Research Article **CODEN: AJPAD7** **ISSN: 2321 - 0923**

Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry

Journal home page: www.ajpamc.com

SYNTHESIS OF METHOTREXATE ALANINE AZO ADDUCT FOR COLON TARGETING

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ABSTRACT

The main purpose of our research work is to prepare methotrexate azo adduct with alanine for colon targeting. Methotrexate alanine azo adduct was synthesized and examine the effect of enzyme azo reductase on the release characteristics of alanine and methotrexate in the gastrointestinal contents of rats. By using this approach two drugs can be targetted at the same time in the colon so as to treat the various diseases of colon such as colitis and colorectal cancer. The in acidic environment of stomach the azo adduct will not release the drugs. After the drug reaches to colon the enzyme azo reductase acts on azo bond and releases the methotrexate and alanine simultaneously. By this approach two drugs can be released at same time in colonic region. The azo adduct was evaluated for its color, solubility, R_f value, melting point, IR, ^{1}HNMR and Mass spectral analysis. By in-vitro method colon targeting property of the drug was evaluated by using rat fecal matter. The cytototoxic and acute toxicity studies of the compound were also performed which reveals that the methotrexate alanine azo adduct is safe for use to colon for the treatment of colorectal cancer.

KEYWORDS

MTT, Methotrexate, Azo adduct, Azoreductase, Colorectal cancer and Alanine.

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INTRODUCTION

Oral colon-specific delivery system is used for the treatment of various diseases of colon such as ulcerative colitis, IBS and colorectal cancer[1-3]. From this approach the high local concentration of drug can be achieved and side effects of drugs can be minimized. Methotrexate is a chemotherapeutic agent which competitively inhibits[4] the binding of folic acid to its cognate enzyme dihydrofolate

reductase. It can be administered in lethal doses, which are then reversed using the folic acid alternate leucovorin[5]. It can be use for the treatment of colorectal cancer. Alanine[6] is an α-amino acid with the chemical formula CH_3CH (NH_2) COOH. The L- isomer contains 20 amino acids in which genetic code is encoded. L-Alanine is second only to leucinein, accounting for 7.8% of the primary structure in a sample of 1,150 proteins[7]. D- Alanine occurs in bacterial cell walls and in some peptide antibiotics. Azo conjugation has been used as tools to deliver the drugs especially to the colon. The azo bond remain intact in the physiological environment of stomach and small intestine but once the dosage form enters the colon, the enzyme azo reductase act on the colon and breaks the azo bond which releases the drug into colon[8-10] Figure No.1.

MATERIAL AND METHODS

Preparation of Alanine Methotrexate Azo adduct was subjected for diazotiazation was carried out by dissolving .05 M Alanine in 20 ml methanol then cool it and maintained the temperature of 0-5°C, then add and 1 ml thionyl chloride, orange diesters was form. Then 10 ml conc. hydrochloric acid and 5ml sodium nitrite (10%) was added to form the diazonium salt of alanine. The diazotized salt was coupled with .05 M Methotrexate and the reaction was carried out in mild alkaline conditions by using Sodium hydroxide (2M). During the experiment the temperature of 0-5 °C was maintained by using crushed ice. The product was stirred for 24 hrs and TLC was taken by using toluene, ethylacetoacetate and formic acid (5:4:1) at definite interval of time and visualization of spots was done by using iodine chamber. After completion of reaction concentrate the product, filter it and recrystallize it by using ethanol.

Characterization

The obtained product was confirmed by m.p. The obtained product was subjected for physicochemical properties. The azo complex formation was confirmed by IR, NMR and Mass spectral studies Figure No.2-4.

Analytical methods

IR Spectra was taken on a Shimadzu Spectrophotometer. ^{1}H NMR spectra were taken on a Bruker Avance II 400 NMR Spectrophotometer. Melting and decomposition points were conducted in a melting point apparatus. The IR spectrum of the synthesized compound was obtained from Laureate Institute; Himachal Pradesh. ^{1}HNMR and Mass spectrum was performed in SAIF, Punjab University.

Acute Toxicity Study

The azo adduct was evaluated for acute toxicity study. The Animal Ethical Committee of Institute approved the protocol. OECD 423 guide-lines were followed in the procedures. Two groups of 6 albino rats, one for test and other for control, were used for the study. The study was performed by administering the methotrexate alanine azo adduct at 2g/kg body weight for the test group animals. The evaluation of acute toxicity study was performed for 14 days. Changes in the skin colouration, observing body weight, corneal reflex, change in behaviour, and convulsions are compared with the control group animals (Madhav and Shankar, 2011[11].

***In vitro* release study**

The derivative was further subjected for in vitro release using a rat fecal material using phosphate buffer of Ph 7.4. Shimadzu 1800 UV spectrophotometer was used for this purpose. 1gm of rat fecal material was taken in 6 test tube. 1ml of drug solution (10 microgram/ml) was added in each test tube. Then 5ml of phosphate buffer was added in each test tube and incubate it for 30 minutes at 37ºC for different interval of time. Filter the solution and absorbance was evaluated using U.V. Spectrophotometer at 299 and 525 for methotrexate and alanine respectively Figure No.5.

Cytotoxicity Studies

Cytotoxic studies was performed in Department of Pharmacology Manipal College of Pharmaceutical Sciences by the below mentioned principle. The colorimetric assay was performed that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT after entering into the cells and passes into the

mitochondria reduced to an insoluble, coloured (purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is measured spectrophoto- metrically. The reduction of MTT only occur in metabolically active cells the level of activity is a measure of the viability of the cells. Following were the result of acute toxicity study in Table No.1. Doxorubicin was used as reference compound.

RESULTS AND DISCUSSION

From the IR spectra of methotrexate alanine azo adduct, the azo peak was observed at 1622.13 cm^{-1} in addition to the peaks originated from alanine and methotrexate. IR (KBr): 3564.45 (O-HStret), 3419.79. (N-H Stret) 1622.13(-N=N- stret), ^1H NMR δ (DMSO): 8.4038, 8.0021(ArH), 4.5031 (ArOH), 2.3786, 2.4334(N-H), 1.9648(R-CH_2). M/Z(%): 92.9(67), 150.9(11), 210.9(21), 229(5), 268.8(100), 270.8(63), 326.8(17.8), 384.8(5), 440.7(5), 560.7(26.4), 618.8(178), M.p-290^{0}C, %Yield-40%, R_f0.43, Colour-light Yellow. The azo adduct did not show any release in HCl buffer pH 1.2. It implies that azo conjugate does not degrade in stomach.

It shows release rate of 84 and 92 release rate for methotrexate and alanine respectively in 30 minutes in rat fecal material. Our cytotoxic studies shows that methotrexate alanine azo adduct show IC50 value >200µ gm/ml when compared to doxorubicin with IC50 value 4.5µgm/ml. So this cleary indicates that methotrexate alanine azo adduct was non toxic even at more than 200µ gm/ml value due to the formation of azo adduct with alanine .The same adduct when entered into colon region is going to release methotrexate and alanine separately in Figure No.1. From the cytotoxic studies it was found that the product is safe for use to colon for the treatment of colorectal cancer in Table No.1.

Table No.1: MTT assay

Cell HCT- Humam colon cancer Line 116 – Cell Line														
Time point of treatment- 48hrs														
MTT Assay														
Group No	**Compound Name**	**Conc (µg/ml)**	**Absorbance at 540nm**			**% Cell Death**			**% Cell Viability**			**Mean Cell Viability**	**IC50 (ug/ml)**	**SEM**
1	Methotrexate Alanine azo adduct	25	0.176	0.203	0.189	22.5	10.6	16.7	77.5	89.4	83.3	83.4	>200	3.4
		50	0.194	0.22	0.22	14.5	3.1	3.1	85.5	96.9	96.9	93.1		3.8
		100	0.204	0.209	0.208	10.1	7.9	8.4	89.9	92.1	91.6	91.2		0.7
		200	0.2	0.182	0.21	11.9	19.8	7.5	88.1	80.2	92.5	86.9		3.6
2	Doxorubicin	0.05	0.205	0.214	0.21	9.7	5.7	7.5	90.3	94.3	92.5	92.4	4.5	1.1
		0.5	0.18	0.145	0.13	20.7	36.1	42.7	79.3	63.9	57.3	66.8		6.5
		5	0.094	0.088	0.099	58.6	61.2	56.4	41.4	38.8	43.6	41.3		1.4
		50	0.079	0.099	0.067	65.2	56.4	70.5	34.8	43.6	29.5	36.0		4.1

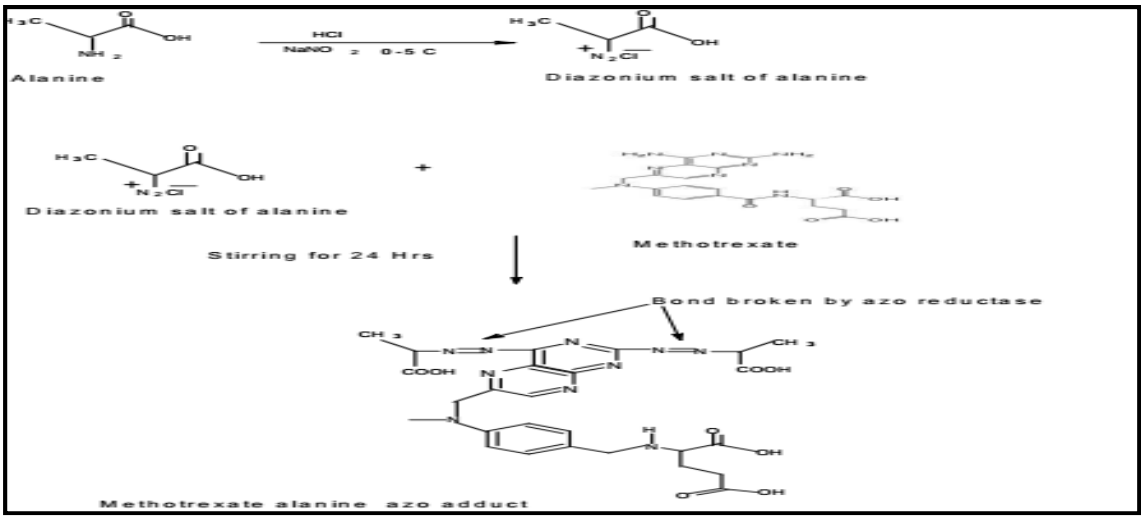

Figure No.1: Synthesis of Methotrexate Alanine Azo Adduct

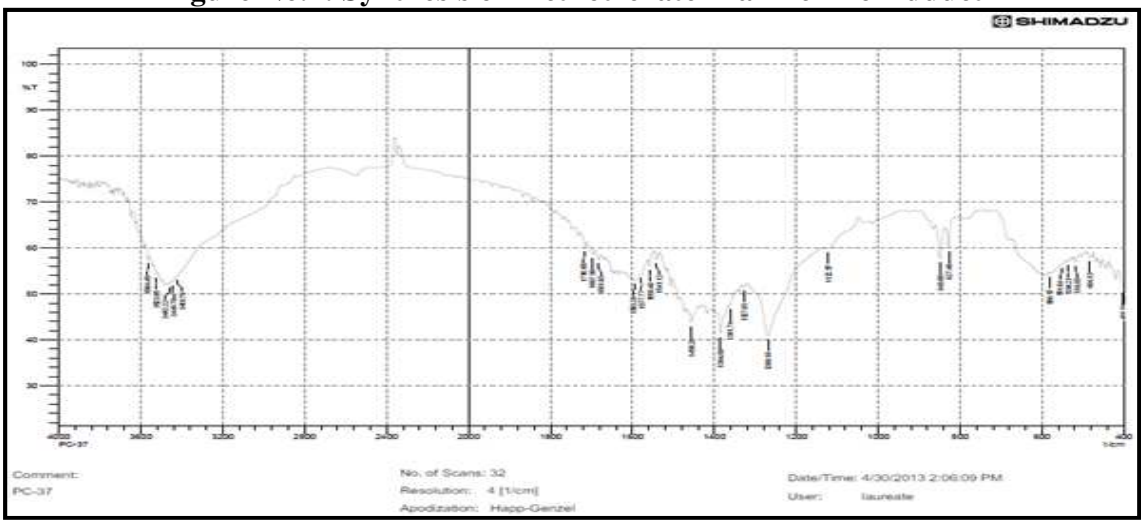

Figure No.2: IR Spectra of Methotrexate Alanine azo adduct

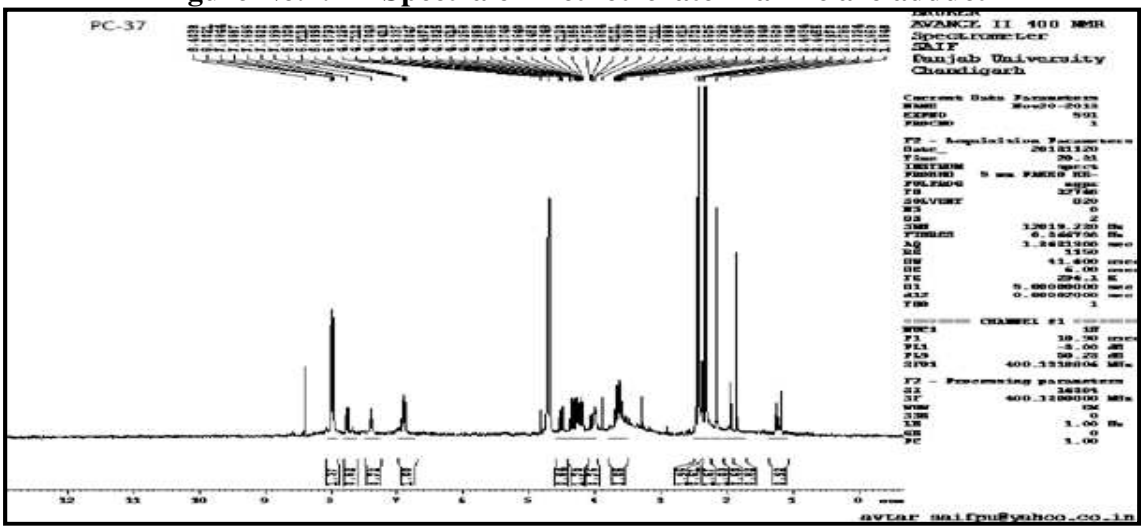

Figure No.3: NMR Spectra of Methotrexate Alanine azo adduct

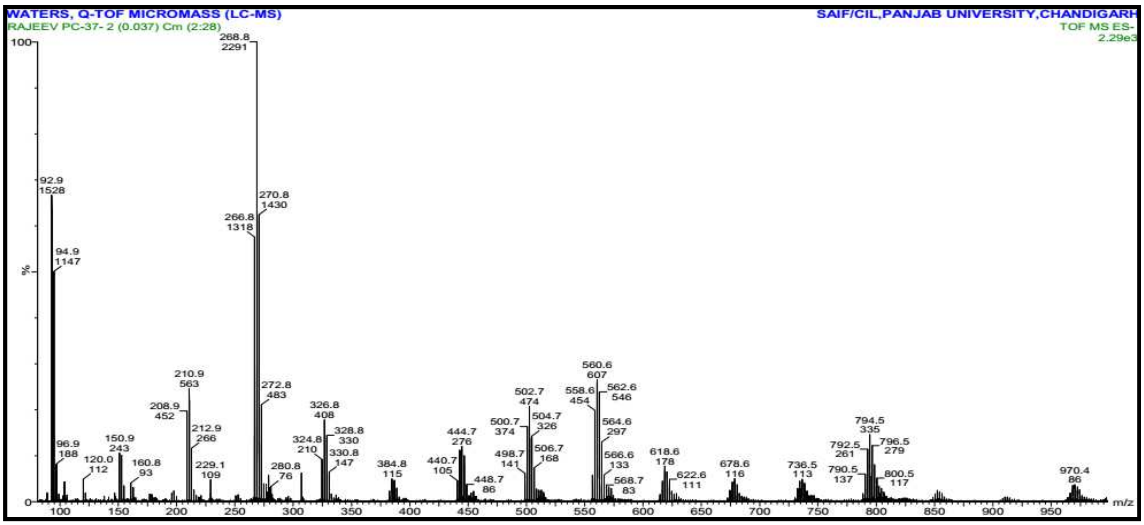

Figure No.4: Mass Spectra of Methotrexate Alanine azo adduct

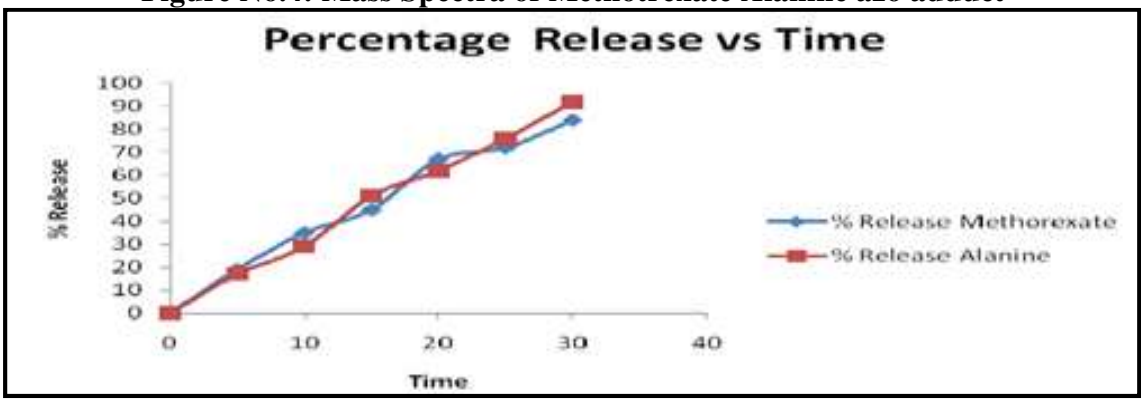

Figure No.5: *In vitro* Release of Methotrexate and Alanine from Azo adduct

CONCLUSION

Our research study result reveals it is having significant colon specificity hence this method is feasible for preparing colon targeted delivery and this complex is used for treating various disease of colon. So we draw the conclusion that this method is so beneficial, economic and patient compatible for targeting drug to colon region in effective manner.

ACKNOWLEDGEMENT

I am extremely thankful to Sharon laboratories for providing me methotrexate as gift sample. I am also thankful to SAIF, Chandigarh and Laureatt Institute of Pharmacy for providing me NMR and Mass spectra. Last but not the least I am also thankful to DIT University for providing me research facility.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: Rajeev Kumar Sharma *et al*. Synthesis of methotrexate alanine AZO adduct for colon targeting, *Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry,* 2(2), 2014, 76-81.