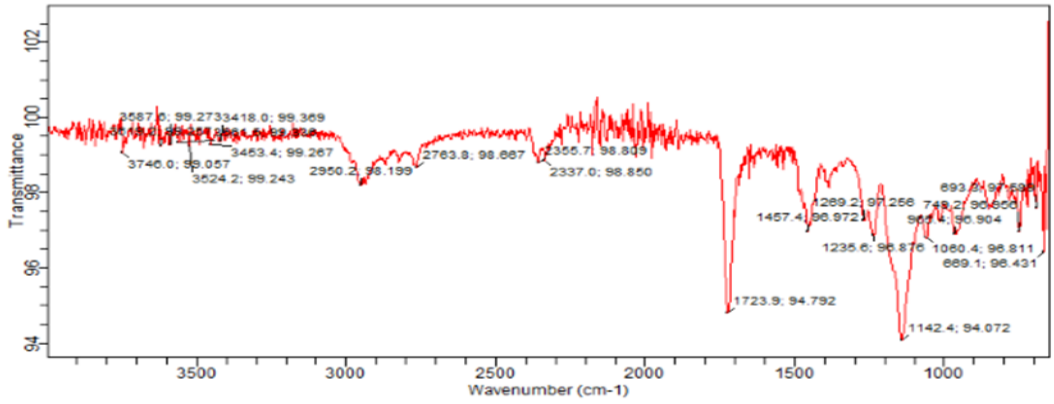


Fig. 19. FTIR spectrum of Azathioprine and Eudragit RL 100

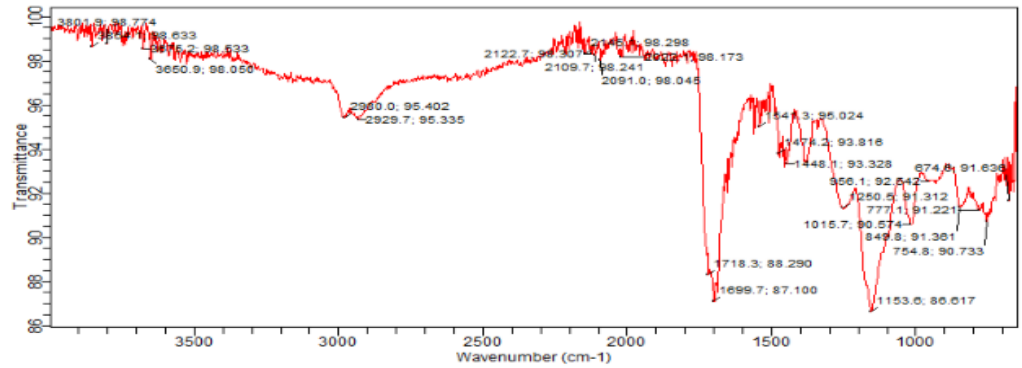
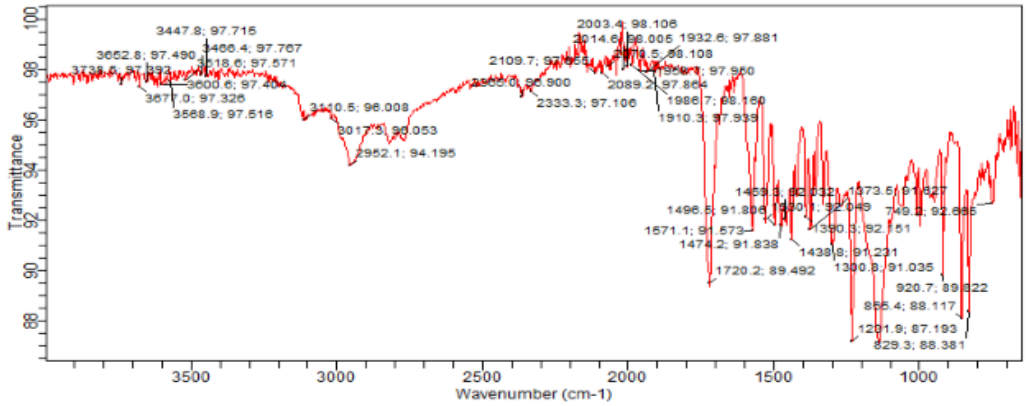


Fig. 20. FTIR spectrum of Azathioprine and Eudragit E 100



DISCUSSION

Solubility

The drug Azathioprine was soluble in dilute solutions of alkali hydroxides.

Standard calibration curve of Azathioprine

The standard calibration curve of drug solution in UV region (200-400nm) was done to find out the wavelength of maximum absorption (λ_{max}). The λ_{max} was found to be at 281 nm. The standard calibration curve of Azathioprine was developed at this wave length 281 nm, was obtained by plotting Absorbance vs. concentration. Table 5 and figure 4 shows the absorbance values of Azathioprine. The standard calibration curve shows the correlation coefficient of 0.9998. The curve was found to be linear in the concentration range of 5-35µg/ml at 281 nm. The calculations of drug content, in vitro drug release studies are based on this calibration curve.

FTIR studies

Compatibility studies were performed using FT-IR spectrophotometer. The IR spectrum of blend of pure drug and polymer were studied. The characteristic peaks of Azathioprine were obtained at different wave numbers in different samples. The peaks obtained in the spectra of each sample of drug and polymer correlates with the peaks of drug spectrum. This indicates that the drug is compatible with the formulation components. The spectra for drug and polymers were shown in figure 16 to 20. All the bands associated with the drug in the spectrum. There is no chemical interaction between drug and polymers. Azathioprine was compatible with polymers used in the formulation and there were no extra peaks observed.

Evaluation parameters of Azathioprine nanoparticles

Drug content

The content uniformity was performed for all formulations and results are tabulated in table 6. Three trials were performed for batches which were analyzed spectrophotometrically. The mean values and standard deviations of all the formulations were calculated. The percentage drug content of the tables was found to be between 84% to 70%ofAERL and 83% to 71% of AEE. The results were within the range and that indicated content uniformity of drug in all formulations.

Particle size

The particle size was performed for all formulations and results are tabulated in table 6. Three trials were performed for batches which were analyzed. The mean values and standard deviations of all the formulations were calculated. The particle size of the tables was found to be between 472±6.4 to 651±1.32 of AERL and 432±5.04 to 568±10.7 of AEE. The results

were within the range and that indicated of drug particle size in all formulations.

Entrapment efficiency

Entrapment efficiency performed for all formulations and results are tabulated in table 6. Three trials were performed for batches which were analyzed. The values of all the formulations were calculated. The percentage entrapment efficiency of the tables was found to be between 62±0.82 to 75±0.42of AERL and 70±0.23 to 81±0.36 of AEE. The results were within the range and that indicated of drug Entrapment efficiency in all formulations.

Drug recovery

Drug recovery performed for all formulations and results are tabulated in table 6. Three trials were performed for batches which were analyzed. The values of all the formulations were calculated. The percentage drug recovery of the tables was found to be between 83% to70 of AERLand84% to 72% of AEE. The results were within the range and that indicated of drug recovery in all formulations.

Percentage yield

Percentage yield calculated for all formulations and results are tabulated in table 6. The values of all the formulations were calculated. The percentage yield of the drug was found to be between 76.12% to 86.28% of AERL and 72.12% to81.08% of AEE. The results were within the range and that indicated percentage of drug in all formulations.

***In vitro* dissolution study**

The *in-vitro* drug release profiles of nanoparticles from each batch were shown in table 7, 8. The drug releases from the nanoparticles were studied by dialysis method. The cumulative percentage release of Azathioprine from Eudragit RL100 nanoparticles varied from 95% to 84% depends on the drug polymer ratio for 8 hr. The cumulative percentage release of Azathioprine from Eudragit E 100 nanoparticles varied from 90%to 79% depends on the polymer drug ratio for 8 hr.

From the *in-vitro* dissolution data it was found that formulation AERL F1 released 95% of drug in 8hr of the study indicating that the polymer amount is sufficient to control the drug release. Out of two polymer formulations Eudragit RL 100 showed better control over drug release indicating that the release was decreased when the concentration of the polymer was increased.

Kinetic studies

Release Kinetics

In case of zero order (C = C0 – k0t) the graph was plotted in cumulative percent of drug release Vs time, and in First order release kinetics (In C = C0–K1t). The graph was plotted in log cumulative percent of drug retained Vs time. For Higuchi model kinetics(C= K2 t1/2) the graph was plotted in cumulative percent drug released Vs square root of time. Korsmeyer-Peppas model (C/C0 = K tn) the graph was plotted in log cumulative percent of drug released Vs log time.

Zero order kinetic model

Here the graph is plotted between cumulative percent drug Vs time. The regression coefficient value of zero order kinetic plot was found to be 0.985.

First order kinetics plot

Here the graph is plotted between log cumulative percent drug remaining Vs time. Regression coefficient is calculated and interpreted. The regression coefficient value of zero order kinetic plot was found to be 0.982 (figure 13).

Higuchi model

In this model, graph is plotted between cumulative percent drug released Vs square root of time. Regression coefficient and slope values are calculated and interpreted. The regression coefficient value of zero order kinetic plot was found to be0.987 (figure 14).

Koresmeyer-peppas model

In this model, graph is plotted between log cumulative percent drug released Vs log time. Regression coefficient and slope values are calculated and interpreted. The regression coefficient value of zero order kinetic plot was found to be 0.985(figure 15).

The release kinetics of the formulation AERLF5 was calculated using zero order, First order Higuchi model and korsemeyer-peppas model. To confirm the exact mechanism of drug release the dissolution data was then fitted to korsemeyer-peppas model. The kinetic values obtained for optimized formulation are tabulated in table 9. And the kinetic plots were shown in fig. 13 to 15.

Korsemeyer-peppas model indicates that release mechanism is not well known and more than one type of release phenomena could be involved. The ‘‘n’’ value could be used to characterize different release mechanisms. The results are reported in table 9. The ‘‘n’’ value is 0.814. So it was concluded that the release occurred via Non-Fickian diffusion.

Stability study

The stability studies of the all formulation were carried out by storing in different storage conditions of (25 ± 2^0C, 30 ± 2^0C, 40 ± 2^0C) for a period of 3 weeks.

Table 11 shows the stability study of Azathioprine nanoparticles with two polymers. There is no significant change observed in the nanoparticles of all formulations. The formulations are in acceptable characteristics.

The promising formulations were subjected to short term stability study by storing the formulation at 25^oC, 30^oC 40^oC up to the three weeks. After three weeks the nanoparticles were again analyzed for particle size, drug content, entrapment efficiency and percentage drug release. At 25^oCand at 30°Cn there is no significant change in drug release. Butat 40^oC, slight change was there in the last week.

CONCLUSION

In the present study novel drug delivery system of Azathioprine nanoparticles were successfully developed and to improve the local action and ultimately its bioavailability. The nanoparticles were formulated using different polymers (Eudragit RL100, Eudragit E100) and surfactant (Tween 80). IR spectra studies revealed that the drug and the polymers used were compatible. The evaluation parameters like drug content, drug recovery, particle size, entrapment efficiency, nanoparticle and percentage yield were within the limits for various batches formulated. *In vitro* dissolution of batch AERLF5 containing Eudragit RL100 showed good drug release rate in comparison to remaining batches containing Eudragit E 100. Formulations subjected to curve fitting analysis shows Zero order drug release and followed Non-Fickian diffusion mechanism. Stability studies showed satisfactory results. From the entrapment efficiency, process yield and *In Vitro* dissolution studies it can be concluded that the formulation AERLF5 is the good one i.e., the formulation containing high concentration of Eudragit RL100 showed that the release decreases as the concentration of the polymer increases.

The major problem in oral drug formulations is low bioavailability which mainly results from poor aqueous solubility. The method of preparation of nanoparticles of Azathioprine was found to be simple and reproducible. The slow and constant release of Azathioprine from nanoparticles maintain constant drug plasma concentration thereby increasing therapeutic efficacy. The developed formulation overcome and alleviates the drawbacks and limitations of Azathioprine sustained release formulations. As a result of this study it may be concluded that the Azathioprine nanoparticles containing Eudragit RL 100 can be used to increase the sustain release. The concept of formulating nanoparticles of Azathioprine offers a suitable and practical approach in serving desired objectives of nanoparticles.

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