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COMPARATIVE IN VIVO STUDY OF PURE DRUG AND FAST DISSOLVING TABLETS OF MEFENAMIC ACID

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ABSTRACT
The objective of this study was to investigate differences in the pharmacokinetic patterns between pure drug and an optimized formulation of fast dissolving tablets of Mefenamic acid. 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 highest mean C_{max} value was observed for optimized fast dissolving tablets (68.33 ± 0.42 ng/ml) compared to pure drug (27.72 ± 0.31ng/ml). The mean time taken to peak plasma concentration for *(Tmax)* following administration of pure drug was 11.53 ± 0.011 hours, while it was 6.09 ± 0.072 hour following administration of selected optimized fast dissolving tablets. The elimination rate constant (Kel) for pure drug and optimized fast dissolving tablets were found to be 0.58 ± 0.012 h^{-1} and 0.53 ± 0.014 h^{-1} respectively. The absorption rate constant (Ka) for pure drug and optimized fast dissolving tablets were found to be 1.68 ± 0.01 h^{-1} and 5.53 ± 0.02h^{-1} respectively. The $AUC_{0-\alpha}$ values observed with optimized fast dissolving tablets686.1.±2.07 ng hr/ml in compared to pure drug values 191 ± 1.43 ng hr/ml. Thus, the results of pharmacokinetic studies indicated rapid and higher oral absorption of Mefenamic acid when administered as its fast dissolving tablets. Both Ka and AUC were markedly increased by fast dissolving tablets.

KEYWORDS: LC-MS/MS, Mefenamic acid, fast dissolving, In-vivo studies, pharmacokinetic parameters, fast dissolving.

INTRODUCTION
Mefenamic acid is a non-steroidal anti-inflammatory drug (NSAID) used for relief of signs and symptoms of rheumatoid arthritis, osteoarthritis and is used in chronic and acute conditions of pain and inflammation. As its serum concentrations and analgesic effect are correlated, rapid absorption of Mefenamic acid could be a prerequisite for the quick onset of its action.[1] The major problems with drug are its very low solubility in biological fluids, gastric irritation and its short biological half-life of 2 h. It is practically insoluble in water and so possesses poor solubility and subsequent poor GI absorption and bioavailability. Thus rapid Mefenamic acid absorption could be a prerequisite for the quick onset of its action.[3] Because of its high membrane permeability characteristic, extent of Mefenamic acid absorption approaches up to 100%. Therefore, dissolution becomes the rate limiting step for absorption and the quick release of Mefenamic acid in the gastrointestinal tract following oral administration is desirable. The major problem of Mefenamic acid is its very low water solubility, which results into poor dissolution rate. The pharmacokinetic performance of Mefenamic acid fast dissolving tablets was studied in a comparison with that of pure drug in rabbits.[4]

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 animal ethics committee was obtained for conduction of experiments (Ref: BCOP /IAEC/I-7/ 2015-2016, Dated 19-2-2016). Pure Mefenamic acid and optimized fast dissolving tablets were selected based on in-vitro release studies and stability conditions were chosen as dosage forms for administration.

Subject selection
The pharmacokinetic performance of Pure Mefenamic acid and optimized Mefenamic acid fast dissolving tablets were studied in a randomized crossover study

design in rabbits. 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 animals. 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.[7,8] The study was conducted in a crossover design with 2 weeks washout periods in between the two experiments. The animal dose of Mefenamic acid was calculated relevant to human dose by using the following formula.[5,6] The above dosage form was administered through gastric intubation method.

Human dose of Mefenamic acid = 100mg

$$\text{Animal dose} = \frac{\text{Human dose} \times \text{Animal weight}}{\text{Human weight}}$$

= 100x3/70= 4.287 mg = 5mg

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 (before drug administration), 0.5, 1.0, 2.0, 3.0, 4.0 and 6.0 hrs after pure drug administration and at 0,1, 2, 4, 6, 8, 12, 16, 20, and 24 hrs after administration of Mefenamic acid fast dissolving tablets. The plasma was immediately separated by aspiration after centrifugation at 4000 rpm for 5 minutes and frozen at -20 ºC until analyzed by LC-MS/MS method.[7,8]

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 ($t_{½}$) and mean residence time (MRT) were calculated using the noncompartmental pharmacokinetics data analysis software PK Solutions 2.0™(Summit Research Services, Montrose, CO, USA).[9] 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 $AUC_{0-\alpha}$ value. All tests were performed at 0.001 level of significance.[10]

Estimation of Mefenamic acid in plasma (LC-MS/MS method[11]

Chromatographic conditions

A summary of the chromatographic and mass spectrometric conditions is as follows:

UPLC : WATERS

Mass spectrometer : API 2000

Ion source : Heated nebulizer

Polarity : Negative

Detection ions

Mefenamic acid : 240.2 * amu (parent), 196.0* amu (product)

Mefenamic acid –d4 : 244.2* amu (parent), 200.2 amu (product)

Column : Phenomenax, synergi 4 µ Polar- RP80 A, 4.6x75mm

Column oven temperature : 35° C

Peltier temperature : 10°C

Mobile phase : Ammonium formate: Acetonitrile (30:70)

Flow rate : 1ml/min.

Volume of injection : 10 µ L

Retention time : Mefenamic acid - 0.5 to 1.20 minutes

: ISTD- 0.5 to 1.20 minutes

Run time : 2.00 minutes

Preparation of working standard solutions

1. Preparation of Mefenamic acid standard stock solution

Mefenamic acid working standard equivalent to 5 mg Mefenamic acid was accurately weighed and transferred into a 5 ml volumetric flask and dissolved in methanol. The solution was made up to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Mefenamic acid. The solutions were labeled and stored in a cold store at 2-8°C.

2. Preparation of internal standard stock solution

0.005 g of Mefenamic acid –d4 was weighed accurately and transferred in to a 5 ml volumetric flask and dissolved in methanol. The solution was made up to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Mefenamic acid –d4. The solutions were labeled and stored in a cold store at 2-8°C. Stock solution was diluted with 60% Acetonitrile in water solution to get a concentration of 75.00µg/ml.

2. Calibration curve standards

1. Preparation of stock dilutions of standard Mefenamic acid solution

Stock solution of Mefenamic acid was diluted with 60% Acetonitrile in water solution to get a concentrations ranging from 1 to 140 µg/ml.

2. Spiking of plasma for calibration curve standards

Concentrations of Mefenamic acid ranging from 50 to 7000 ng/ml were prepared with plasma and labeled them as CC1 to CC8. The calibration curve standards were prepared freshly for each validation run.

3. Extraction procedure

Step 1: Blank, calibration curve standards and the subject samples were withdrawn from the deep freezer and allowed them to thaw. The thawed samples were vortexed to ensure complete mixing of contents. To 0.25 ml of plasma sample in a ria vial, 25ul of Mefenamic acid –d4 standard (75µg/ml) was added. To plasma blank, 25 ul of 60% Acetonitrile in water solution was added and vortexed the samples to ensure complete mixing of contents.

Step 2: Add 2.5 ml of tertitary butyl methyl ether, place on a shaker for 15 minutes and centrifuge for 10 minutes at 4000rpm at 20^{O}C. Transfer supernatant (organic layer) into the another rial vial. Evaporate this layer under a stream of nitrogen at 45^{O}C. The residue was reconstituted with 0.5 ml of mobile phase and vortexed. The samples were transferred in to auto-injector vials and loaded the vials in to auto sampler. 10 ul of sample was injected in to LC-MS/MS system. Analyte Concentrations of stock dilutions of standard Mefenamic acid solution with plasma were shown in Table 1.

4. Data processing

The chromatograms were obtained by using the computer-based Analyst 1.4.2 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 Mefenamic acid peak area and ISTD peak area (analyte area / ISTD area); x = Concentration of Mefenamic acid; m = Slope of the calibration curve; c = y-axis intercept value. Linear regression analysis equation of stock dilutions of standard Mefenamic acid solution with plasma is, y = 0.000136x + 0.000454.

RESULTS AND DISCUSSION

The *in vivo* experiments were conducted as per the protocol and procedure described earlier. 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 Mefenamic acid in plasma. Plasma concentrations of Mefenamic acid 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 indicated that the parameters significantly differed following optimized fast dissolving tablets administration, compared to pure drug administration. The highest mean C_{max} value was observed for optimized fast dissolving tablets (68.33 ± 0.42 ng/ml) compared to pure drug (27.72 ± 0.31ng/ml). The mean time taken to peak plasma concentration for *(Tmax)* following administration of pure drug was 11.53 ± 0.011 hours, while it was 6.09 ± 0.072 hour following administration of selected optimized fast dissolving tablets. The elimination rate constant (Kel) for pure drug and optimized fast dissolving tablets were found to be 0.58 ± 0.012 h^{-1} and 0.53 ± 0.014 h^{-1} respectively. The absorption rate constant (Ka) for pure drug and optimized fast dissolving tablets were found to be 1.68 ± 0.01 h^{-1} and 5.53 ± 0.02h^{-1} respectively. The $AUC_{0-\alpha}$ values observed with optimized fast dissolving tablets 686.1.±2.07 ng hr/ml in compared to pure drug values 191 ± 1.43 ng hr/ml.

Table 1: Analyte Concentrations of Stock Dilutions of Standard Mefenamic acid 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	Plasma Blank	0	0	0	0	N/A	N/A
2	Blank+ISTD	0	254	513643	0	N/A	N/A
3	CC1	50.05	3879	528938	0.01	50.407	100.71
4	CC2	100.15	6997	506198	0.01	97.939	97.79
5	CC3	250.300	17284	484351	0.04	258.119	103.12
6	CC4	500.650	34646	504084	0.07	500.215	99.91
7	CC5	1001.250	77371	577631	0.13	978.007	97.68
8	CC6	2002.500	134349	520675	0.26	1887.085	94.24
9	CC7	5006.250	345626	529110	0.65	4782.401	95.53
10	CC8	7008.750	578109	544120	1.06	7780.670	111.01

Table 2: Plasma Concentrations of Mefenamic acid following oral administration of Pure Mefenamic acid and optimized fast dissolving tablets.

Time (h)	Plasma concentration (ng/ml) (Mean ± s.d)	
	Pure drug	Mefenamic acid fast dissolving tablets
0	0	0
0.5	08.51 ±1.86	28.23 ±1.36
1	11.35±1.74	36.44±1.78
1.5	12.51±1.52	42.20±1.56
2	14.61±1.14	45.24±1.24
3	16.75±1.64	49.62±1.12
4	18.81±1.35	54.12±1.65
5	19.62±1.43	61.18±1.67
6	21.93±1.24	68.33±1.85
8	22.76±1.43	62.15±1.72
10	24.90±1.24	57.42±1.22
12	27.72±1.27	49.23 ±1.36
14	24.64±1.36	42.20±1.43
16	21.96±1.53	35.24±1.64
18	18.84±1.32	28.53±1.18
20	15.92±1.21	23.12±1.62
24	12.28±1.39	17.18±1.47

Table 3: Statistical Treatment of Pharmacokinetic Parameters (Mean ± S.D.) of following oral administration of Pure Mefenamic acid and optimized fast dissolving tablets.

Pharmacokinetic parameter	Pure Drug	Optimized fast dissolving tablets	Calculated value of 't'
C_{max} (ng/ml)	27.72 ± 0.31	68.33 ± 0.42	26.70***
$t_{1/2}$ (h)	11.53 ± 0.011	6.09 ± 0.072	40. 75***
K_{el} (h^{-1})	0.58 ± 0.012	0.53 ± 0.014	6.87***
K_a (h^{-1})	1.68 ± 0.01	5.53 ± 0.02	19.67***
$AUC_{0-\square}$ (ng h/ml)	191 ± 1.43	686.1.±2.07	256.60***
Null hypothesis (H_o): There is no significant difference between the pharmacokinetic parameters of mefenamic acid obtained with pure drug and optimized fast disolving tablets. Table value of 't' with 10 DF at the 0.001 level is 4.587.			
Result: H_o is not accepted as the calculated 't' value more than the table Value of't' with 10 DF at 0.001 levels of significance. It was therefore concluded that there was significant difference between the pharmacokinetic parameters of obtained with pure drug and optimized fast disolving tablets.			

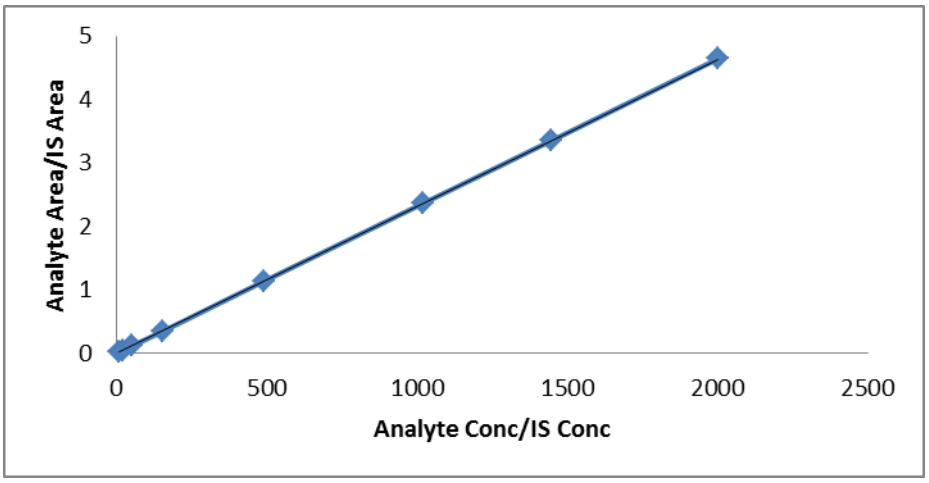


Figure 1: Calibration Curve for Estimation of Mefenamic acid in Plasma.

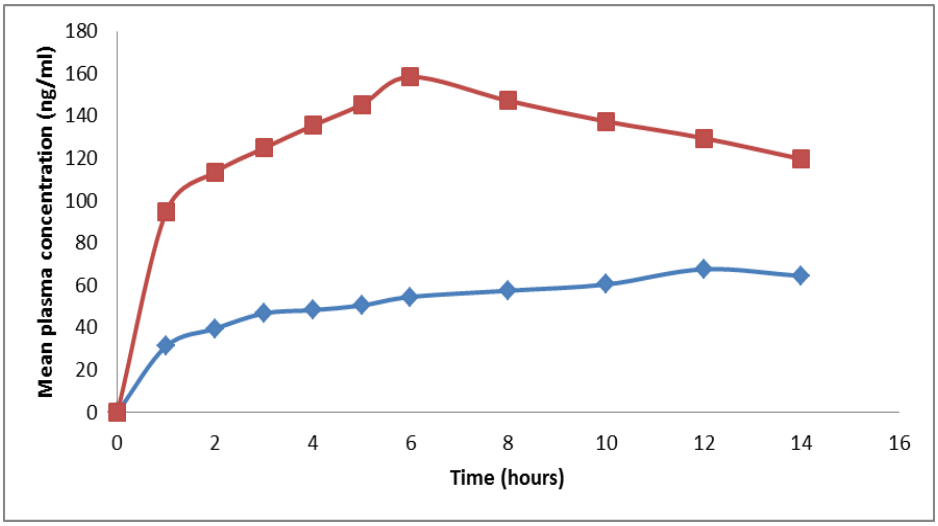
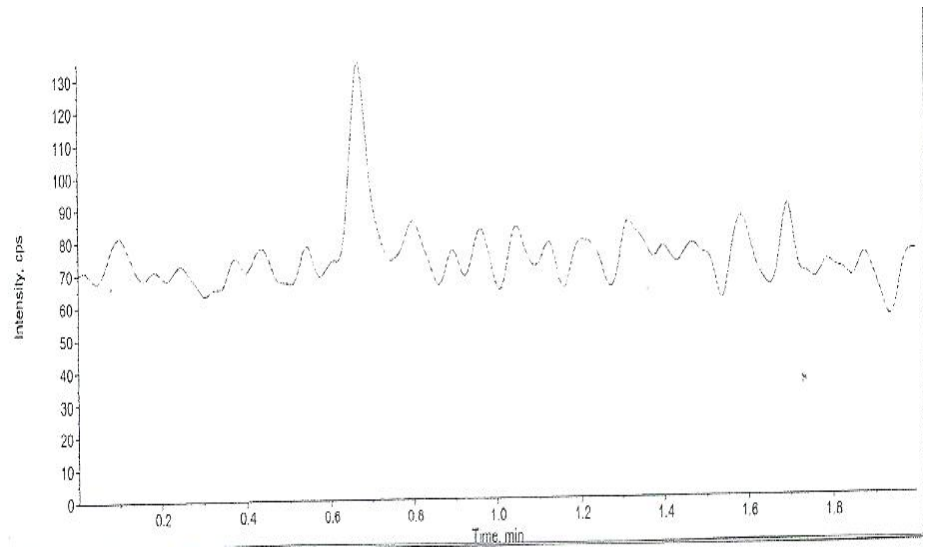


Figure 2: Concentration-Time Curve of Mefenamic acid following oral administration of Pure Mefenamic acid and optimized fast dissolving tablets.
(-♦-)plasma Concentration -Time Curve of mefenamic acid following pure drug administration
(-■-)plasma Concentration -Time Curve of mefenamic acidfollowing optimized fast dissolving tablets administration.

Figure 3: Chromatograms of reserve stock solution of Standard Mefenamic acid Solution with Plasma.

Sample Name:Plasma blankMefenamic acid Mass(es):240.2/196.0amu Concentration: 0.000 ng/ml Calculated conc:N/A ng/ml Proc.Algorithm: Analyst classic Bunching factor:2 Noise Threshold:10.00 Area Threshold :100.00 Num Smooths :10 Sep .Width :0.20 Sep. Height:0.01 Exp .Peak Ratio:5.00 Exp.Adj Ratio:4.00 Exp .Val Ratio :3.00 RT Window :30.0 Expected RT:2.00 Use Relative RT:False Int. Type: No Peak Retention Time:0.00 Area: 0 Height:0 Area Ratio:0.000 Start Time:0.00 End Time:0.00	

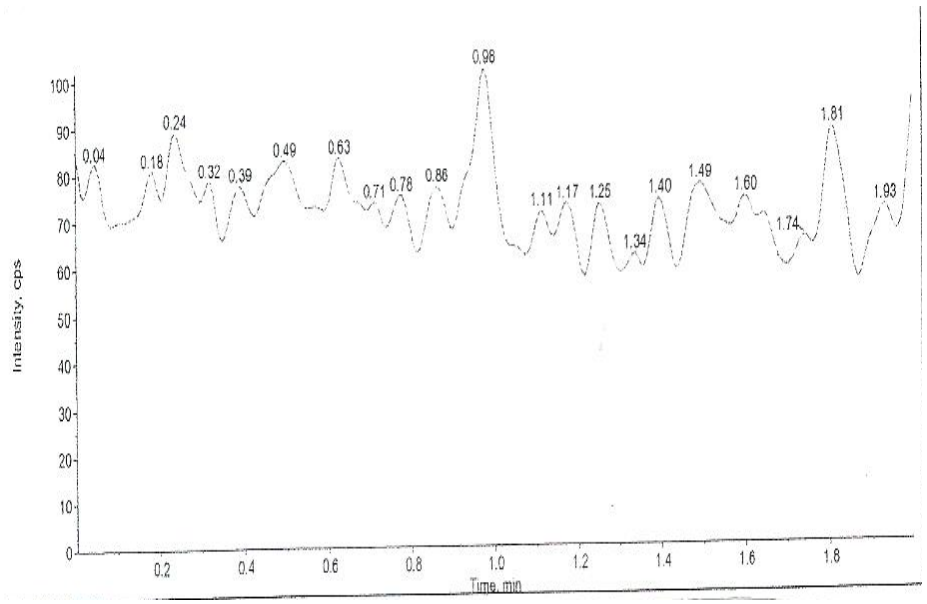
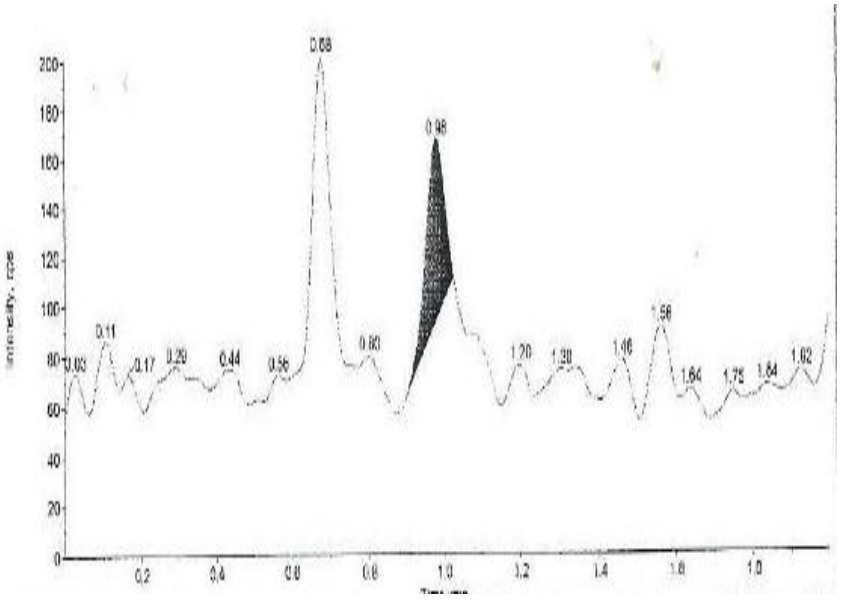
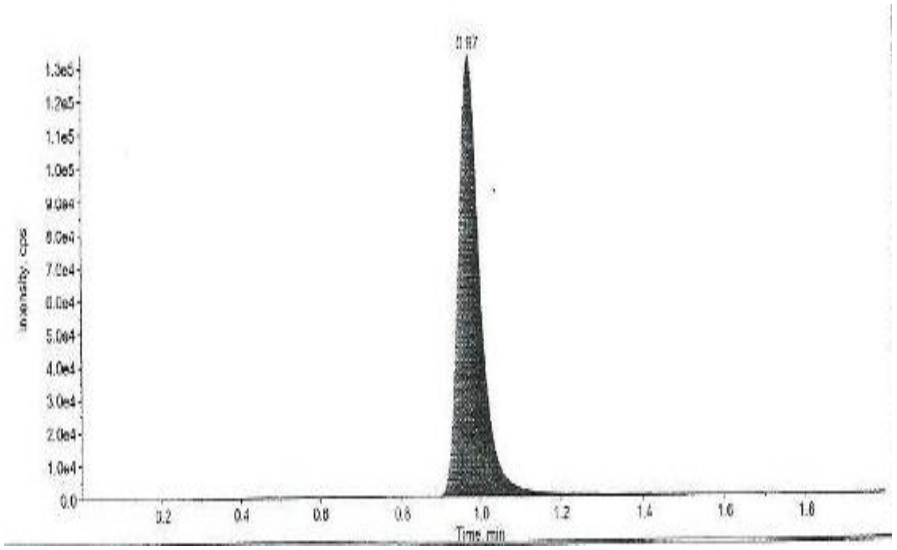
Sample Name: Plasma blank-Mefenamic acid-d4 Mass(es):244.2/200.2 amu Concentration: N/A ng/ml Calculated conc: N/A ng/ml Proc.Algorithm: Analyst classic Bunching factor:1 Noise Threshold:20.00 Area Threshold :200.00 Num Smooths :10 Sep .Width: 0.20 Sep. Height:0.01 Exp .Peak Ratio: 5.00 Exp.Adj Ratio: 4.00 Exp .Val Ratio :3.00 RT Window : 30.0 Expected RT: 1.80 Use Relative RT:False Int. Type: Base To Base Retention Time:1.85 Area:416 Height:125 Start Time:1.79 End Time:1.90	

Figure 4: Chromatograms of Plasma blank and internal standard (Mefenamic acid-d4)

Sample Name: Plasma blank+ISTD Mass(es): 240.2/196.0amu Concentration: 0.000 ng/ml Calculated conc:N/A ng/ml Proc.Algorithm: Analyst classic Bunching factor: 2 Noise Threshold: 10.00 Area Threshold :100.00 Num Smooths :10 Sep .Width: 0.20 Sep. Height: 0.01 Exp .Peak Ratio: 5.00 Exp.Adj Ratio: 4.00 Exp .Val Ratio :3.00 RT Window :30.0 Expected RT: 2.00 Use Relative RT:False Int. Type: No Peak Retention Time:0.00 Area: 0 Height: 0 Area Ratio: 0.00 Start Time:0.00 End Time:0.00	

Sample Name: Plasma blank+ISTD - Mefenamic acid-d4 Mass(es): 244.2/200.2 amu Concentration: 1.000 ng/ml Calculated conc:N/A ng/ml Proc.Algorithm: Analyst classic Bunching factor:1 Noise Threshold:20.00 Area Threshold :200.00 Num Smooths :10 Sep .Width:0.20 Sep. Height:0.01 Exp .Peak Ratio:5.00 Exp.Adj Ratio:4.00 Exp .Val Ratio :3.00 RT Window :30.0 Expected RT :0.89 Use Relative RT:False Int. Type: Base To Base Retention Time:0.97 Area: 513643 Height: 1.3e5+0.005 Start Time:0.89 End Time: 1.03	

CONCLUSION

The results of pharmacokinetic studies indicated rapid and higher oral absorption of Mefenamic acid when administered as its fast dissolving tablets. Both Ka and AUC were markedly increased by fast dissolving tablets.

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