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Determination of Pharmacokinetic Parameters of Sulfasalazine Enteric Coated Tablets In Human Healthy Volunteers

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ABSTRACT

To establish an HPLC method for the determination of sulfasalazine in human plasma and to study the pharmacokinetics in Indian healthy volunteers following oral administration of sulfasalazine enteric-coated tablets. In the study, 08 volunteers were administered with single oral dose of 500mg of sulfasalazine enteric-coated tablets with marketed reference product. The plasma concentrations of sulfasalazine were determined by HPLC-UV method. Chromatography was carried out on C-18 column with mobile phase comprising methanol and ammonium acetate buffer (pH 7.0) in the ratio of 48:52 pumped at a flow rate of 0.8 ml min^{-1}. The retention time for sulfasalazine were 12.2±0.05 min, the pharmacokinetic parameters were calculated with aid of the software DAS2.1.1. The calibration curve of sulfasalazine was linear in the range from 1.00 to 10.00µg/ml (r^2=0.998). The main pharmacokinetic parameters of sulfasalazine enteric coated tablets with marketed reference product were as follows: half life were (7.51±0.54) and (7.32±0.72) h, C_{max} were (7.7125±1.0125) and (7.6±0.30)µg/ml, T_{max} were (6.38±0.62) and 6.38±0.62)h, AUC(0~t) were (84.92±20.25) and (79.39±19.45)µg·h/ml, respectively.

Keywords: Sulfasalazine, methanol, ammonium acetate buffer, HPLC-UV method, $t_{1/2}$, C_{max}, T_{max}, and AUC (0~t).

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INTRODUCTION

To compare the relative bioavailability and characterize the pharmacokinetic profile of test product with respect to the Reference product in normal, healthy, adult, human male subjects under fasting conditions, based on the statistical results of 90% confidence interval for the ratio of the geometric least squares means for In-transformed pharmacokinetic parameters i.e. C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ conclusion would be drawn for the bioavailability of Test Product-T Vs Reference Product-R under fasting condition. Bioequivalence of the test product with that of the reference product under fasting conditions will be concluded if the 90% confidence interval falls within the acceptance range of 80.00–125.00% for In-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$.

Sulfasalazine, chemically2-Hydroxy-5-[(*E*)-2-{4-[(pyriyl)sulfamoyl]phenyl} diazen-1-yl]-benzoic acid.[1] Sulfasalazine is the most used drug for the treatment of inflammatory bowel disease, the long term treatment of ulcerative colitis and chron's disease. It's recommended dose starts at 500 mg once daily and is increased up to 2–3 g per day divided into 2–3 doses over 3–11 months to achieve therapeutic efficacy. Sulfasalazine is a conjugate of 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP) linked by an azo bond. Following oral administration, sulfasalazine is metabolized by bacterial azoreductase enzymes in the colon, reducing the azo bond and releasing these two components. Sulfasalazine itself may serve only as a prodrug to deliver the metabolic products, 5-ASA (a possible anti-inflamatory agent) and SP (an antibacterial agent), to the colon.[2]

MATERIALS AND METHOD

In-vivo Bioavailability study of Sulfasalazine Enteric coated tablets in healthy human subjects:

This part of the work demonstrates the Bioavailability study for the comparison of Sulfasalazine 500 mg Enteric coated tablets with respect to the Reference formulation azulfidine (Sulfasalazine 500 mg EN tablets) in normal, healthy, adult, human male subjects under fasting conditions. The study was conducted at S.R.L institute of pharmaceutical sciences, Warangal, Telangana, India. Before conducting the study, a detailed design protocol was approved by IEC (Independent Ethical Committee). Analysis of Sulfasalazine was conducted using validated HPLC analytical method.

Selection of subjects

Eight non-smoker healthy adult, human male subjects between 18 and 45 years of age, having a Body Mass Index (BMI) between 18.5 and 30.0kg/m^2 and body weight not less than 50 kg were selected who are living in India. Healthy individuals were evaluated by personal history, medical

history and general clinical examination. Vital parameters such as BP should be within the range of 100-139 mmHg systolic and 60–89 mmHg diastolic, Pulse rate should be within the range of 60–100/min, Oral temperature between 97.8˚F-99.0˚F (to be performed on the day of check-in during each period).

Subjects were excluded from the study if subjects were having history of any major surgical procedure in the past 3 months, history of diabetes mellitus, tuberculosis and systemic hypertension, history disorders of cardiac, gastrointestinal, respiratory, hepatic, renal, endocrine, neurological, metabolic, psychiatric or hematological systems, subjects who had participated in any other clinical study during the last 3 months and history of hypersensitivity to Sulfasalazine and related drugs or other excipients in the formulation.

Study design and conditions:

This was a single dose, randomized, open label, balanced, two treatments, two periods, two sequences, two ways cross over study. The comparative bioavailability study was conducted under fasting condition with a washout period of 7days.

The order of receiving the test and reference product for each subject during the two treatment period of the study are determined according to a randomization. The randomization of subjects under fasting conditions was given in Table 1.

Table 1: Randomization of subjects

Subject No	Sequence	Period I	Period II
1001	TR	T	R
1002	RT	R	T
1003	TR	T	R
1004	RT	R	T
1005	TR	T	R
1006	TR	T	R
1007	RT	R	T
1008	RT	R	T

Blood sampling:

The blood samples were collected through indwelling cannula placed in a forearm/arm vein of the subjects. A total of 13 blood samples (10 ml of blood sample) was collected from each subject in each phase of study i.e. pre dose 0.0 and at 1.00, 2.00, 3.00, 4.00 5.00, 6.00, 7.00, 8.00, 12.00, 18.00, 24.00, 36.00 and 48.00 hours after dosing in heparinized tubes and immediately placed on ice. Sulfasalazine from the plasma was extracted using protein precipitation extraction method. The samples were centrifuged within 30mins of sample collection at 3000rpm for 5mins at room temperature. The resulting plasma samples were stored at -20^{0}C until analysis.

Preparation of Internal standard:

Piroxicam was used as internal standard and it was prepared by dissolved in a 1:1 methanol-ethanol solution to prepare 5µg/ml stock solution. The methanol-ethanol mixture also served as deproteinizing solvent for the plasma samples.[3]

Plasma sample preparation:

Samples for drug analysis were prepared by transferring 100µl of plasma and 150µl of internal standard (Piroxicam) solution into micro centrifuge tubes. The tubes were vortex-mixed for 5 minutes and the extracts centrifuged at 14000 rpm for 10 minutes. The clear supernatants were aspirated and filtered through a 0.20 µm syringe filter followed by direct injection onto the HPLC system.

Method Development:

Various chromatographic conditions were experimented to achieve better efficiency of the chromatographic system. Conditions such as mobile phase composition, wavelength of detection, column, column temperature, pH of mobile phase, and diluents were optimized. Several proportions of buffer, and solvents (water, methanol and ammonium acetate buffer) were evaluated in order to obtain suitable composition of the mobile phase. Selection of retention time, tailing, theoretical plates, and run time were the major tasks while developing the method. [4]

The column INERTSIL C_{18} (250mm X 4.6mm, 5µ) has shown peak broadening. Chromolith TM Performance RP-18e (50mm×4.6mm, 5µ) column had shown good resolution and efficacy.[5, 6]

The composition of mobile phase 0.01M Ammonium acetate of pH 5.0 & Acetonitrile with flow rate of 1ml/min of runtime of 15 min yielded peaks with non-sink in the base line with unstable retention times.

At 48:52 (methanol: Ammonium acetate buffer pH 7.0) ratio of the mobile phase, a perfect peak was eluted. Thus the mobile phase ratio was fixed at 48:52 (methanol: Ammonium acetate buffer) in an isocratic mobile phase flow rate.[7, 8]

Initially 1.0 ml/min flow rate was tried but the resolution was poor. Finally 0.8 ml/min flow rate has yielded good result. The optimized conditions were illustrated in Table 2.

Table 2: Optimized chromatographic conditions for proposed method

Parameters	Method
Stationary phase (column)	Chromolith TM Performance RP-C_{18} (50mm×4.6mm, 5 µ) column
Mobile Phase	methanol: Ammonium acetate buffer P^H 7.0 (48:52 % v/v)
pH	7.0 ± 0.02
Flow rate (mL/min)	0.8

Run time (minutes)	15.0
Retention Time (R_t) (minutes)	12.8
Column temperature (°C)	30
Volume of injection loop (µL)	20
Internal standard	Piroxicam
Diluent	Mobile phase

Analysis of Plasma Samples:

Plasma samples of Sulfasalazine were analyzed by using validated HPLC method.

Reagents and standards used:

The reagents and standards used for the analysis of Sulfasalazine were Ammonium acetate, Acetonitrile, methanol & sulfasalazine. All the other reagents were used of analytical grade.

Instrumentation:

A waters alliance 2695 XE separation module with a UV detector was used. The data was acquired and processed by means of Empower chromatography software. Chromatographic separation was achieved by a Chromolith TM Performance RP-18e 50mm×4.6mm column protected by a Chromolith TM Guard Cartridge RP-18e 5mm×4.6 mm. For the mobile phase, a mixture of methanol: Ammonium acetate buffer (48:52, v/v) adjusted to pH 7.0 by Tri ethanol amine was delivered in isocratic mode at 0.8 ml/min flow rate.

Preparation of calibration curve:

Starting from pooled stock solution of Sulfasalazine (10mg/ml) in water, standards were prepared using pooled human drug free plasma obtained from healthy volunteers as diluent. The calibration curve was performed with standards of the final concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/ml in human plasma. The concentrations of calibration curve standards and corresponding peak area ratio (PAR) were given in Table 3 and the Graph of Concentration Vs peak area ratio (PAR) was plotted and shown in Figure 1 and chromatogram of Sulfasalazine was shown in Figure 2

Table 3: Calibration curve data for Sulfasalazine

Concentration (µg/ml)	Peak area ratio (PAR)
0	0
1	77
2	143
3	235
4	320
5	394
6	455
7	536
8	621
9	698

10	802
Correlation Coefficient	0.998

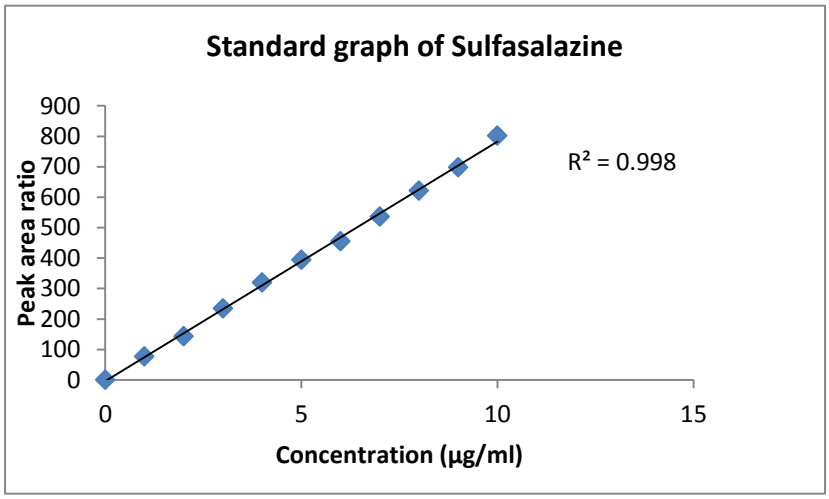

Figure 1: Standard curve for Sulfasalazine

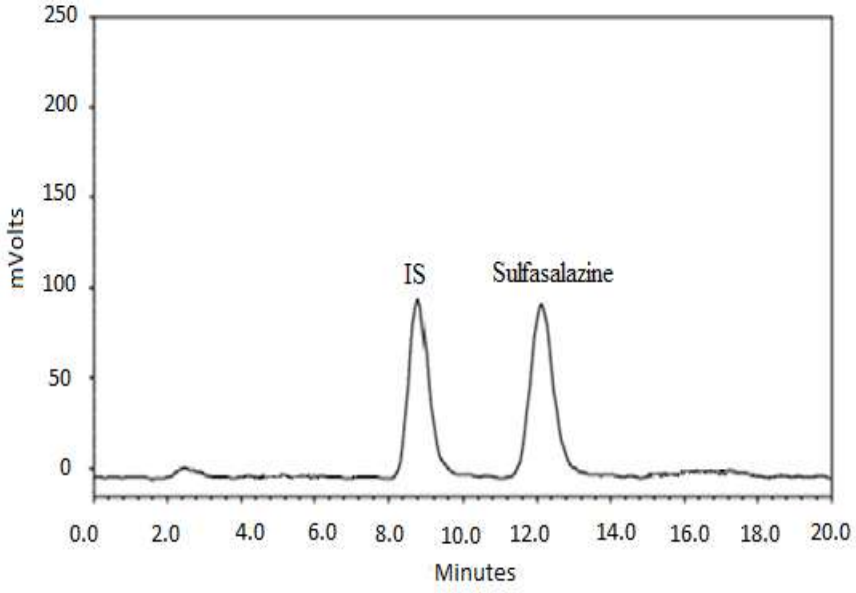

Figure 2: Chromatogram of Sulfasalazine

Pharmacokinetic data analysis:

Sulfasalazine plasma concentration-time data were analyzed for each subject using non-compartmental model of Winnonlin version 5.3 for each period. Basic pharmacokinetic parameters are required for the establishment of bioequivalence, such as Maximum measured plasma concentration following each treatment (C_{max}), The area under the plasma concentration versus time curve from time zero to the last measurable concentration (AUC_{0-t}), The area under the plasma concentration versus time curve from time zero to infinity ($AUC_{0-\infty}$) and time to reach maximum plasma concentration (T_{max}).

RESULTS AND DISCUSSION

The stable optimized formulation was selected for Bioavailability study in human healthy subjects

The drug-plasma concentrations obtained from the bioavailability study for Test and Reference products were analyzed in each subject by HPLC method and calculated the basic Pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{½}$. The individual plasma concentrations and means of Test and Reference products in each subject were given in Table 4-5. The plots of comparative individual plasma concentrations of Test and Reference products in each subject were shown in Figure 3-10. Descriptive statistics of formulation means for Sulfasalazine were given in Table 6 and plot of comparative mean plasma concentrations of Test and Reference products was shown in Figure 11. Comparison of relative bioavailability for Test and Reference products were given in Table 7.

The arithmetic mean (SD) of C_{max} for the Test and Reference products were 7.7125 (1.0125) and 7.6 (0.30) µg/ml, T_{max} for the Test and Reference products were 6.38 (0.62) and 6.38 (0.62) hours, AUC_{0-t} for the Test and Reference products were 84.92 (20.25) and 79.39 (19.45) µg.h/ml and $AUC_{0-\infty}$ for the Test and Reference products were 102.47 (25.65) and 94.87 (18.33) µg.h/ml. Bioequivalence of the test product with that of the reference product under fasting conditions was concluded by the calculation of 90% confidence interval for the In-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ for Sulfasalazine. The 90% confidence intervals for C_{max} were 98.45-112.58, for AUC_{0-t} were 95.32-115.33 and for $AUC_{0-\infty}$ were 94.14-111.46. The calculated confidence intervals were meeting the bioequivalence criteria (within the limit of 80 - 125) with respect to the rate and extent of absorption for Sulfasalazine under fasting conditions.

Table 4: Individual and Mean plasma concentrations of Sulfasalazine after administration of Test product-T in each subject

Individual and Mean Plasma Concentration of Sulfasalazine After Administration of Test Product - T Concentration (µg/ml)															
Subject	**Sequence**	**Period**	**Time (hrs)**												
			0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**12**	**18**	**24**	**36**	**48**
1001	TR	1	0	0.05	0.07	1.2	2.1	4.8	6.7	8.6	8.4	6.5	5.4	3.2	1.6	0.4
1002	RT	2	0	0.04	0.05	1.1	4.6	6.5	8.1	7.8	6.9	6.1	4.5	2.9	1.4	0.2
1003	TR	1	0	0.02	0.03	0.9	3.9	5.1	6.2	6.9	6.6	5.7	4.9	3.2	1.6	0.3
1004	RT	2	0	0.03	0.05	1.2	4.2	8.4	7.6	7.1	6.6	4.9	4.1	3.3	1.8	0.4
1005	TR	1	0	0.01	0.02	0.9	2.9	4.8	6.7	6.5	6.1	4.8	3.9	2.7	1.6	0.2
1006	TR	1	0	0.02	0.04	1.6	2.7	4.5	5.9	7.5	7.1	5.7	4.5	3.3	2.1	0.5
1007	RT	2	0	0.02	0.06	1.8	3.7	5.6	8.2	7.8	6.9	5.4	4.3	3.1	2.3	0.4
1008	RT	2	0	0.01	0.03	1.7	2.9	4.8	6.1	7.3	6.9	5.1	3.8	2.9	1.2	0.1
N			8	8	8	8	8	8	8	8	8	8	8	8	8	8
Mean			0	0.02	0.0438	1.3	3.375	5.563	6.938	7.438	6.938	5.525	4.425	3.075	1.7	0.313
SD			0	0.01	0.01	0.12	0.06	0.68	0.84	1.83	1.54	1.31	1.26	1.19	0.17	0.12
Min			0	0.01	0.02	0.9	2.1	4.5	5.9	6.5	6.1	4.8	3.8	2.7	1.2	0.1
Median			0	0.03	0.04	1.2	3.7	6.5	6.7	7.3	7.1	5.1	4.5	2.9	1.8	0.3
Max			0	0.05	0.07	1.8	4.6	8.4	8.2	8.6	8.4	6.5	5.4	3.3	2.3	0.5

Table 5: Individual and Mean plasma concentrations of Sulfasalazine after administration of Reference product-T in each subject

Individual and Mean Plasma Concentration of Sulfasalazine After Administration of Reference Product - T Concentration (µg/ml)															
Subject	**Sequence**	**Period**	**Time (hrs)**												
			0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**12**	**18**	**24**	**36**	**48**
1001	TR	1	0	0.01	0.04	0.9	1.8	4.2	5.9	7.9	7.1	5.6	4.1	2.2	1.1	0.2
1002	RT	2	0	0.01	0.03	0.8	2.7	5.4	7.7	6.8	6.1	5.4	3.9	2.3	1.1	0.1
1003	TR	1	0	0.01	0.03	0.7	3.6	5.5	6.8	7.5	6.9	5.5	4.6	2.8	1.3	0.2
1004	RT	2	0	0.01	0.02	1.1	3.9	7.7	6.9	6.3	5.7	4.3	3.6	2.5	1.5	0.2
1005	TR	1	0	0.02	0.03	1.2	3.1	5.3	7.5	7.1	6.4	4.9	3.6	2.3	1.4	0.1
1006	TR	1	0	0.01	0.03	1.3	2.3	4.1	5.7	7.4	6.6	5.1	4.1	2.9	1.7	0.3
1007	RT	2	0	0.01	0.04	1.7	3.5	5.4	7.6	7.1	6.1	4.8	3.7	2.5	1.7	0.1

1008	RT	2	0	0.01	0.02	1.5	2.7	4.4	6.3	7.5	6.5	4.5	3.3	2.3	0.8	0.1
N			8	8	8				8	8	8	8	8	8	8	8
Mean			0	0.01	0.03	1.15	2.95	5.25	6.8	7.2	6.425	5.013	3.863	2.475	1.325	0.163
SD			0	0.01	0.01	0.12	0.07	0.68	0.92	1.77	1.58	1.29	1.18	1.17	0.15	0.13
Min			0	0.01	0.02	0.7	1.8	4.1	5.7	6.3	5.7	4.3	3.3	2.2	0.8	0.1
Median			0	0.01	0.03	1.1	3.1	5.4	6.8	7.1	6.5	4.9	4.1	2.5	1.1	0.2
Max			0	0.02	0.04	1.7	3.9	7.7	7.7	7.9	7.1	5.6	4.6	2.9	1.7	0.3

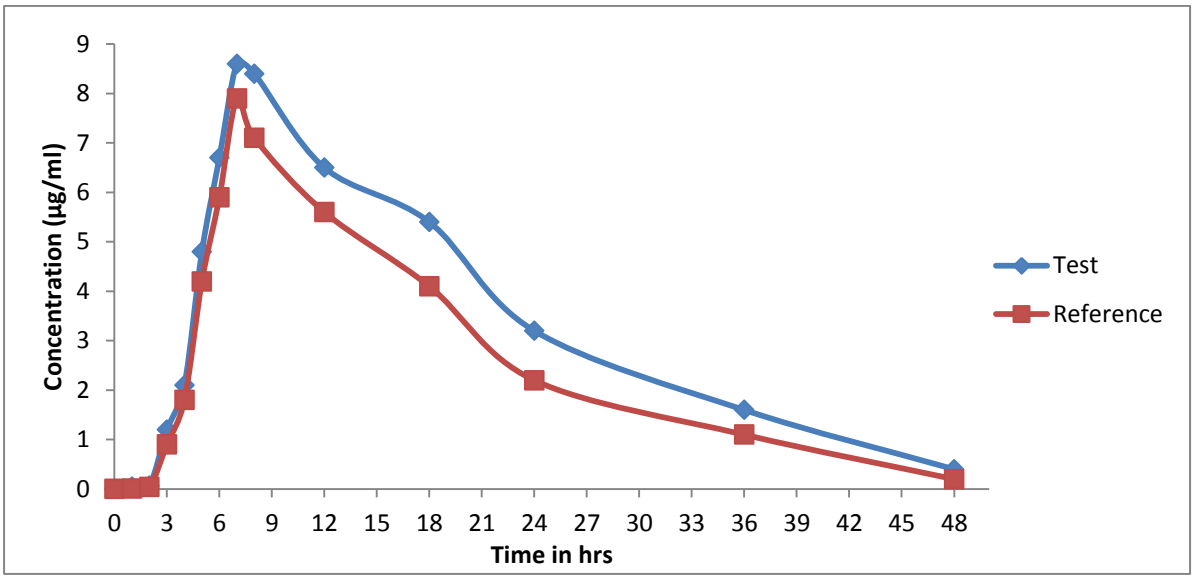

Figure: 3. Individual plasma concentrations of test and reference in subject 1001

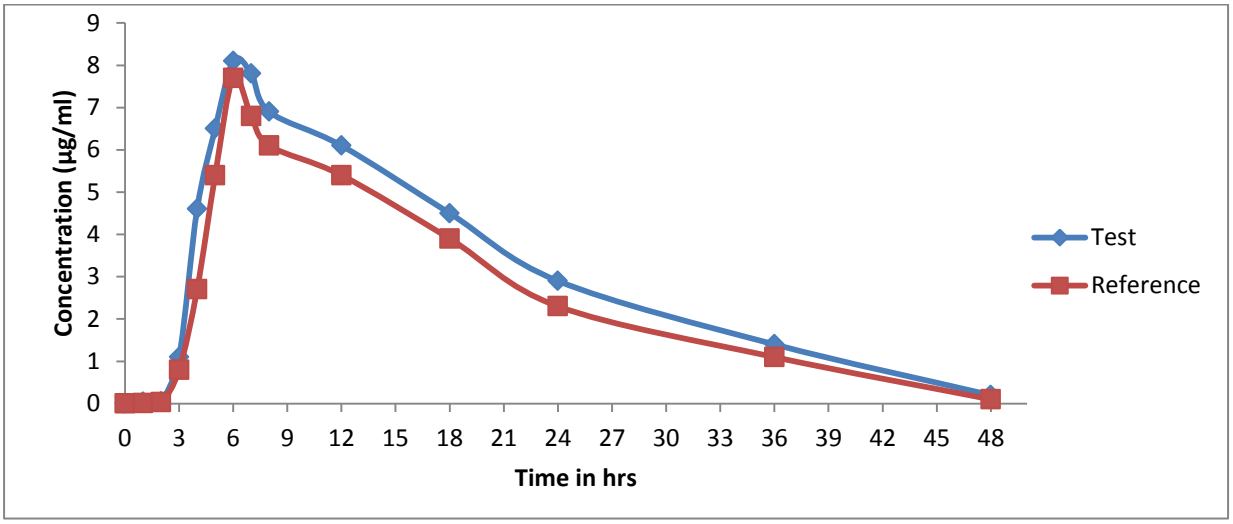

Figure: 4. Individual plasma concentrations of test and reference in subject 1002

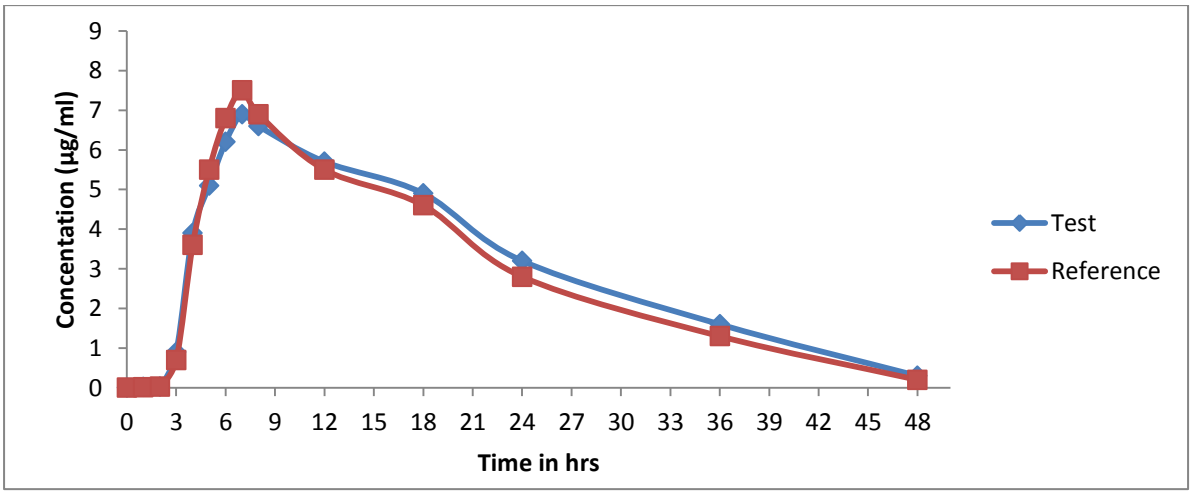

Figure: 5. Individual plasma concentrations of test and reference in subject 1003

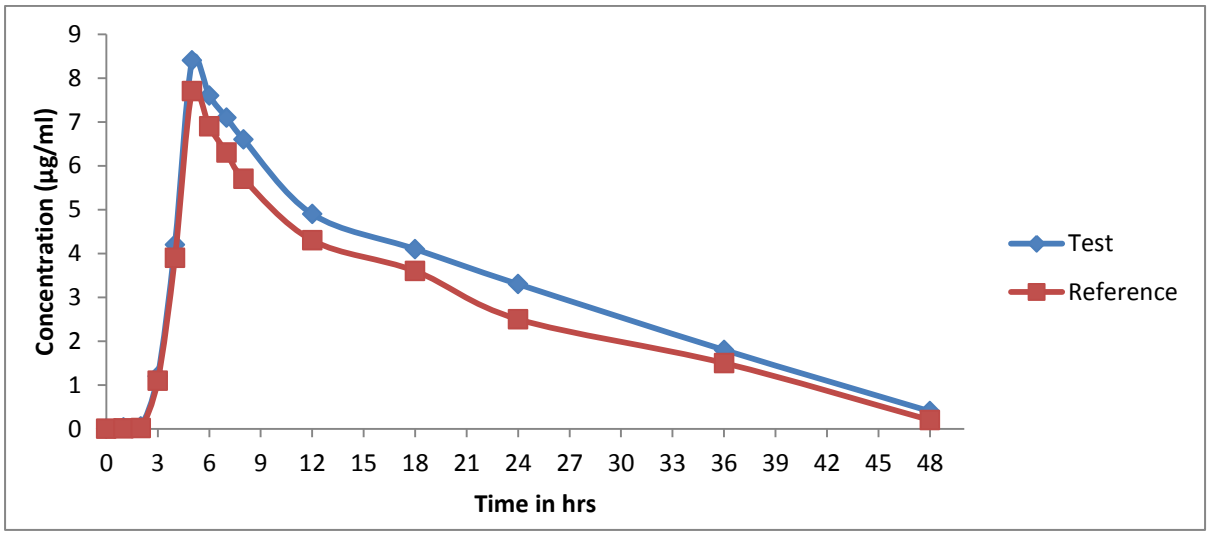

Figure: 6. Individual plasma concentrations of test and reference in subject 1004

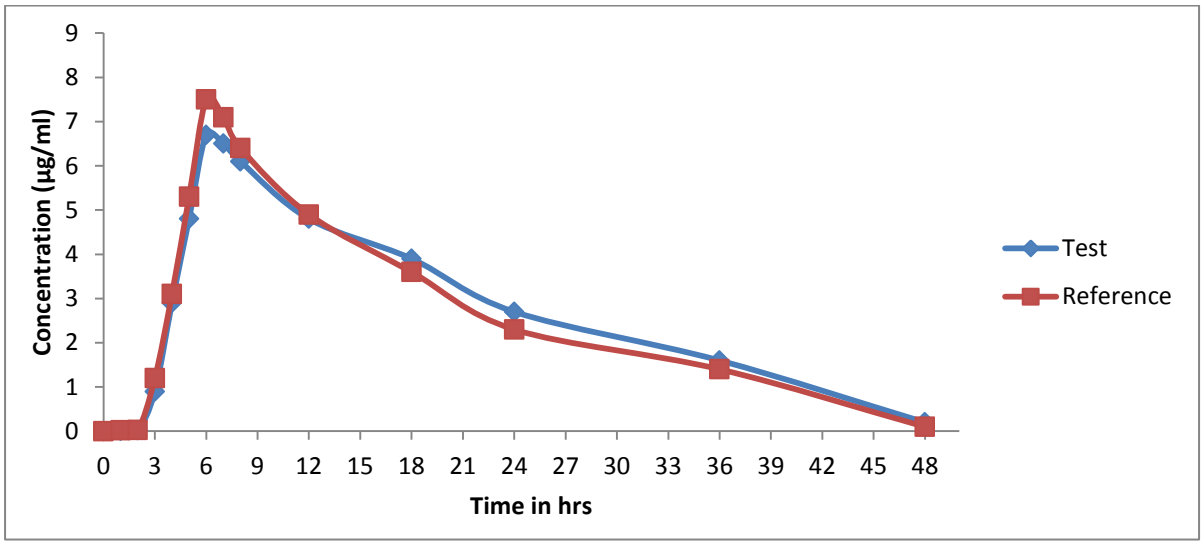

Figure: 7. Individual plasma concentrations of test and reference in subject 1005

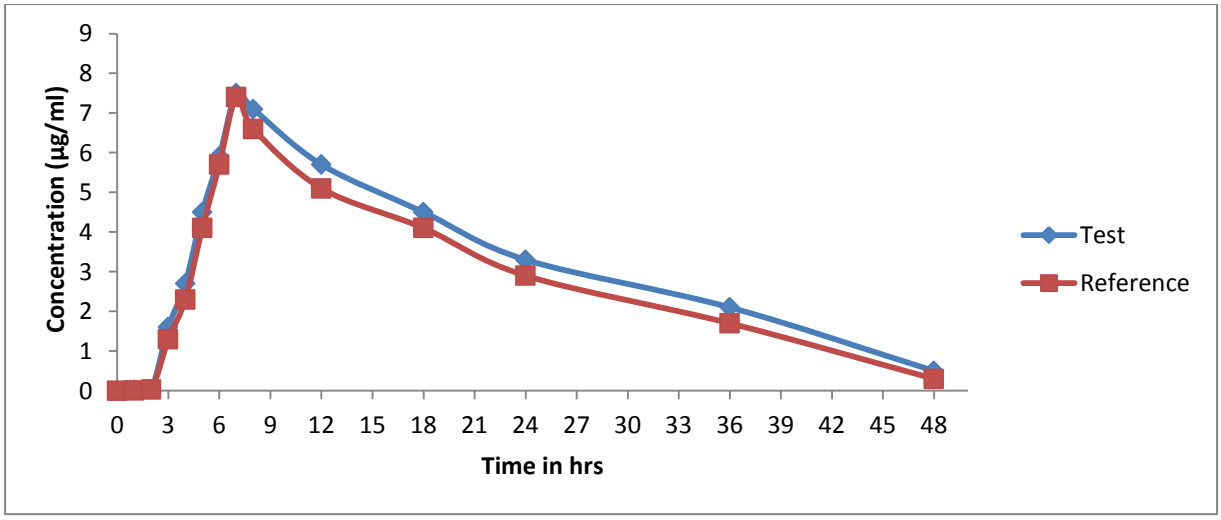

Figure: 8. Individual plasma concentrations of test and reference in subject 1006

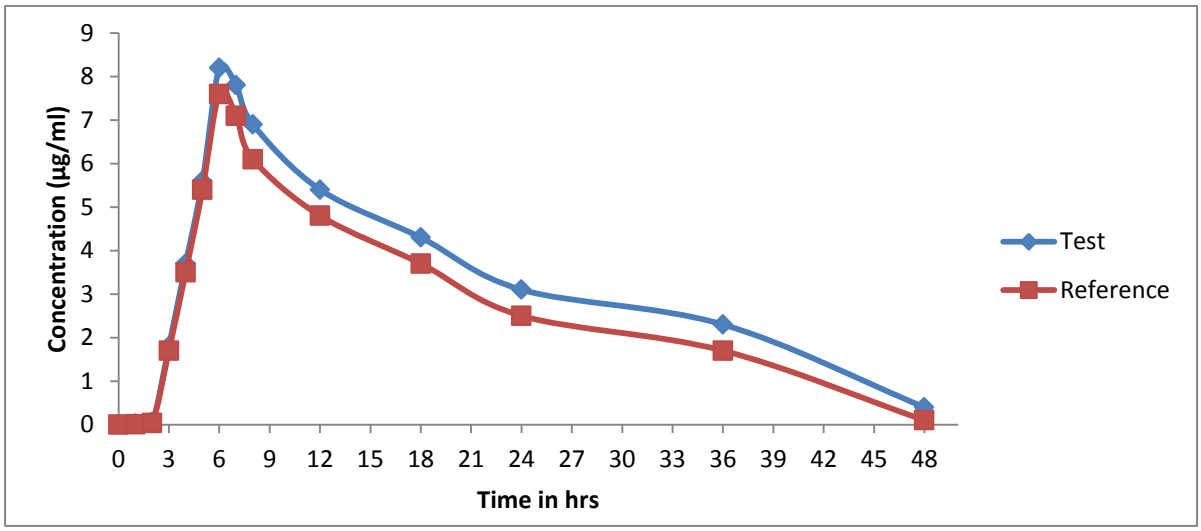

Figure: 9. Individual plasma concentrations of test and reference in subject 1007

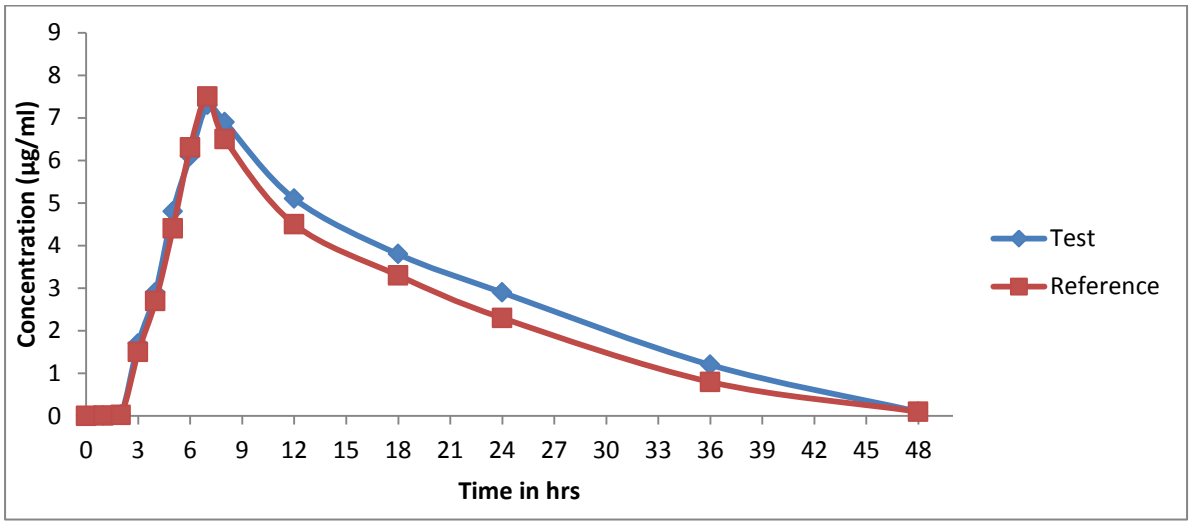

Figure: 10. Individual plasma concentrations of test and reference in subject 1008

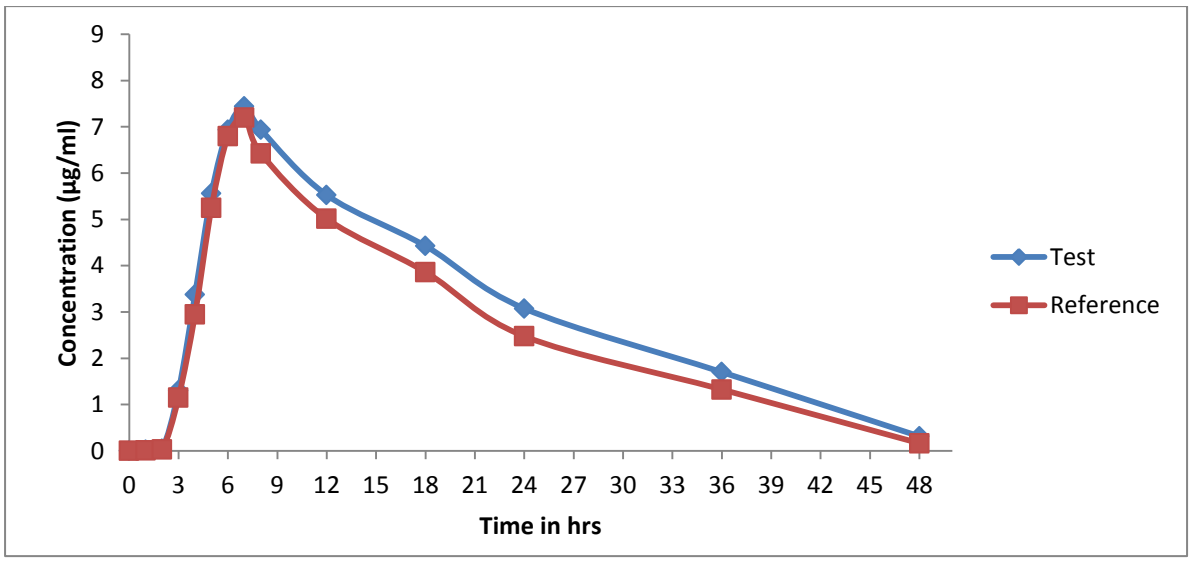

Figure: 11. Comparative mean plasma concentrations of Test Vs Reference

Table: 6. Descriptive Statistics of Formulation Means for Sulfasalazine hydrochloride (n=8)

Parameters (Units)	Mean ± SD (Un – transformed)	
	Test product-T	Reference product
C_{max} (µg/ml)	7.7125±1.0125	7.6±0.30
AUC_{0-t} (µg.h/ml)	84.92±20.25	79.39±19.45
$AUC_{0-\infty}$ (µg.h/ml)	102.47±25.65	94.87±18.33
T_{max} (h)	6.38±0.62	6.38±0.62
t ½ (h)	7.51±0.54	7.32±0.72

Table: 7. Ratios, 90% Confidence Interval and Power for Sulfasalazine

Parameters (Units)	Ratio	90% Confidence Interval*		Power
	[A/B]%	Lower	Upper	
C_{max} (µg/ml)	104.23	98.45	112.58	100.00
AUC_{0-t} (µg.h/ml)	109.92	95.32	115.33	100.00
$AUC_{0-\infty}$ (µg.h/ml)	108.87	94.14	111.46	100.00

** 90% Confidence Interval calculated for ln- transformed data*

CONCLUSION:

The HPLC-UV method was convenient, precise and reproducible. It can be used in the determination of sulfasalazine in human plasma and clinical pharmacokinetics studies. The statistical analysis of pharmacokinetic parameters of colon release tablets was performed by paired *t*- test. From the results there was significant difference in the Cmax, T_{max} and AUC_{0-t} between optimized formulation and marketed product, indicating that colon targeting influence the peak plasma concentration. The AUC0-∞ was significantly different from each other indicating that the administration of drug as colon targeted tablet affected the extent of absorption (slow and complete).

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