

Comparative bioavailability evaluation of Azathioprine after oral and core in cup tablets administration in rabbits

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ABSTRACT

Azathioprine is the drug of choice for treatment of active inflammatory bowel disease (IBD). Core-in-cup tablets have been developed based on combination of hydrophobic polymers and a gelling hydrophilic polymer, microcrystalline cellulose, to achieve a prolonged release formulation of Azathioprine tablets using Cellulose acetate phthalate as coating polymer to produce a delivery system in which the release of drug is modulated. The objective of this study was to investigate differences in the pharmacokinetic patterns between an optimized core in cup tablet formulation and pure drug of Azathioprine. The formulations were administered to 2 groups of white New Zealand rabbits (n=6) following cross over design pattern and the plasma levels were measured using LC-MS/MS method. Pharmacokinetic parameters were determined for each formulation. The comparison of the plasma time curves of the dosage forms showed that each dosage form caused significant differences in the drug plasma levels. The optimized core in cup tablet formulation showed some lag phase initially before releasing the drug. The mean residence time of core in cup tablet formulation (21.59 ± 0.036 hrs) was found to be more than pure drug of Azathioprine (2.66 ± 0.02 hrs). Core in cup tablet formulation alleviating the conditions of experimental model of colitis, if the time of administration and pulse time are adjusted to the circadian pattern. From the above results, it can be concluded that the prepared core in cup tablet can be considered as one of the promising formulation technique for chronotherapeutic management of inflammatory bowel disease.

KEYWORDS: LC-MS/MS, Azathioprine, core in cup tablet, In-vivo studies

I. INTRODUCTION

Azathioprine is widely used as an immunosuppressant and drug of choice in the treatment of inflammatory bowel disease, especially in the treatment of ulcerative colitis and Crohn's disease.¹ The bioavailability of Azathioprine upon oral administration is limited to an extent of 41-50%. Inflammatory bowel diseases can be treated more effectively by local delivery of drug targeted to the colon. Colonic drug delivery is also useful for enhanced systemic absorption of drugs because of less hostile environment existing in the colon compared to stomach and small intestine. Azathioprine undergoes approximately 50% first pass metabolism.² To overcome this drawback, the present study was undertaken to investigate the colon targeted drug delivery system of Azathioprine through core in cup tablet.³ Due to the distal location of the colon in the gastrointestinal tract, pulsatile drug delivery should prevent drug release in the stomach and small intestine and produce a gradual onset of drug release upon entry into the colon.⁴ Hence in the present study, core in cup tablet of Azathioprine was designed with the intention of delivering the drug in the colon region for effective treatment of inflammatory bowel disease. Optimized tablet formulations demonstrated good potential to deliver the drug to the colon by successfully exhibiting a lag time of 5 h during in vitro drug release study.⁵ An in vivo evaluation study conducted to ascertain pharmacokinetic parameters in rabbits revealed that the onset of drug absorption from the Core-in-cup tablets was significantly delayed compared to that from the Marketed Azathioprine SR formulation.

II. MATERIALS AND METHODS

The in vivo study of the optimized formulations were performed as per the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on

Animals (CPCSEA), Ministry of social Justice and Empowerment, Government of India. Prior approval by Institutional animals ethics committee was obtained for conduction of experiments (Ref: IPT /IAEC/1053/PO/Re/S/076/CPCSEA, Dated 27-12-2020).Marketed Azathioprinepure drug and optimized core in cup tablet prepared in the laboratory conditions and chosen on the basis of lag time achieved, in-vitro release studies and stability conditions were chosen as dosage forms for administration.

Preparation of Azathioprine core tablets: After preliminary experiments, the optimum formulation of core tablets was obtained. The core tablets were prepared by wet granulation method. The required quantities of Azathioprine, PVPK-30 (as a binder), Moringa olifera gum(as a polymers) and lactose (as a diluent) were weighed and mixed uniformly and prepared a wet mass by addition of binder solution. The wet mass was passed through sieve number #12 and allowed to drying for 30 minutes in a tray dryer for 600C. The dried granules were passed through the sieve number #16 and finally lubricated with talc and magnesium stearate. The obtained dry granules were weighed into individual tablets and finally compressed into the tablet by 16 station rotary tablet compression machine using 9mm flat punches.

Preparation of cup tablet: The cup formulations were formulated by direct compression technique. In which the required quantities of Eudragit RS100 and microcrystalline cellulose, were weighed and mixed uniformly and finally the powder mixture was compressed by 16 station rotary tablet compression machine by using special punch designed and fabricated, to prepare cup tablets. The newly designed upper 12 mm punch has protrusion and lower punch (12mm) remains flat faced .

Preparation of core in cup tablet: The cups were placed in a 12mm die cavity and core tablet was inserted into the cups and compressed with 12mm flat faced punches⁶.

Enteric coating : Core in cup tablet were further coated with enteric coating polymer(cellulose acetate phthalate) by spray coating method. 9% cellulose acetate phthalate in 8:2 (v/v) mixture of acetone: ethanol plasticized with dibutylphthalate (0.75%), was used as a coating solution. Talc (0.1% w/v) was added as antiadherant and the solution was stirred for 15 min. Placed the core in cup tablets into a coating pan, the coating solution was sprayed over the tablets by R&D coater, rotating with a speed of 15 rpm, the pressure of the

spray gun was maintained at 0.1 M.Pa and the air temperature was maintained at 35-40⁰C. The tablets were coated to a 5%, 7.5 % and 10% w/ w total weight gain.

In Vivo Evaluation :

Subject selection: Twelve New Zealand healthy rabbits with a mean age of 10±2 weeks and with a mean body weight of 3±0.2 kg were used in this study. Each group consisted of six rabbits (n=6) each and were subjected for overnight fasting, it was taken care that there was no stress on the animals⁷. Rabbits were randomly divided into two groups for different sampling time and each group was housed in one cage. Food and water were available ad libitum at all times during the experiment. The study was conducted in a crossover design with 2 weeks washout periods in between the two experiments. The animal dose of Azathioprine was calculated relevant to human dose by using the following formula. The above dosage form was administered through gastric intubation method⁸.

Human dose of Azathioprine = 50 mg.

$$\text{Animal dose} = \frac{\text{Human dose} \times \text{Animal weight}}{\text{Human weight}}$$
$$= \frac{50 \times 3}{70} = 2.142 \text{ mg} = 2.5 \text{ mg}$$

Blood sampling: About 1 ml of blood samples were collected from the tracheal lobular vein of the rabbit using and the blood was stored in screw top heparinized plastic tubes, the sampling time for blood was done at 0 mins (Predose), 1 hr, 2 hr, 4 hr, 6 hr, 8hr, 10 hr, 12 hr, 14 hr, 16 hr, 18 hr, 20 hr, 24hr and 48 hr. The plasma was immediately separated by aspiration after centrifugation at 4000 rpm for 5 minutes and frozen at -20°C until analyzed by LC-MS/MS method⁹.

Determination of Pharmacokinetic Parameters

Various pharmacokinetic parameters such as peak plasma concentration (C_{max}), time at which peak occurred (T_{max}), area under the curve (AUC), elimination rate constant (K_{el}), biological half-life (t_{1/2}) and mean residence time (MRT) were calculated using the noncompartmental pharmacokinetics data analysis software PK Solutions 2.0™(Summit Research Services, Montrose, CO, USA). The pharmacokinetic parameters of the tested formulations were statistically analyzed using paired sample's t-test for normal distributed results of C_{max}, K_a, K_e, MRT and AUC_{0-∞} value. All tests were performed at 0.001 level of significance¹⁰.

Estimation of Azathioprine in plasma (LC-MS/MS method)

Chromatographic conditions

A summary of the chromatographic and mass spectrometric conditions is as follows¹¹⁻¹²:

HPLC: Agilent Series 1100

Mass spectrometer: API 4000 (MDS SCIEX LC-MS/MS)

Ion source: Turbo Ion Spray

Polarity : Positive ion mode

Column : Atlantis[®] dC18, 4.6 x 50mm, 3 μ

Column oven temperature : 40 $^{\circ}$ C

Autosampler temperature : 5 $^{\circ}$ C

Mobile phase: 10mM Ammonium formate buffer (pH 3.0 \pm 0.1)

Acetonitrile (100 % v/v)

Flow rate: 0.8 mL/min

Retention time: Azathioprine - 2.46 min

: Caffiene (ISTD) - 2.45 min

Set point (Horizontal): 5.0

Set point (Vertical): 5.0

Injection Volume : 5 μ L

Run time: 3.50 minutes

MRM transitions:

Azathioprine: 278.1 amu (parent), 142.1 amu (product)

Caffiene (ISTD): 194.7 amu (parent), 138.1 amu (product)

MRM Conditions

Curtain Gas (CUR): 20.0 PSI

Collision Gas (CAD): 6.0 PSI

Temperature (TEM): 550.0 $^{\circ}$ C

Ion Spray Voltage (IS): 5500V

Ion Source Gas (GS1): 30 PSI

Ion Source Gas (GS2): 30 PSI

Preparation of working standard solutions

1. Preparation of Azathioprine standard stock solution :

Azathioprine working standard 10 mg was accurately weighed and transferred into a 2 ml volumetric flask and dissolved in 0.200 mL DMSO (Dimethyl Sulfoxide) The solution was made up to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Azathioprine. The solutions were labeled and stored in a cold store at 2-8 $^{\circ}$ C.

2. Preparation of internal standard stock solution :

2 mg of Caffiene was weighed accurately and transferred in to a 2 ml volumetric flask and dissolved in 1 mL water. The solution was made up

to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Caffiene. The solutions were labeled and stored in a cold store at 2-8 $^{\circ}$ C.

2. Calibration curve standards: Stock solution of Azathioprine was diluted with 50% methanol in water solution to get a concentrations ranging from 10 to 10000 ng/ml. Concentrations of azathioprine ranging from 1 to 1000 ng/ml were prepared with plasma and labeled them as CC1 to CC-8. The calibration curve standards were prepared freshly for each validation run. Concentrations of stock dilutions of standard azathioprine solution with plasma were shown in Table 1.

3. Extraction procedure: Withdraw plasma sample and thaw at room temperature. Vortex for proper mixing. Pipette 0.045 mL of plasma sample into micro tube; add 5 μ L of internal standard (3 μ g/mL) and vortex for 30 sec. Then add 5 μ L of respective working standard and vortex for proper mixing. To the above micro tube add 150 μ L of Acetonitrile drop by drop while vortexing for 1.0 min. Centrifuge at 4000 rpm for about 10.0 min at 10 $^{\circ}$ C. Transfer the clear solution into the labeled vials and inject 5 μ L into chromatographic system and fill the sample processing and drug extraction Precipitation form .

4. Data processing: The chromatograms were obtained by using the computer-based Analyst 1.6.2 version software supplied by the Applied Biosystems, Canada. The concentrations of the unknown samples were calculated from the equation using regression analysis of spiked plasma calibration standard with $1/x^2$ as weighting factor. $y = mx + c$, Where, y = Ratio of azathioprine peak area and ISTD peak area (analyte area / ISTD area); x = Concentration of azathioprine; m = Slope of the calibration curve; c = y-axis intercept value.

III. RESULTS AND DISCUSSION

In the present study , pH-dependent polymer (Eudragit RS100) with a overcoat of Cellulose acetate phthalate was suitable for adequately sustained drug release and to protect Azathioprine from being released in the upper region of the GI system. The in vitro drug release studies indicate that the optimized formulation was a promising system targeting Azathioprine to the colon. Tablet with a coating level of 9 % w/w showed a lag time of 5 hr corresponds to time required to reach colonic region. The obtained results showed the capability of the system in delaying drug release for a programmable period of time and the possibility of exploiting such delay to

attain colon targeting. The in vivo experiments were conducted as per the protocol and procedure described earlier. The ability of core in cup tablet as a drug delivery system to release drugs in a predetermined time release manner was investigated in rabbits after oral administrations was investigated. Bioanalytical methods employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) play a significant role in evaluation and interpretation of pharmacokinetic data. For the successful conduct of pharmacokinetic study, the development of selective and sensitive bioanalytical methods plays an important role for the quantitative evaluation of drugs and their metabolites (analytes). The LC-MS/MS methods were highly sensitive and suitable for the detection of drug in plasma even in low concentrations. Calibration curves were constructed from blank sample (plasma sample processed without IS), blank+IS samples and eight point calibration standards for Azathioprine in plasma. Plasma concentrations of Azathioprine at different times were calculated and are shown in Table 2 and in Fig 2. Pharmacokinetic parameters such as absorption rate constant, elimination rate constant,

half life, AUC, and MRT were calculated from the plot of time versus plasma concentration and subjected to statistical analysis and the results were shown in Table 3. The results from the oral administration of Azathioprine pure drug indicated the maximum plasma concentration (C_{max}) 33.3 ± 0.21ng/ml at 4hr (t_{max}) while pulsatile formulations administration exhibited the maximum plasma concentration (C_{max}) of 56.5± 0.14ng/ml after an initial lag time of 5 hrs. The oral administration of Azathioprine resulted in a low and quite variable AUC of 312.9 ± 1.23ng/ml/hr, whereas the optimized core-in-cup tablets resulted in AUC of 1261.9±1.46ng/ml/hr. The mean residence time of optimized core -in-cup tablets administration (21.59 ± 0.036hrs) was found to be more than oral administration (2.66± 0.02hrs). In conclusion, optimized core-in-cup tablets formulation shown drug release over a period of 5-17 hrs, consistent with requirements for chronopharmaceutical drug delivery, was achieved. Thus, core-in-cup tablets formulation parameters could be modified to modulate the drug release time in accordance with chronotherapeutic objectives.

Table 1: Composition of optimizedAzathioprine Core tablets

Ingredients	Core tablet
Azathioprine	2.5
Povidone	10
Moringa olifera gum	75
Lactose	108.5
Magnesium stearate	2
Talc	2
Total	200

Table 2: Composition of optimizedAzathioprine Cup tablets

Ingredients	Cup tablets
Eudragit RS100	400
MCC	42
Mg. stearate	4
Talc	4
Total	450

Table: 3. Analyte Concentrations of Stock Dilutions of Standard Azathioprine Solution with Plasma

S.No	Sample name	Analyte Concentration (ng/mL)	Analyte peak area	IS Peak Area	Area Ratio	Calculated Concentration (ng/mL)	Accuracy (%)
1	Aqueous mixture	N/A	254680	52036	4.894	505.623	N/A
2	Plasma blank	0	0	0	0	N/A	N/A
3	Blank + ISTD	0	0	34323	0	N/A	N/A
4	CC1	1.000	721	49719	0.015	0.963	96.29
5	CC2	2.000	1329	50809	0.026	2.167	108.33
6	CC3	5.000	2684	51083	0.053	4.897	97.94
7	CC4	10.000	5404	53318	0.101	9.944	99.44
8	CC5	100.000	52960	53349	0.993	102.128	102.13
9	CC6	500.000	239277	52002	4.601	475.325	95.06
10	CC7	800.000	409439	54294	7.541	779.361	97.42
11	CC8	1000.000	483491	48340	10.002	1033.850	103.38

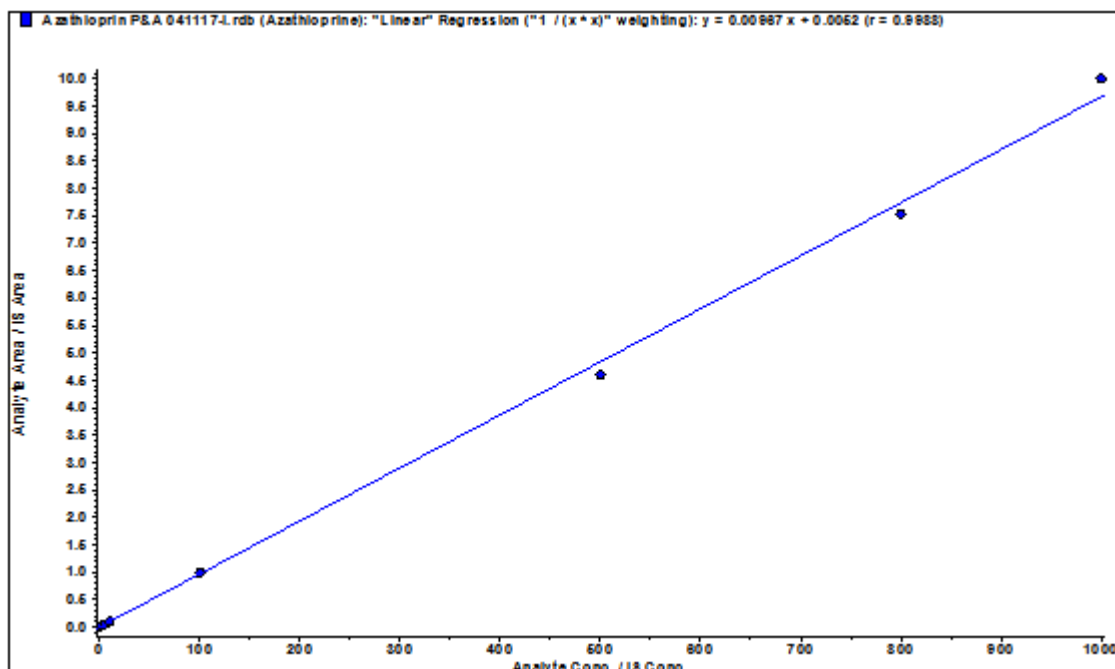


Figure 1: Calibration Curve for Estimation of Azathioprine in Plasma

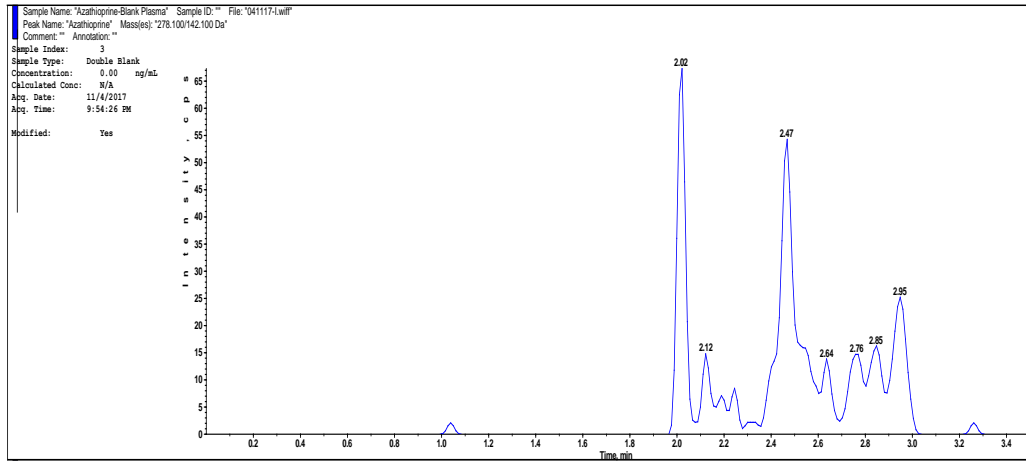


Figure 2. Chromatograms of blank Plasma

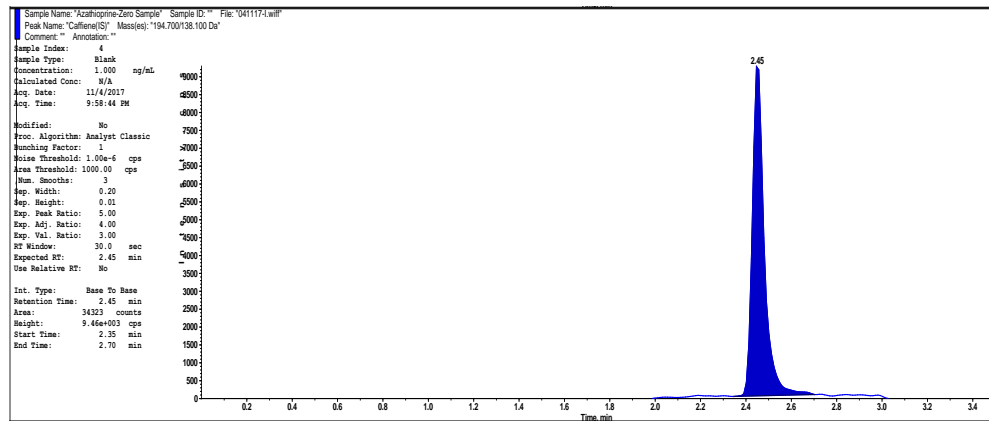


Figure 3. Chromatograms of blank and internal standard

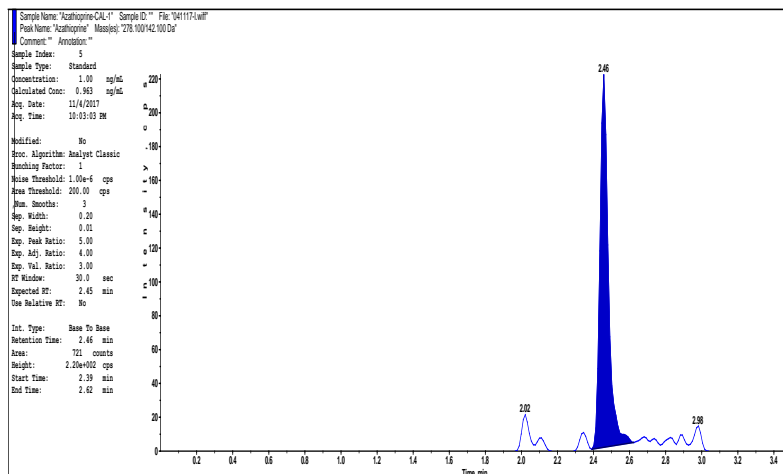


Figure 4. Chromatogram of stock solution of Standard Azathioprine Solution with Plasma

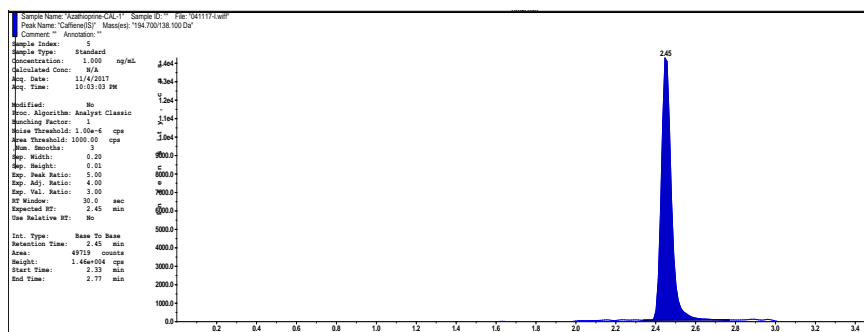


Figure 5. Chromatogram of stock solution of internal standard Caffeine Solution with Plasma

Table 4: Plasma Concentration of Azathioprine following pure drug administration and Azathioprine core in cup tablets administration

Time (h)	Plasma concentration (ng/ml) (Mean ±s.d)	
	Pure drug	Azathioprine core in cup tablets
0	0	0
0.5	05.12 ±0.13	0
1	16.34±0.18	0
1.5	25.31±0.52	0
2	29.37±0.43	0
4.0	33.29±0.12	0
6.0	29.27±1.13	12.31 ±0.49
8.0	26.18±1.34	21.45±0.57
10.0	22.23±0.94	40.87±1.11
12.0	18.23±0.73	56.45±1.08
16.0	14.90±0.86	48.34±1.39
20	11.72±1.11	32.27 ±1.27
24.0	8.64±0.57	20.34±1.23
32.0	7.0±0.43	12.32±0.36
48	3.8±0.34	6.45±1.08

Table 5. Statistical Treatment of Pharmacokinetic Parameters (Mean ± S.D.) of following oral administration of pure drug and core-in-cup tablets of Azathioprine

Pharmacokinetic parameter	Pure Drug	core-in-cup tablets	Calculated value of 't'
Cmax(ng/ml)	33.3 ± 0.21	56.45±1.08	23.70***
MRT (h)	2.66± 0.02	21.59 ± 0.036	40.75***
t1/2(h)	2.11 ± 0.014	9.21 ± 0.011	6.87***
Kel(h-1)	1.41 ± 0.008	0.336± 0.05	19.67***
Ka(h-1)	7.9± 0.011	14.3± 0.04	31.75***
AUC0-∞(ng h/ml)	312.9 ± 1.23	1261.9.±1.46	256.60***

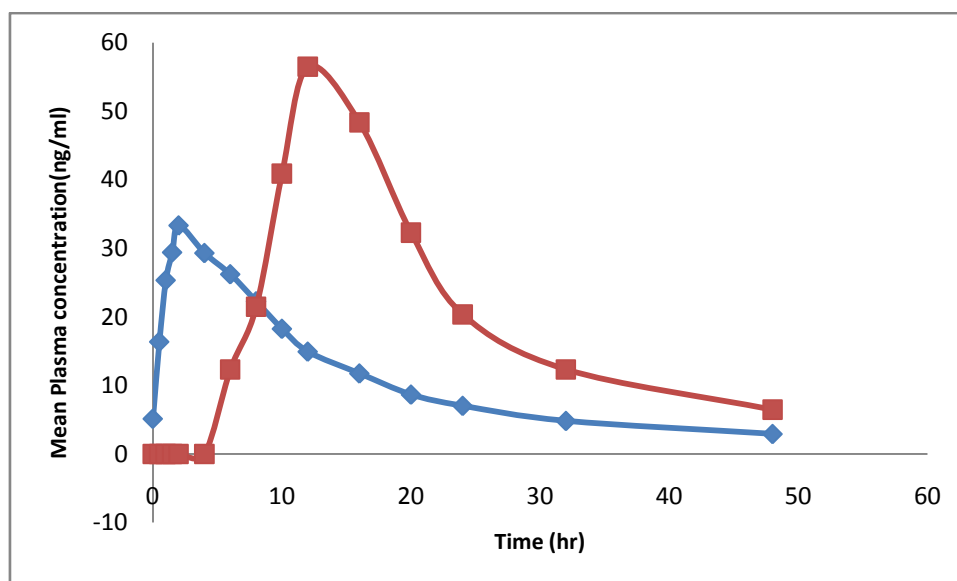


Figure: 4. Concentration-Time Curve of Azathioprine following of core-in-cup tablets and pure drug

(●)Azathioprine following oral administration of core-in-cup tablets

(●)Azathioprine following oral administration of pure drug

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