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SYNTHESIS OF METHOTREXATE ALANINE AZO ADDUCT FOR COLON TARGETING

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ABSTRACT

The current aim of our research work is to prepare methotrexate azo adduct with alanine for colon targeting. We synthesized alanine methotrexate azo adduct 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 azo adduct did not release drug in acidic environment of stomach, but when drug reaches to colon the enzyme azo reductase acts on azo bond and releases the methotrexate and alanine simultaneously. By using this approach dual drugs can be released at same time. The azo adduct was evaluated for its color, solubility, R_f value, melting point, IR, ¹HNMR and Mass spectral analysis. It was further subjected for evaluating its colon targeting property by in-vitro method using rat fecal matter. The cytotoxic and acute toxicity studies of the compound were also performed which reveals that the alanine methotrexate azo adduct is safe for use to colon for the treatment of colorectal cancer.

KEY WORDS

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 cancer¹⁻³. 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 inhibits⁴ 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 leucovorin⁵. It can be used for the treatment of colorectal cancer. Alanine⁶ is an α -amino acid with the chemical formula $\text{CH}_3\text{CH}(\text{NH}_2)\text{COOH}$. The L-isomer is one of the 20 amino acids encoded by the genetic code. L-Alanine is second only to leucine in rate of occurrence, accounting for 7.8% of the primary structure in a sample of 1,150 proteins⁷. 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 remains intact in the physiological environment of stomach and small intestine but once the dosage form enters the colon, the enzyme azo reductase acts on the colon and breaks the azo bond which releases the drug into colon⁸⁻¹⁰ Figure No.1.

MATERIALS 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 formed. 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, ethylacetate 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. ¹H NMR spectra were taken on a Bruker AvanceII 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. ¹H NMR and Mass spectrum was performed in SAIF, Punjab University.

Acute Toxicity Study

The azo adduct was evaluated for acute toxicity study. The protocol was approved by the Institutional Animal Ethical Committee. 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 acute toxicity study was evaluated for a period of 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, 2011¹¹).

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. .1 gm of rat fecal material was taken in 6 test tube. 1 ml 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 37 °C 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. This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial

succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg.isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can 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-CH₂).

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-290⁰C,% Yield-40%,R_f 0.43, 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 reveals that methotrexate alanine azo adduct show IC50 value >200µgm/ml when compared to doxorubicin with IC50 value 4.5 µgm/ml. So this clearly indicates that methotrexate alanine azo adduct was non toxic even at more than 200µgm/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)	SE M	
1	Methotrexate	25	0.176	0.203	0.189	22.5	10.6	16.7	77.5	89.4	83.3	83.4	>200	3.4	
	Alanine azo adduct	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

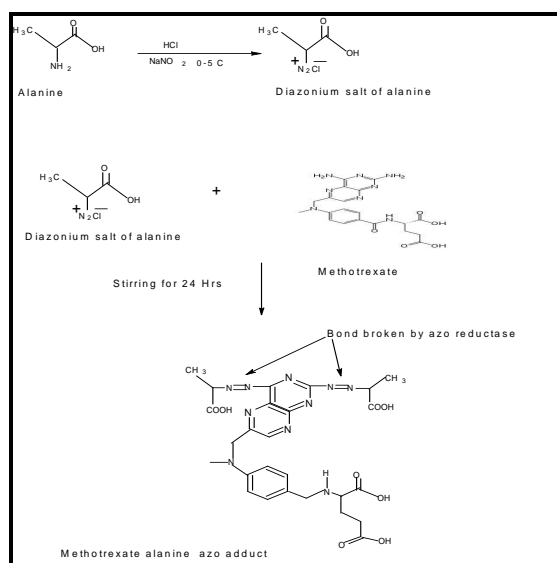


Figure No.1: Synthesis of Methotrexate Alanine Azo Adduct

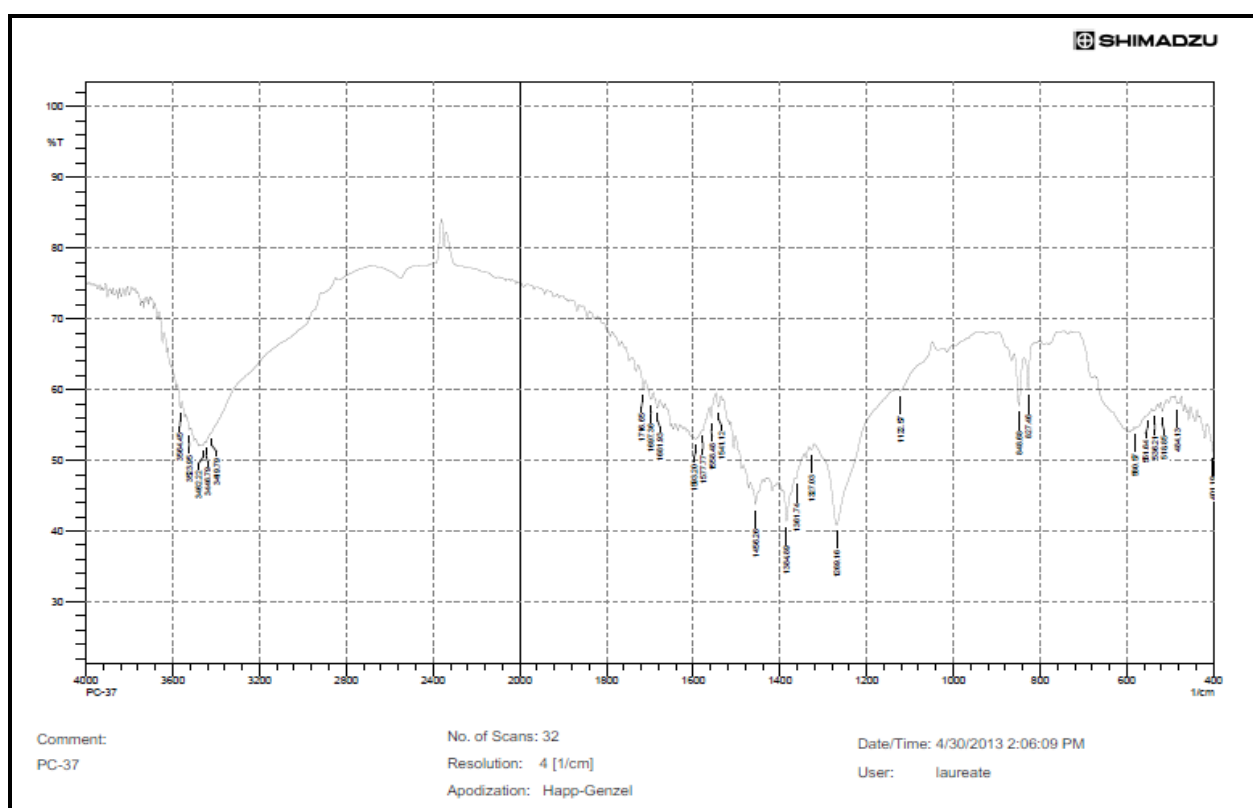


Figure No.2: IR Spectra of Methotrexate Alanine azo adduct

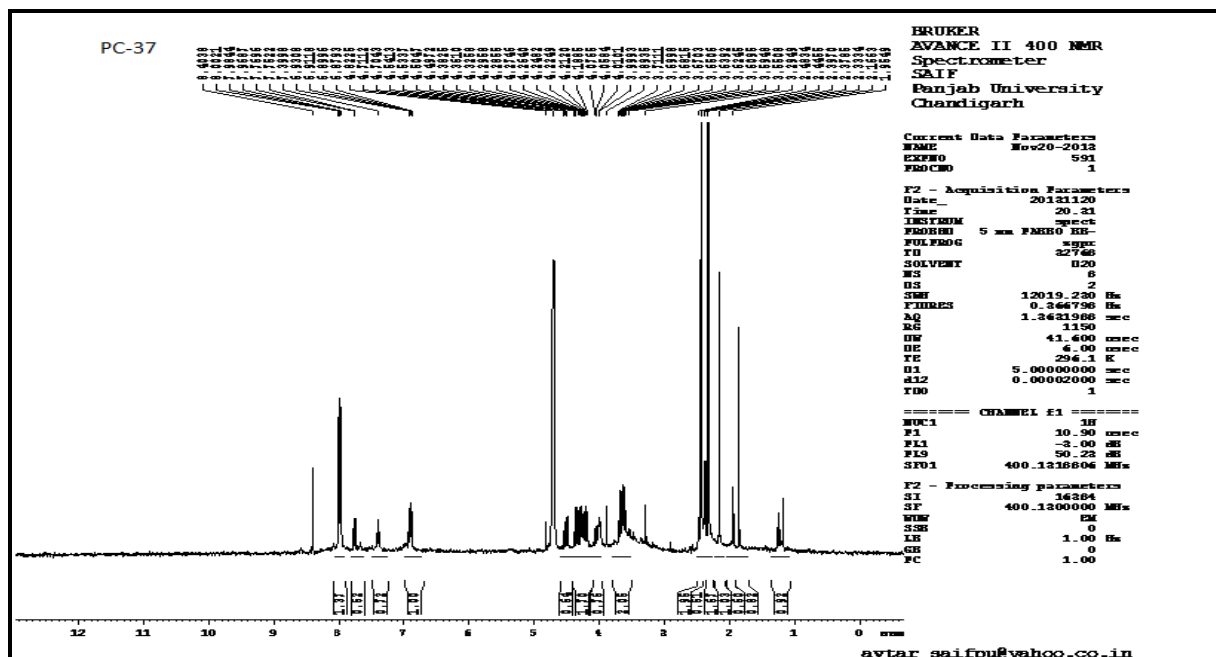


Figure No.3: NMR Spectra of Methotrexate Alanine azo adduct

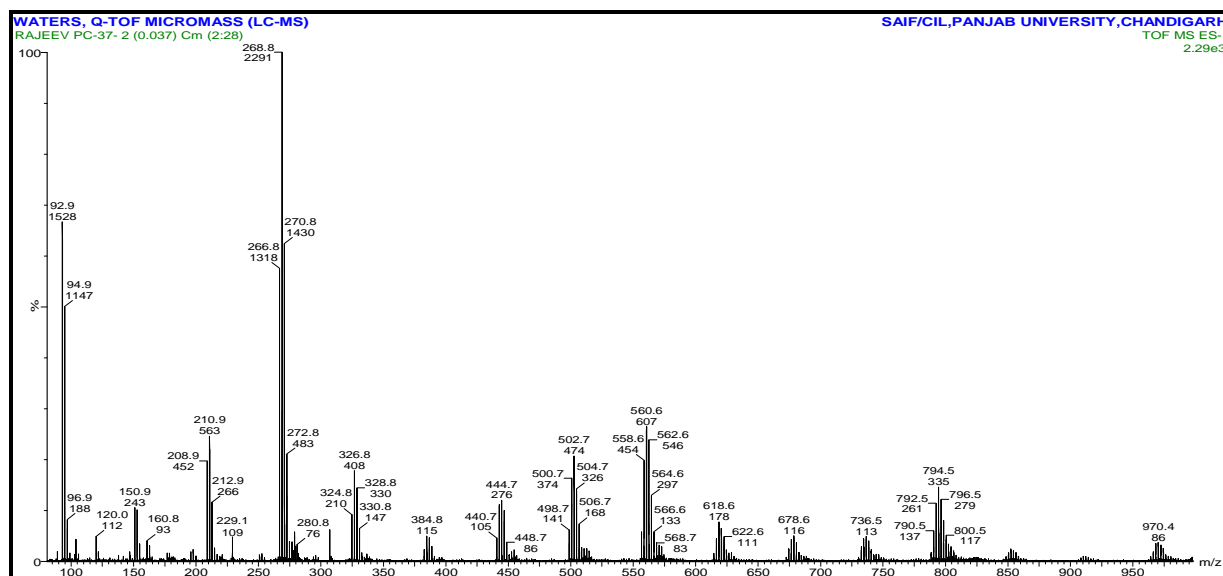


Figure No.4: Mass Spectra of Methotrexate Alanine azo adduct

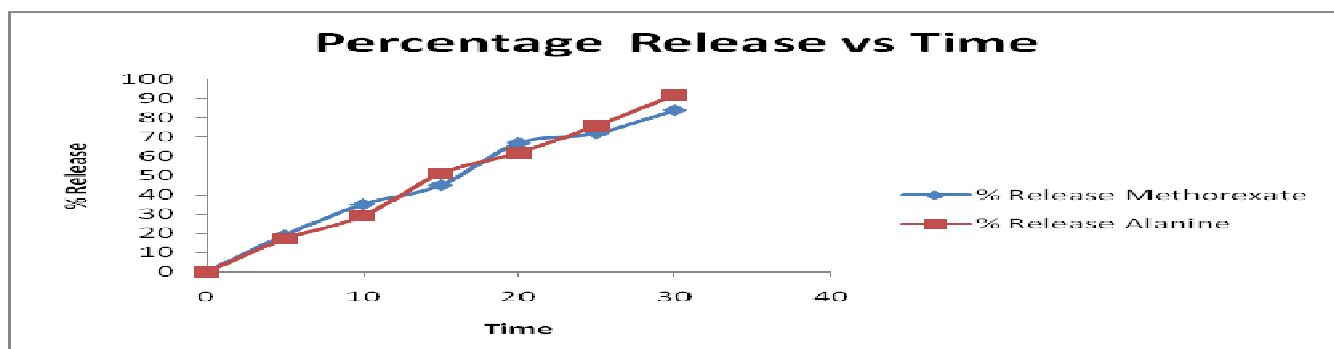


Figure No.5: In vitro Release of Methotrexate and Alanine from Azo adduct

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. The conclusion was drawn that this method is so beneficial, economic and patient compatible for targeting drug to colon region in effective manner.

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