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FORMULATION AND *INVIVO* EVALUATION OF METHOTREXATE HOT MELT EXTRUDES USING KOLLIDON VA 64 & PEG 1500

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ABSTRACT
Methotrexate is an effective anticancer agent which acts by competitively inhibiting dihydrofolate reductase (DHFR). The drug belongs to BCS class II due to which the bioavailability of drug is very less. In the present investigation a total of 12 physical mixtures and hot melt extrudes of the drug is prepared using Kollidon VA 64 and PEG 1500 at various ratios. The formulations are evaluated for dissolution studies. From the % drug dissolved formulation M:Kollidon VA 64: PEG 1500 at a ratio of 1:4:0.4 is selected. The formulation is further evaluated using invivo evaluation. The plasma samples are evaluated using HPLC Agilent 1200 with PDA detector. The samples were analyzed for various kinetic parameter such as Ka, Ke, Cmax, Tmax and AUC. From the evaluation studies a significant increase in all kinetic parameters were observed. The AUC of formulation increased 6.2 folds when compared to pure drug and the Ka increased about 8 folds.

KEY WORDS: Hot Melt Extrusion, Cmax, AUC, Invitro and Invivo evaluation.

MATERIALS AND METHODS

Materials
Methotrexate (Gift sample from Ipca Laboratories, Mumbai), Amino acetophenone (Gift sample from Dr.Reddy's Laboratories, Hyderabad), Acetonitrile (HPLC grade, Qualigens).

Methods
Hot-melt extrusion (HME) is a simple technique widely used in the plastics, rubber and food industry; whereas its application in the field of pharmaceutical industry became more popular in the recent years. HME is a solvent-free, continuous processing technique used to produce a variety of dosage forms. During the hot-melt extrusion process, drug(s), binder and other excipients are fed into the heated barrel, mixed by the rotating screw element and extruded through the die attached at the end of the barrel. The materials inside the barrel are heated mainly by the heat generated due to the shearing effect of the rotating screw and the heat conducted from the heated barrel. Due to intense mixing and elevated temperatures that are present during the processing, active ingredients are very uniformly dispersed in the hot-melt extrudates forming solid dispersions or solid solutions, depending on the miscibility with the binder selected for the extrusion process. Obtained extrudates can be transformed into powders by milling or cut to short lengths and then further pelletized. It has been proven that HME technology may improve the dissolution rate of poorly water-soluble drugs by forming solid dispersions and solid solutions, control or modify drug release and mask the bitter taste of drugs.

Methotrexate is an effective anticancer agent which acts by competitively inhibiting dihydrofolate reductase (DHFR). The drug belongs to BCS class II due to which the bioavailability of drug is very less. [1] The drug is studied by formulating hot melt extrudes.

Preparation Of Physical Mixtures Of Methotrexate With Various Carriers
Physical mixtures of methotrexate were prepared by mixing different ratios of excipients like Kollidon VA 64 (1:1, 1:2, 1:3 & 1:4), and PEG 1500(1:1, 1:2, 1:3 & 1:4) show below **table no 1 & 2.** The defined ratios of APIs and excipients were taken into mortar and pestle and mixed for about 10 min until a homogeneous mixture was obtained. The physical mixture was evaluated.

Table No.1 . Physical Mixtures of Methotrexate with Various Carrier Ratios

S.no	Physical mixture combination	Physical mixture ratios			
1	M :Kollidon VA64	1:1	1:2	1:3	1:4
2	M :PEG 1500	1:1	1:2	1:3	1:4

Table no. 2. Physical Mixtures of Methotrexate with Various Carrier Ratios Dual Combination

S. No.	Physical Mixture Combination	Physical Mixture Ratios			
2	M :Kollidon VA64:PEG 1500	1:4:0.1	1:4:0.2	1:4:0.3	1:4:0.4

Preparation Of Hot Melt Extrudes Of Methotrexate With Various Carriers

Hot Melt Extrusion (HME)[2, 3]

Hot Melt Extrudes of Methotrexate and Methotrexate with Various Drug: Carrier ratios are shown in Table no:3. The extrudes are prepared using R&D model Twin Screw Thermo Scientific™ Extruder. The carrier and Drug at required ratios as per formulation table is weighed, mixed thoroughly and transferred into the hopper of the extruder. The powder is passed through the heater where the temperature was maintained 10^{O} C above the melting point of the mixture. The melted Drug: Carrier mixture is passed through a twin blade extruder. The melt extrudes via twin screw and exits on to a belt conveyor. The melted extrude solidifies during conveying which is collected in stainless steel tray as long strands. The prepared hot melt extrudes were milled to required size and kept for further analysis. All extrudes were prepared in the same process. The whole process is given in the following **Figures.2a-2g.**

Fig:2a, 2b. Thermoscientific Hot Melt Extruder

Fig: 2c. Drug: Carrier Mixture in Hopper

Fig:2d. Drug: Carrier Hot Melt

Fig:2e. Hot Melt Extuding on to conveyor

Fig:2f. Hot Melt Extudes collecting in to SS tray

Fig:2g. Hot Melt Extrude Of The Drug & Carrier

Table No. 3. Hot Melt Extrudes of Methotrexate with Various Carriers Single and Dual Combination

S.No.	Hot Melt Extrudes	Hot Melt Extrudes Ratios			
2	M : Kollidon VA64	1:1	1:2	1:3	1:4
4	M : PEG 1500	1:1	1:2	1:3	1:4
6	M : Kollidon VA64:PEG 1500	1:4:0.1	1:4:0.2	1:4:0.3	1:4:0.4

Evaluation Of Hot Melt Extrudes Of Methotrexate & Methotrexate

Dissolution Study of Prepared Hot Melt Extrudes

In - vitro dissolution study can be performed using the standard paddle apparatus describe in the pharmacopeia. Mainly paddle type dissolution apparatus is used for the dissolution test of Hot Melt Extrudes. In vitro dissolution can be performed by using USP- Type II apparatus (Disso 2000 with auto sampler) containing 0.1M HCl at the speed of 50 rpm with 37.5 ± 0.5^{o} C. Dissolution carried for 2 hr at intervals of 0, 5, 10,20,30,45, 60, 90 and 120 min. 5ml of sample taken at each interval into beaker and dilute to required dilution and finally place sample in UV-Visible spectrophotometer and tabulated absorbance values. From that values to calculate percent drug release of Methotrexate.

Table:4. Dissolution Parameters for Methotrexate or Methotrexate HME.

S.NO.	DISSOLUTION PARAMETERS	
1	Type of Apparatus	USP-Type II (Paddle)
2	Rotational speed	50 rpm
3	Medium for Methotrexate	0.1 M HCl
4	Medium for Methotrexate	0.1 M HCl
5	Volume of Medium Taken	900ml
6	Sample Volume	5ml
7	Temperature	37.5 ± 0.5^{O}C
8	Sampling Time Intervals	0,5,10,20,30,45, 60, 90 and 120 min.

Table 5. Dissolution Data of Methotrexate Pure Drug & Kollidon VA 64 HOT MELT EXTRUDES

Time (min)	Percent Methotrexate Dissolved (x ±s.d., n = 3)				
	M	M:Kollidon VA64 1:1	M:Kollidon VA64 1:2	M:Kollidon VA64 1:3	M:Kollidon VA64 1:4
5	2.53±0.3	52.96±0.33	65.66±0.30	74.51±0.30	81.16±0.21
10	5.36±0.8	56.06±0.22	76.34±0.42	79.64±0.22	84.66±0.87
20	9.83±0.6	67.46±0.42	78.46±0.22	84.32±0.42	90.92±0.76
30	14.35±0.7	77.53±0.51	81.66±0.51	92.32±0.51	96.96±0.76
45	17.46±0.3	85.36±0.62	89.56±0.42	93.40±0.62	98.97±0.88
60	19.66±0.4	86.44±0.41	91.66±0.40	97.77±0.40	99.82±0.48
90	23.98±0.5	87.93±0.41	92.46±0.81	98.65±0.41	99.90±0.78
120	24.76±0.3	89.96±0.52	95.33±0.52	98.95±0.52	100.02±0.50

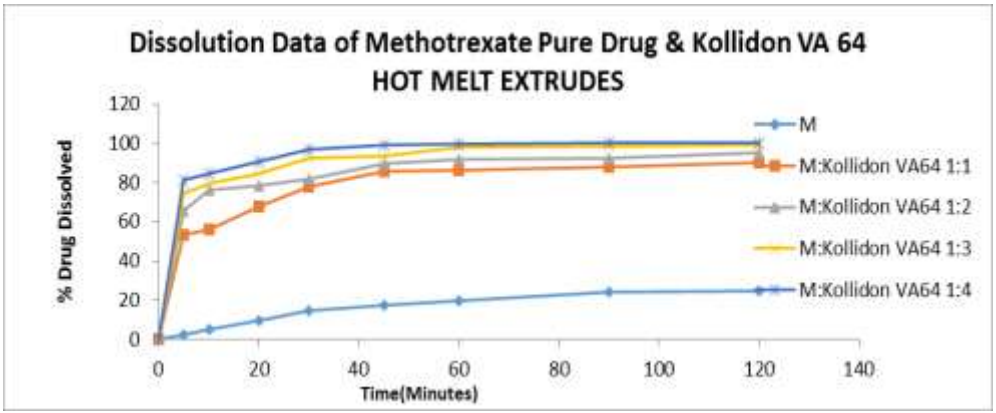

Fig:3. Dissolution data of pure drug and Hot Melt Extrudes of Kollidon VA 64

Table 6. Dissolution Data of Methotrexate Pure Drug & PEG 1500 HOT MELT EXTRUDES

Time (min)	Percent Methotrexate Dissolved (x ±s.d., n = 3)				
	M	M:PEG 1500 1:1	M:PEG 1500 1:2	M:PEG 1500 1:3	M:PEG 1500 1:4
5	2.53±0.3	31.6±0.58	52.93±0.52	60.12±0.67	68.10±0.88
10	5.36±0.8	46.5 ±0.66	57.94±0.62	66.55±0.65	71.36±0.86
20	9.83±0.6	52.2±0.95	58.62±0.46	70.28±0.89	84.66±0.87
30	14.35±0.7	53.6±0.42	65.62±0.89	78.28±0.99	90.92±0.76
45	17.46±0.3	59.3±0.28	76.04±0.77	84.55±0.76	96.96±0.76
60	19.66±0.4	68.4±0.75	78.09±0.65	86.34±0.65	98.97±0.88
90	23.98±0.5	77.7±0.57	80.01±0.86	93.13±0.49	99.82±0.48
120	24.76±0.3	78.87±0.53	80.05±0.76	94.22±0.89	99.90±0.78

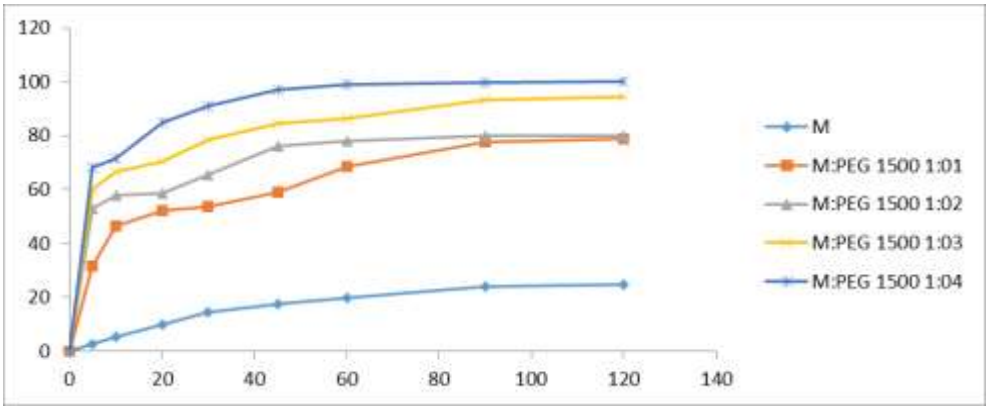

Fig:4. Dissolution data of pure drug and Hot Melt Extrudes of PEG 1500

Table: 6. Dissolution Data of Methotrexate Pure Drug & M: Kollidon VA64: PEG 1500 HOT MELT EXTRUDES.

Time (min)	Percent Methotrexate Dissolved (x ±s.d., n = 3)				
	M	M:Kollidon VA64:PEG1500 1:4:0.1	M:Kollidon VA64:PEG1500 1:4:0.2	M:Kollidon VA64:PEG1500 1:4:0.3	M:Kollidon VA64:PEG1500 1:4:0.4
5	2.53±0.3	50.5±0.66	58.6±0.68	87.93±0.41	88.1±0.38
10	5.36±0.8	58.9±0.42	78.7±0.56	89.96±0.52	97.2 ±0.66
20	9.83±0.6	62.2±0.81	85.1±0.55	92.46±0.81	99.2 ±0.35
30	14.35±0.7	66.5±0.32	85.9±0.42	95.33±0.52	100.1±0.22
45	17.46±0.3	80.9±0.73	86.9±0.18	97.77±0.40	100.1±0.55
60	19.66±0.4	84.5±0.76	89.1±0.15	98.65±0.41	100.1±0.75
90	23.98±0.5	88.5±0.95	94.9±0.37	98.95±0.52	100.1±0.87
120	24.76±0.3	99.3±0.67	99.7±0.23	100.9±0.22	100.1±0.63

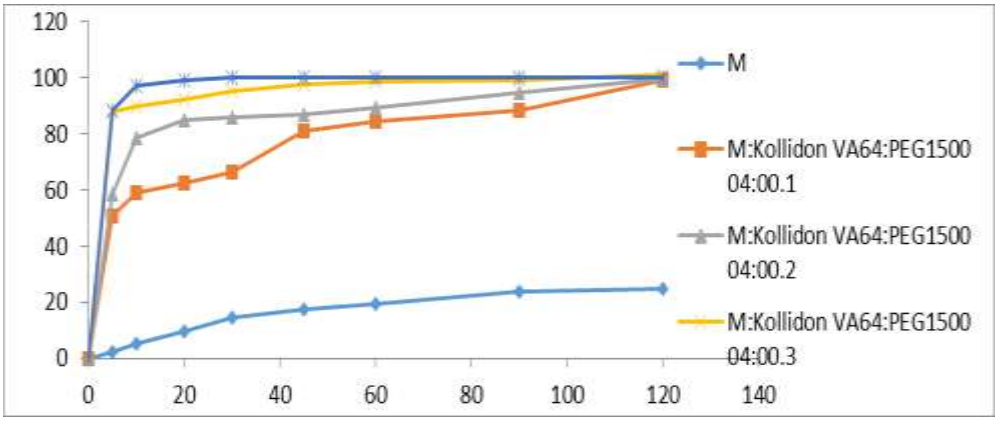

Fig:6. Dissolution data of pure drug and Hot Melt Extrudes of M:Kollidon VA 64: PEG 1500

IN VIVO STUDY PROTOCOL[4]

Pharmacokinetic And Bioavailability Evaluation Of Methotrexate

Hot Melt Extrudes of Methotrexate exhibited markedly higher dissolution rates and dissolution efficiency values when compared to the corresponding pure drugs and were found suitable for formulation into compressed tablets by direct compression technique. Methotrexate Hot Melt Extrudes were further subjected to *in vivo* pharmacokinetic and bioavailability assessment in rabbits. The following products were tested for in vivo performance.

1. Methotrexate
2. Methotrexate-M:KOLLIDON VA 64:PEG 1500Hot Melt Extrudes (1:4:0.4)

Calculation of Animal Equivalent Dose from Human Dose

As per the Guidelines to industry for conducting clinical trials in humans the first step towards the dose fixing is NO OBSERVED ADVERSE EFFECT LEVEL DETERMINATION (NOAEL) from which the human dose is calculated. The NOAEL studies are generally calculated using suitable animals such as Rat, Mice, or rabbits. From the obtained animal dose the Human Equivalent Dose (HED) is calculated using following equation.

$$HED = \frac{\text{Animal Dose (mg/kg) X Animal Weight (kg)}}{\text{Human Weight (kg)}^{0.33}}$$

To Calculate Animal Equivalent dose (AED) from Human Dose we can rewrite the above equation as follows

$$AED = \frac{\text{Human Dose (mg/kg)}}{\text{Animal weight (kg) X Human Weight (kg)}^{0.33}}$$

Using Above equation considering the average human weight as 70 kg, animal equivalent dose calculations were carried out

Weight of rabbits in kg = 1.5- 2.5

Human Dose of Drugs in mg: Methotrexate 10mg.

Calculated Animal Equivalent Dose (AED):

Methotrexate = 0.8mg/kg

Healthy rabbits of either sex (weighing 1.5 – 2.5 kg) were fasted overnight. Methotrexate and its Hot melt extrudes were administered at dose equivalent to 0.8 mg/kg of Methotrexate. Each product was repeated 4 times (n = 4). The *in vivo* experiments were conducted as crossover study as follows.

A group of 4 rabbits were given Methotrexate initially and after a washout period of one month, they were given with Methotrexate Hot Melt Extrude. After collecting the zero hour blood sample (blank), the product in the study was administered orally in a capsule (size 5) shell with 10 ml water. Blood samples (0.5ml) were collected from marginal ear vein at 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 12.0 h after administration. The blood samples were allowed to clot and centrifuged at 5000 rpm and the serum separated was collected into dry tubes. All the samples were stored under refrigerated conditions prior to assay. Serum concentration of the Methotrexate was determined by the HPLC methods. From the time versus serum concentration data various pharmacokinetic parameters such as peak concentration (C_{max}) , time at which peak occurred (T_{max}) area under the curve (AUC), elimination rate constant (K_{el}), biological half-life ($t_{½}$), percent absorbed to various times and absorption rate constant (K_a) were calculated in each case. The results are given in Table.

Estimation of Methotrexate Serum Samples

Methotrexate in serum samples was estimated according to High Performance Liquid Chromatographic (HPLC) method of Abdolhosein Moghbel *et. al.*,

Materials

1. Methotrexate (Gift sample from Ipca Laboratories, Mumbai)
2. Amino acetophenone (Gift sample from Dr.Reddy's Laboratories, Hyderabad)
3. Acetonitrile (HPLC grade, Qualigens)
4. Methanol (HPLC grade, Qualigens)
5. Glacial acetic acid (Excelar, Qualigens)
6. Distilled water (Tripel glass distilled)

Chromatographic Conditions

Instrument: Agilent 1200 Infinity series with PDA detector
Column : C-18 RP (ODS-A) 250 x 4.6 mm I.D; Particle size : 5 µm
Mobile Phase : Acetonitrile : water (55:45 v/v), pH adjusted to 2.95 with glacial acetic acid
Flow Rate: 1.5 ml/min
Injection volume: 20 µl
Detector: UV-VIS Spectrophotometric detector at 254 nm

Extraction Procedure

To 50 µl of internal standard (Aminoacetophenone, 5 *µg/ml* in methanol) and 4.45 ml of methanol was added and the tubes were vortex-mixed for 15 min and centrifuged at 5000 rpm for 10 min. The supernatant was transferred to clean tubes and evaporated to dryness. The residue was reconstituted with 0.5 ml mobile phase and 20 *µl* of the solution was injected into the HPLC system after filtering through 0.2 µ nylon membrane filter.

Calibration Curve

Standard solutions containing 0.5, 1.0, 2.0 3.0 and 4.0 µg/ml of Methotrexate were prepared in methanol. To each 50 µl of standard solution, 50 µl of internal standard [5] (Aminoacetophenone, 5 µg/ml in methanol), 500 µl of blank serum and 4.4 ml of methanol were added and the tubes processed as above. Standard curve was obtained by plotting peak area ratio of Methotrexate to internal standard vs. concentration. The results are given in **Table7 and Fig.7**.

Table:7. Calibration Curve for the Estimation of Methotrexate in Plasma by HPLC

Sr. No.	Amount of (ng) Methotrexate added to 1.0 ml serum	Mean (n = 5) ratio of peak area of Methotrexate to peak area of internal standard	Coefficient of variation (%)
1	50	0.420	1.10
2	100	0.845	1.42
3	200	1.679	0.99
4	300	2.514	1.87
5	400	3.487	1.88

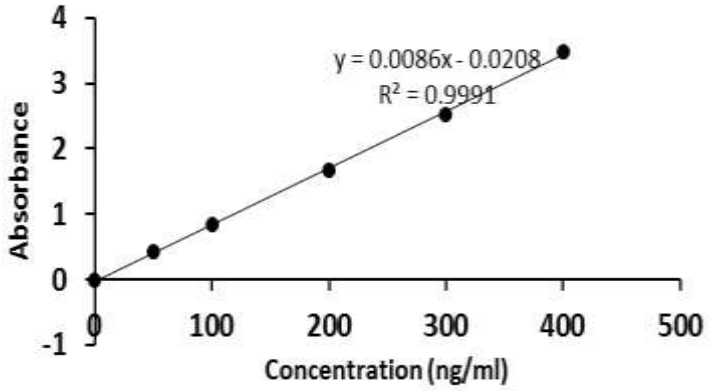

Fig.7. Calibration Curve for the Estimation of Methotrexate in Serum by HPLC

The serum concentration data following the administration of various products are given in **Table 8**. From the time versus serum concentration data various pharmacokinetic parameters such as peak concentration (C_{max}), time at which peal occurred (T_{max}), area under the curve AUC, elimination rate constant Kel, biological half life ($t_{½}$), percent absorbed to various times and absorption rate constant (K_a) were calculated in each case.

Determination Of Various Pharmacokinetic Parameters

Determination of C_{max} and T_{max}

From the time versus serum concentration curves, peak serum concentration (C_{max}) and time at which peak occurred (T_{max}) were recorded.

Determination of Elimination Rate Constant (K_{el}) and Biological Half-Life ($t_{½}$)

Time versus serum concentration data was plotted on a semi logarithmic graph paper as shown in Fig.7.2. The

elimination rate constant (K_{el}) was calculated from the slope of the linear line in the elimination phase (the best fit linear regression line for the points in the elimination phase was drawn by the method of least squares). The corresponding biological half-life was calculated using the equation $t_{1/2} = 0.693/K_{el}$.

Determination of Percentage Absorbed to Various Times and Absorption Rate Constant (K_a)

Percentage absorbed to various times and absorption rate constant (Ka) were calculated from serum concentration data by the method described by the Wagner and Nelson[2.3.] The equation developed for the determination of absorption rate from blood data is

$dA/dt = V_d . dC_b/dt + K_{el}.C_b$

Where dA/dt = Absorption rate

V_d = Apparent volume of distribution

dC_b/dt = Rate of change of blood concentration (C_b) at time t and

K_{el} = Elimination rate constant.

The equation may be integrated between the limits of t = 0 and t = T and divided by V_d to give,

$At/V_d = C_T + K_{el}. \quad Cb\ dt$

$At/Vd = C_T + K_{el}. \quad [AUC]$

Where A_t = Amount of drug absorbed to time t.

C_T = blood Concentration at time t and the quantity under the integral sign in the area under the blood concentration versus time curve between the indicated limits. When the successive values of At/ Vd are calculated, a maximum or asymptotic value $[A_T/V_d]_\infty$ is obtained. The maximum asymptotic value is divided into successive values of AT/Vd to yield percentage absorbed data i.e.

$(A_T/V_D) / [A_T/V_d]_\infty \ x\ 100$ as a function of time.

Estimation of Area Under the Curve [AUC]

The area under the time versus serum concentration by applying trapezoidal rule. The remaining area from 12 hours to ∞ time was calculated using the following equation,

$[AUC]_{12-\infty}$ = Concentration at 12th hour / K_{el}

Then $[AUC]_{0-\infty} = [AUC]_{0-12}\ hr + [AUC]_{12-\infty}\ hr$

Table: 8. Serum Concentration of Methotrexate and Methotrexate Hot Melt Extrudes Following their Oral Administration in Rabbits (n = 4)

Time (hr)	Serum Concentration (ng/ml) of Methotrexate (x ± s.d.,)	
	Methotrexate	Methotrexate Hot Melt Extrudes
0.25	2.80±1.12	64.48±2.31
0.50	4.70±1.25	73.98±2.65
1.0	6.68±2.35	75.59±3.02
2.0	8.30±3.02	71.90±3.54
3.0	10.19±3.54	66.86±2.89
4.0	12.43±2.89	61.69±3.54
6.0	9.49±2.84	49.95±2.87
8.0	7.09±1.76	37.59±2.69
12.0	3.68±2.38	19.24±2.57

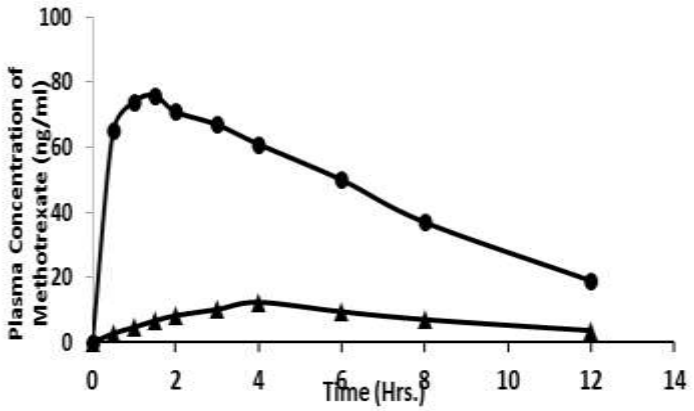

Fig: 8. Serum Concentration of Methotrexate and Methotrexate Hot Melt Extrudes Following their Oral Administration in Rabbits (n = 4)

Table: 9. Summary of Pharmacokinetic Parameters estimated following Oral Administration of Methotrexate and its Products

Sr. No.	Pharmacokinetic Parameter	Methotrexate Pure Drug	Methotrexate Hot Melt Extrudes
1	C_{max} (ng/ml)	12.09	76.21
2	T_{max} (hr)	4.29	1.02
3	K_{el} (hr^{-1})	0.159	0.158
4	$T_{½}$ (hrs)	4.35	4.38
5	$(AUC)_{0\rightarrow12}$ ng-hr/ml	91.55	588.31
6	$(AUC)_{0\rightarrow\infty}$ ng-hr/ml	114.33	709.31
7	K_a (hr^{-1})	0.4135	3.560

RESULTS AND DISCUSSION

Preparation of Hot Melt Extrudes

The Hot melt extrudes of methotrexate with various carrier ratios are prepared using R&D model Twin Screw Thermo Scientific™ Extruder. Methotrexate and carriers such as PEG 1500 and Kollidon VA 64 as per formulation **table 3** are mixed and heated to a temperature of 130^{O} C to ensure complete melting of drug & carriers. The molten mixture is then passed through twin screw extruder gradually to get drug carrier extrudes. The extrudes were obtained as long strands with white glossy appearance. All extrudes were prepared for about 100 doses. The obtained extrudes were collected in stainless steel trays, powdered using ball mill and kept in desiccator until further use. All extrudes with various ratios of drug and carrier is prepared with same procedure. Various step wise procedure involved in preparation of Hot Melt Extrudes were given in fig 2.

Dissolution Study of Prepared Hot Melt Extrudes

The hot melt extrudes of all formulation are evaluated for invitro dissolution studies. With both the carriers i.e . kollidon VA 64 and PEG 1500 with increase in carrier concentration the % drug dissolved increased significantly when compared to pure drug. Among the two carriers HME prepared using PEG 1500 at 1:4 ratio is 71.36% at the end of 10 minutes. where as in case of HME prepared using kollidon VA 64 at 1:4 ratio the % drug dissolved is 84.66%. in order to check the interaction effect of Kollidon VA 64 and PEG 1500 various formulations are prepared. Among all the formulations HME of methotrexate prepared using Kollidon VA 64 and PEG 1500 at ratio of 1:4:0.4 showed maximum % of drug release. The Q value of the formulation was achieved within 5 minutes and 100% drug release is achieved within 30 minutes. From the dissolution studies it is understood that the combination of two carriers Kollidon VA 64 and PEG 1500 has synergistic effect over dissolution of Methotrexate Hot Melt Extrudes. The formulation is further analyzed using invivo method and drug kinetics was studied in comparison with the pure drug.

In Vivo Study

The studies are carried out as per the guidelines given under NOAEL. A group of 4 rabbits were selected weighing between 1.5 to 2kg. The rabbits were suitable acclimatized by maintaining 20-25^{O} C of room temperature. The rabbits were kept above ground level in separate cages. After overnight fasting, after zero hour blood samples, pure drug was administered to all four rabbits. Suitable dose adjustments were made according to weight of the animal. . Blood samples (0.5ml) were collected from marginal ear vein at 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 12.0 h after administration. The blood samples were allowed to clot and centrifuged at 5000 rpm and the serum separated was collected into dry tubes. All the samples were refrigerated until assay.

After suitable wash out period (1 month) the same group of rabbits were given with best hot melt extrude (Methotrexate: Kollidon Va64 : PEG 1500, 1:4:0.4). same procedure was followed for collection of blood samples and analysis. Suitable HPLC method was used to analyze plasma concentration of methotrexate from pure drug and formulation. The method was developed using acetoaminophenone as internal standard. After suitable analysis of samples the plasma concentration of drug is estimated using method described in the procedure. From the results obtained various kinetic parameters such as Cmax (ng/ml), Tmax (hr), Kel (hr -1), T½ (hrs), (AUC)0→12 ng-hr/ml, (AUC)0→∞ ng-hr/ml, and Ka (hr-1) were estimated. The details of results were given **in table 9.** . From the evaluation studies a significant increase in all kinetic parameters were observed. The AUC of formulation increased 6.2 folds when compared to pure drug and the Ka increased about 8 folds.

CONCLUSION

The objective of present investigation was to sufficiently amplify dissolution rate and bioavailability of Methotrexate using Hot Melt Extrusion Method. Novel carriers such as kollidon VA 64 and PEG 1500, at least possible concentrations were used in successful preparation of Hot Melt Extrudes.

The dissolution study reveal that Hot Melt Extrudes follow first order drug release due to presence of more drug in solution. The bioavailability studies by *In-Vivo* method conclude that enhancing dissolution rate enhance bioavailability. From the *In-vivo* studies, the absorption of drug follows first order. Hence Hot Melt Extrudes of Methotrexate have met the objectives of the study. From the above it was concluded that the above mentioned

method hold promise for the enhancement of solubility and consecutive formulation.

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