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# Magnetic and Compartmental Targeting of Azathioprine using Gelatin Microspheres: Formulation, Physicochemical Characterization and *In Vitro* Release Studies

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**ABSTRACT**

Azathioprine loaded gelatin microspheres were formulated to control/target drug release in an arthritic joint by magnetic force or intra-articular injection. Glutaraldehyde cross linked microspheres were characterized for drug loading, entrapment efficiency, gas chromatography, magnetite content, particle size, scanning electron microscopy (SEM), Fourier-Infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC) and *in vitro* release studies. The gelatin microspheres loaded with azathioprine showed 17% of drug loading with 82% of entrapment efficiency. Gelatin magnetic microspheres showed 13% of loading with 87% of entrapment efficiency. Gas chromatography confirms absence of residual glutaraldehyde in the microspheres. The magnetite content of the azathioprine loaded gelatin magnetic microsphere was 25.7% w/w. The average particle sizes of gelatin nonmagnetic and magnetic microspheres were 41 and 54 µm, respectively. SEM confirms the spherical nature of the microspheres. FT-IR revealed the absence of drug polymer interaction, and DSC suggested amorphous nature of entrapped drug in the microspheres. The formulated microspheres could release the drug over a period of 48 h.

## INTRODUCTION

Azathioprine is an immune suppressant (Dollery, 1999) indicated for many disease conditions, which requires immune suppression such as rheumatoid arthritis, organ transplantation, crohn's diseases, chronic active hepatitis, systemic lupus erythematosus, polyarteritis nodosa and other similar conditions. Apart from its therapeutic benefit, azathioprine has various toxic (Dollery, 1999) effects that include suppression of haemopoietic system, hepatotoxicity and teratogenicity.

Plasma half-life of azathioprine ranges (Dollery, 1999) from 3 to 5 h and because of short biological half-life; it has to be administered frequently to maintain effective plasma concentration. Drug delivery systems that control and localize azathioprine plasma concentration would be useful in minimizing the dose induced adverse reactions and provides the better therapeutic effect. Hence in the present work gelatin micro-spheres are formulated to control and localize azathioprine at the desired site of action. Very little information is available in the literature about azathioprine controlled drug delivery (Glulati *et al.*, 1998; Singh and Kim, 2007).

In the present investigation, azathioprine was encapsulated in gelatin magnetic microspheres so that it can be localized with help of an external magnetic field. For e.g. in arteries which supplies blood to diseased organ or transplanted organ or in intestine in case of ulcerative colitis.

Nonmagnetic gelatin microspheres were also formulated for intra-articular administration (compartmental targeting) to treat rheumatoid arthritis. Azathioprine loaded gelatin microspheres were formulated by emulsification and cross linking technique using glutaraldehyde.

The drug loaded microspheres were characterized by drug content, entrapment efficiency, magnetite content, particle size analysis, scanning electron microscopy (SEM), Fourier transformed infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), and *in vitro* release studies.

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## MATERIALS AND METHODS

### Materials

Gelatin, type-B, 300 bloom strength was purchased from Sigma Chemicals, U.S.A. Magnetite powder, less than 5 μm was purchased from Sigma-Aldrich Chemie GmbH, Germany. Azathioprine was a gift from Arvind Remedies, India. Ethanol, toluene, span 80 and glutaraldehyde were purchased from S.D. Fine Chemicals Ltd, Boisar, India. All other reagents used were of analytical grade.

### Method

#### *Preparation of gelatin microspheres loaded with azathioprine*

As shown in the Table 1, 750 mg of gelatin was dissolved in 3 ml of water preheated to 50ºC. 200 mg of azathioprine was dissolved separately in 1 ml of 0.01N NaOH preheated to 50ºC and mixed with the gelatin solution. 375 mg of magnetite was wetted with 1 ml of ethanol and added to the gelatin/azathioprine solution, triturated briskly in a mortar and pestle to get a lump free suspension. Then the mixture was added drop wise to 100 ml of sesame oil with 0.1% w/v span 80 and emulsified by stirring with the help of a hand blender (10,000 rpm/2 min).

Then the stabilized emulsion was stirred at room temperature using a mechanical stirrer (Remi, India, approx.1000 rpm). 1 ml of glutaraldehyde-saturated toluene solution was added successively at each hour, and the stirring was continued at room temperature for 5 h. The cross-linked microspheres were collected by filtration using Whatman filter paper (No: 41). After filtration, the microspheres were washed thrice with 10 ml of 5% w/v sodium metabisulphite. Finally, the microspheres were washed twice with 10 ml of ethanol. After washing, the microspheres were dried at 40ºC over night, transferred to glass vials and stored in a desiccator.

#### *Test for residual glutaraldehyde*

Glutaraldehyde residue present in the microspheres determined by a gas chromatography technique as reported by Saravanan *et al.,* 2008. Briefly, aqueous extract of microspheres injected into the gas chromatographic system (Chemitto-GC 8610) fitted with carbowax column (Carbowax–20M, 10%, 3 m, 1/8", 80/100 mesh) for the detection of glutaraldehyde. Flame ionization detector and nitrogen as carrier gas was used in the analysis. Oxygen and hydrogen used for ignition purpose. The temperature of oven, injector and detector, fixed at 140, 165 and 200˚C, respectively.

#### *Determination of drug loading and entrapment efficiency*

Drug loaded microspheres (100 mg) were digested with 10 ml of 1N sodium hydroxide at room temperature for 12 h. After filtration and suitable dilution, azathioprine present in the solution was determined at 280 nm using a UV visible spectrophotometer (Shimadzu 1601). Drug loading in the microspheres was estimated by using the formula (Saravanan *et al.,* 2008):

$$L = Qm/Wm \times 100$$

Where, L is the percentage loading of microspheres. Qm is the quantity of the azathioprine present in Wm g of microspheres.

#### *Determination of magnetite content*

The magnetite content in microspheres was estimated quantitatively by hydrolyzing an aliquot of the microspheres in concentrated hydrochloric acid and assaying the resultant hydrolysate for iron by atomic absorption spectroscopy (Gupta *et al.,* 1988; Saravanan *et al.,* 2008) at 248 nm using AAS/127 instrument (electronic corporation, India).

#### *Particle size analysis*

The particle size of microspheres was measured by optical microscopy. The microspheres were dispersed in water, and a smear was made on a glass slide and the size of 300 particles was measured by using a micrometer (Saravanan *et al.,* 2008) attached with a microscope. The average particle size was calculated.

#### *Scanning electron microscopy*

The sample for the SEM analysis was prepared by sprinkling the microspheres on to one side of double adhesive stub. The stub was then coated with gold using Jeol JFC 1100 sputter coater. The SEM analysis of the microspheres was carried out by using Jeol JSM 5300, Japan. The microspheres were viewed at an accelerating voltage of 15-20 kV.

#### *Fourier transform infrared spectroscopy and differential scanning calorimetry*

Infrared spectra of azathioprine and formulated microspheres were taken by using KBr pellet technique and were recorded on BOMEM MB–II FT–IR spectrometer. DSC of azathioprine and formulated microspheres were performed using Perkin-Elmer DSC–7 model. The instrument was calibrated with indium. All the samples (≈5 mg) were heated in aluminum pans using dry nitrogen as the effluent gas. The analysis was performed with a heating range of 50-350°C and at a rate of 20°C $min^{-1}$.

**Table. 1:** Physicochemical parameters of gelatin microspheres loaded with azathioprine.

| Batch No. | Gelatin (mg) | Azathioprine (mg) | Magnetite (mg) | Percentage of drug loading | | Percentage of drug entrapment | Average particle size μm n=300 | Magnetite content | | Percentage of magnetite entrapment |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Theore-tical | Actual[a] | | | Theore-tical | Actual[a] | |
| 1 | 750 | 200 | - | 21 | 17.2 ± 1.3 | 81.9 | 41.78 | - | - | - |
| 3 | 750 | 200 | 375 | 15 | 13.1 ± 0.92 | 87.3 | 54. 34 | 28.3 | 25.7 ± 1.8 | 94.7 |

[a]Values are mean ± SE (n=3), -Not applicable.

**In vitro *release***

The *in vitro* release studies of drug loaded microspheres were carried out at 37°C in the phosphate buffer (pH 7.4). Each batch of microspheres, equivalent to 100 mg of azathioprine, was individually added to 200 ml of phosphate buffer (pH 7.4) in flasks. The flasks were shaken (60 oscillations/min) in an incubator (Saravanan *et al.,* 2008) (Remi, India) at 37°C. One ml of sample was withdrawn at regular time intervals and the same volume of phosphate buffer was replaced. After suitable dilution, azathioprine content was estimated at 280 nm using a UV visible spectrophotometer (Shimadzu 1601).

## RESULTS AND DISCUSSION

**Preparation of Microspheres**

The formulated microspheres showed a good drug loading and entrapment efficiency as shown in Table 1. The gelatin microspheres loaded with azathioprine showed 17% of drug loading with 82% of entrapment efficiency. Gelatin magnetic microspheres showed 13% of loading with 87% of entrapment efficiency. Glutaraldehyde was used as cross-linking agent and it must be completely removed from the microspheres as it is irritant and toxic to cells. The formulated microspheres were free from residual glutaraldehyde and solvent. The treatment of sodium metabisulphite was efficient in removing residual glutaraldehyde.

**Magnetite content**

It has been suggested that 21% w/w magnetite is sufficient to achieve 100% retention of the magnetic carrier with a mean size of 1 µm using an 8000 G magnet (Gupta and Hung 1989) in an artery. This percentage is required to overcome blood pressure or force exerted in the artery by the magnet of 8000 G. Hence, the microspheres were formulated with theoretical content of 28% magnetite to have better magnetic localization. The actual magnetite content of the azathioprine magnetic microsphere was 25.7% w/w which was well above the desired concentration. Apart from intra-arterial administration same magnetic microspheres can also be useful in localization in the intestine by keeping a suitable magnet externally near the desired target site for targeted delivery in ulcerative colitis. In our previous work (Saravanan *et al.,* 2009) we have localized ranitidine hydrochloride in the stomach by using the same technique. The magnetite content of the formulated gelatin microspheres are good enough to retain by the 8000 G magnet.

**Size distribution**

The size distributions of microspheres were ranged from 10 to 80 µm. The average particle sizes of gelatin nonmagnetic and magnetic microspheres were found to be 41 and 54 µm, respectively. Presence of magnetite increased the particle size. The particle size should be within the range which can be injected using a conventional needle and should be accommodated within the synovium. Particles in the range of 1 to 70 µm have been employed in intra-articular delivery and larger size particles have shown better retention time/sustained effect (Larsan *et al.,* 2008). Based on this information we have attempted to formulate gelatin microspheres in the range of 10-80 µm to get maximum retention at the injected joint and to have prolonged drug release. In general, a particle size of less than 125 microns is used for the intra-arterial route. Particles of this size can be administered easily by suspending them in a suitable vehicle and injecting them using a conventional syringe (Tice *et al.,* 1990) with an 18- or 20-gauge needle. All formulated microspheres showed acceptable size range of their intended use.

**Scanning electron microscopy**

As shown in Figure 1, SEM pictures of microspheres confirm the spherical structure of the formulated microspheres. The surface of the drug loaded azathioprine were smooth (Fig. 1A and B), whereas the surface of magnetic microspheres was rough showing magnetite particles on the surface (Fig. 1C and D).

**FT-IR analysis**

FT-IR spectrum of azathioprine was compared with reference spectrum given in British Pharmacopoeia and found to be identical. Most of the drug peaks also appeared in the drug loaded microspheres (Figure 2) which indicated no drug-polymer interaction and effective encapsulation of the azathioprine in the formulated microspheres. Particularly, a peak at 1575 $cm^{-1}$ representing purine ring (Singh and Kim, 2007) was found to present in drug loaded microspheres, which confirmed stable nature of the drug. Azathioprine loaded gelatin microspheres also showed similar observations.

**DSC**

The DSC of azathioprine showed (Figure 3) an exothermic peak at 267° C indicated crystalline nature of the drug. The physical mixture of gelatin magnetic microspheres and azathioprine also showed an exothermic peak at 267° C. The drug peak was disappeared in drug loaded gelatin magnetic microspheres and suggested amorphous form of entrapped drug. Similar observations also seen in azathioprine loaded gelatin microspheres.

**In vitro *release***

No information is available in the literature about the selection of the dissolution medium for azathioprine controlled release formulations. Since azathioprine is very less soluble in water and in acidic pH, we have used phosphate buffer pH 7.4 as a release medium in order to maintain sink conditions. The formulated microspheres could release the drug more than 40 h in a sustained manner. The release was much slower in magnetic microspheres, and it could be due to low drug loading (13.1% w/w) than the nonmagnetic microspheres (17.2% w/w). The release was very fast at initial stage and about 75-85% of the drug was released by the end of 12 h. Later the release was slow and extended up to 48 h.

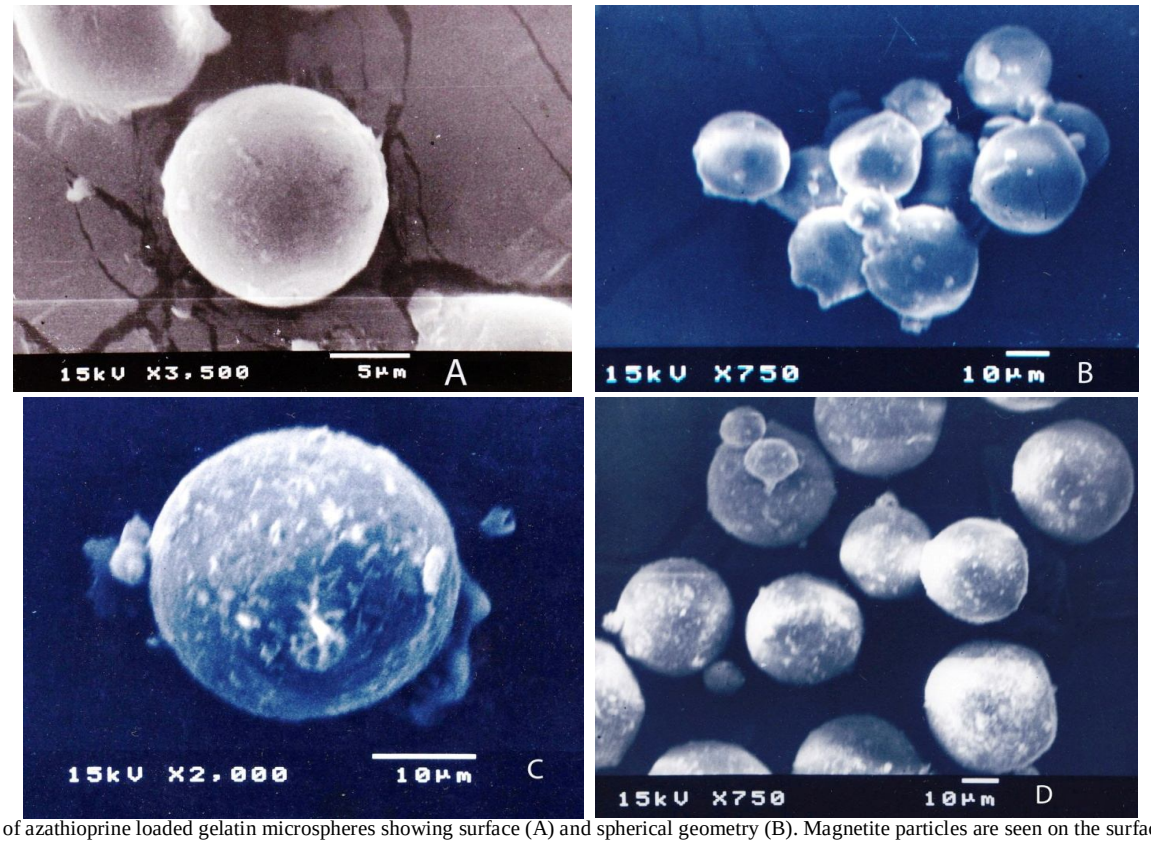


**Fig. 1:** SEM of azathioprine loaded gelatin microspheres showing surface (A) and spherical geometry (B). Magnetite particles are seen on the surface of azathioprine loaded gelatin magnetic microspheres (C and D).

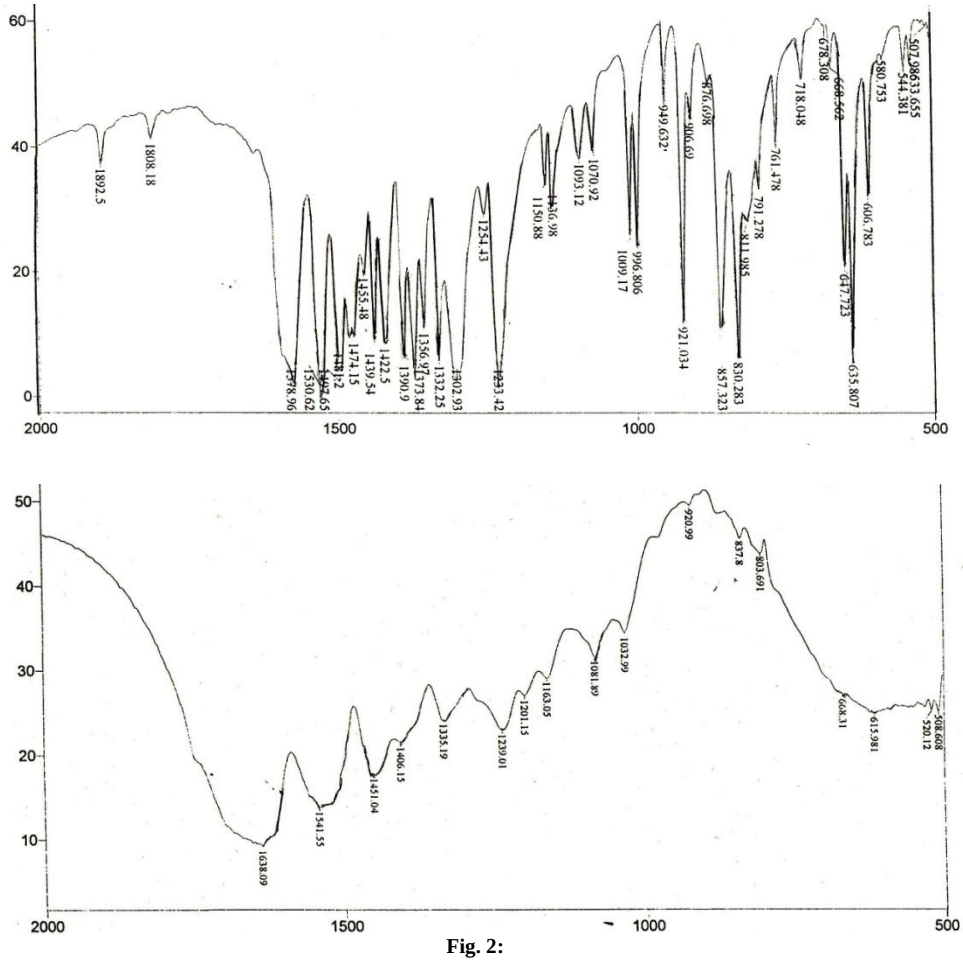


**Fig. 2:**

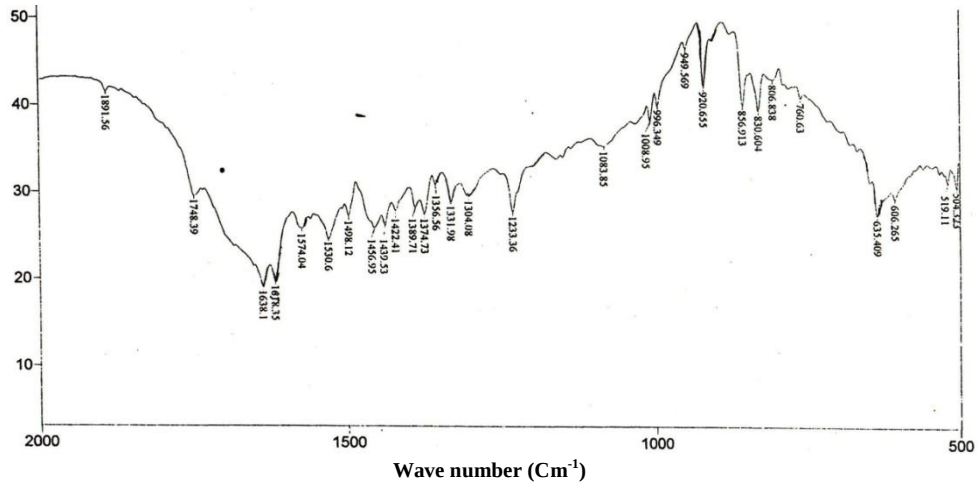


**Fig. 2:** FT-IR spectrum of azathioprine (top), unloaded gelatin magnetic microspheres (middle) and azathioprine loaded gelatin magnetic microspheres (bottom).

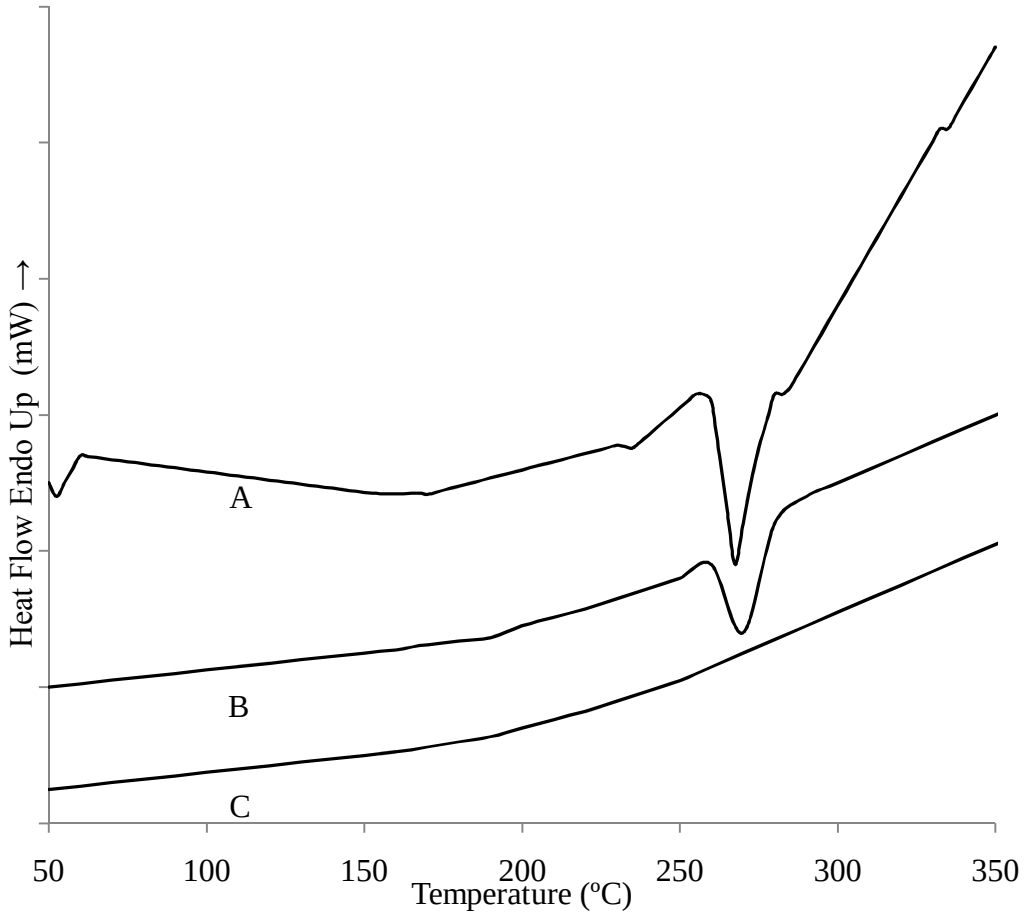


**Fig. 3:** DSC thermograms of azathioprine (A), physical mixture of azathioprine and gelatin magnetic microspheres (B) and azathioprine loaded gelatin magnetic microspheres (C).

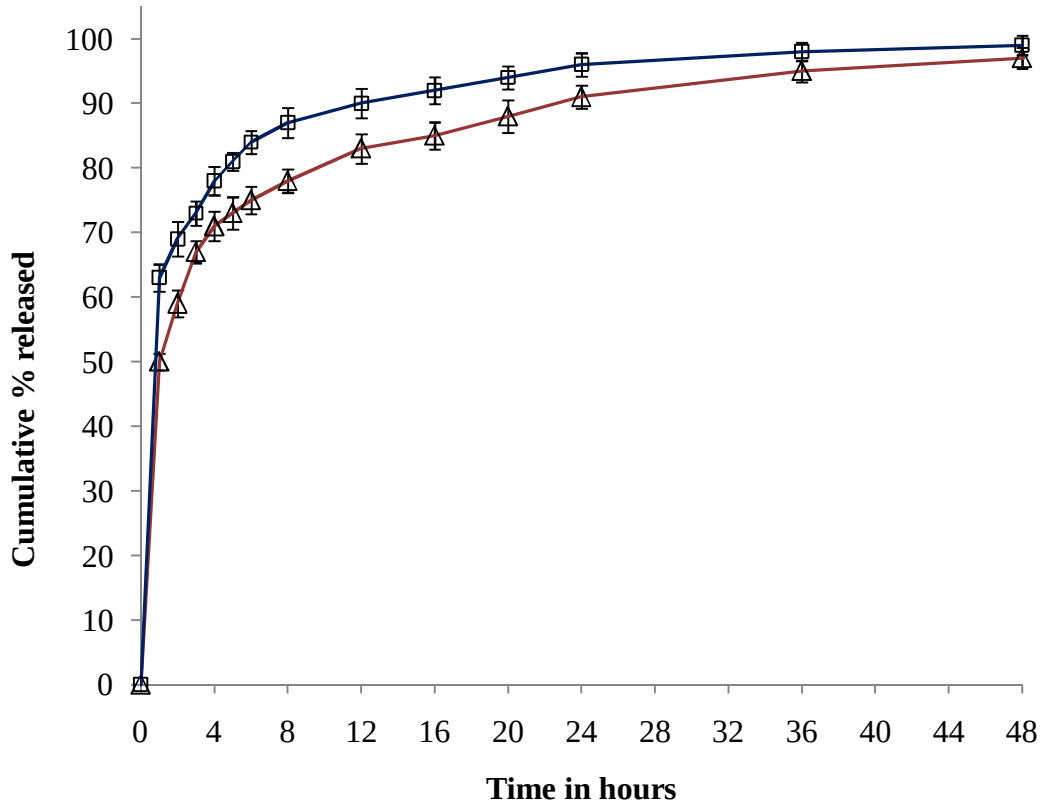


**Fig. 4:** *In vitro* release of azathioprine from gelatin microspheres (□) and magnetic microspheres (Δ) in phosphate buffer (pH 7.4). Values represent mean of three determinations and bars represent ± SE.

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