RESEARCH PAPER

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Sensitive HPLC Method for Bioavailability and Bioequivalence Studies of Theophylline SR Formulations

C. J. SHISHOO* AND S. S. SAVALE
Dept. of Quality Assurance, L. M. College of Pharmacy,
Navrangpura, Ahmedabad - 380 009.

As part of ongoing programme on the development of a sustained release (SR) formulation of theophylline (200 mg capsule) and determination of its bioavailability, a specific, sensitive and simple HPLC method has been developed for the estimation of lower µg levels of theophylline in plasma. 8-Chlorotheophylline was used as an internal standard. With simple extraction of theophylline from plasma, the method has a linearity range of 0.5 to 30 µg/ml and an average recovery of 79.32% for theophylline and 88.49% for the internal standard, respectively. Theophylline plasma concentration versus time data is presented for the test formulation (T) (SR capsule, 200 mg) and a standard formulation (S) (SR tablet, 200 mg). Different pharmacokinetic parameters for formulation T and formulation S were estimated and compared statistically for determination of their bioequivalence.

Theophylline (THP) inhibits adenosine-induced bronchoconstriction and is used commonly as a bronchodialator in asthamatic complications[1]. It has a narrow therapeutic window (therapeutic concentration in plasma, 10 to 20 µg/ml[2, 3]) and hence, requires close therapeutic monitoring of the drug levels in the body[1]. Therefore it is very important to ensure that the therapeutic concentration is maintained in the body over a suitable period. Maintenance of therapeutic level of drug in body is very critical for safety and efficacy of sustained-release (SR) formulations. To determine the lower µg levels of theophylline in plasma, a sensitive, precise and accurate analytical method became necessary. Several analytical methods that include UV-Vis spectrophotometry[4-6], GC[7] and HPLC[8-17], involving various sample preparation methods, for quantitation of theophylline in biological fluids (mainly plasma, serum and urine) have been reported.

As a part of an ongoing programme on the development of sustained-release formulation of theophylline, a simple, rapid, specific and sensitive HPLC method with UV detection has been developed for the determination of plasma levels of theophylline. 8-chlorotheophylline (8-ClTHP) was used as an internal standard. The method developed has been used to determine bioavailability as well as bioequivalence of the test formulation T in comparison with a standard formulation S.

*Corresponding Author
Email: cjsbcs@netscape.net

EXPERIMENTAL

Analytically pure sample of THP was gifted by Core Healthcare Ltd., Ahmedabad. Methanol and tetrahydrofuran (HPLC grade, Spectrochem), dichloromethane and 2-propanol (Extra pure, Ranbaxy Chem.), potassium dihydrogen phosphate (EP, E. Merck), orthophosphoric acid (LR, S. D. Fine Chem.), 8-ClTHP (G. D. Searle and Co.) and triple glass-distilled water, filtered through 0.45 µm filter, were used. Formulation (S) was a SR tablet dosage form, an established brand, obtained from the market while formulation (T) was the test formulation as a SR capsule dosage form. Both the formulations contained 200 mg of theophylline.

Chromatographic conditions:

The chromatographic system consisted of Shimadzu LC10 isocratic solvent delivery system fitted with a universal injector-Rheodyne 7125 model with 20 µl loop, LC10A multiwavelength UV-Vis detector and Shimadzu

CR10 software for Integration of data. The analytical column used was ET 250/4 NUCLEOSIL® 100-5 C18 (10μm, 25 cm X 4 mm) supplied by Macherey Nagel, Germany, along with a guard column filled with C18. Flow rate of the mobile phase was maintained at 1.1 ml/min. The mobile phase was prepared by mixing 0.05 M potassium dihydrogen phosphate buffer (pH: 4, adjusted with 50%v/v phosphoric acid), methanol and tetrahydrofuran in the proportion of 83, 15 and 2% v/v. It was filtered through 0.45 μm filter and was degassed by ultrasonication.

Solutions and solvent systems:

Standard stock solution (1 mg/ml) of THP was prepared in water and solution of 8-CITHP was prepared in methanol. THP solution was further diluted with water to obtain the concentration of 0.1 μg/ml (diluted stock solution). 8-CITHP was used as an internal standard. Twenty microlitres of 8-CITHP solution (1 mg/ml) was added to 1 ml of samples (untreated plasma) separately. A mixture of dichloromethane:2-propanol (9.5:0.5 v/v) was used as an extraction solvent to extract THP and 8-CITHP from plasma.

Plasma spiking studies:

To 1 ml of untreated human plasma, a definite volume (5, 10 μl of diluted stock solution (0.1 mg/ml) and 5, 10, 15, 20, 25 and 30 μl of standard aqueous solution of THP (1 mg/ml)) was added and mixed well for 5 s on a vortex mixer. It was allowed to stand for 5 min. Fifty microlitres of 1 M HCl solution was added to each test tube and vortexed for 10 s (plasma pH ≅ 4). Twenty microlitres of 8-CITHP solution (1 mg/ml) was then added to individual test tubes and mixed well (vortex mixing for 5 s). The plasma was treated with 5 ml of the extraction solvent and agitated by vortexing for 1 min (full speed). The organic layer was separated by centrifugation (3000 rpm, 10 min). Four millilitres of this layer was transferred into a test tube containing anhydrous sodium sulphate and then into an amber colored vial. The plasma sample was extracted again with 3 ml of the extraction solvent following the above described procedure. The organic extracts were combined in the amber colored vial and evaporated to dryness in a hot water bath (about 70°, under a gentle stream of nitrogen). The residue after evaporation was reconstituted with 500 μl of mobile phase. The resulting solution was filtered through 0.45 μm filter paper. Twenty microlitres of the filtrate was injected into the HPLC system and ultraviolet absorbance was measured at 272 nm. Linearity of the analytical method was determined by preparing a calibration curve by plotting the peak area ratios (ratio of the area of peak corresponding to THP to the area of peak corresponding to 8-CITHP) against the concentration of THP (0.5, 1, 5, 10, 15, 20, 25 and 30 μg/ml of plasma).

Recovery Studies:

Drug free plasma samples spiked with theophylline in the range 0.5-30 μg/ml were subjected to the extraction and assay procedure described above and the peak areas for THP were compared to the peak areas obtained from their corresponding standard solutions, containing internal standard, prepared in mobile phase. Recovery of the internal standard is determined in a similar manner as in the case of THP.

***In vivo* study of the SR formulations of THP:**

A preliminary *in vivo* (bioequivalence) study was carried out for SR capsule formulation (T) and SR tablet formulation (S) containing 200 mg of THP in a two treatment two period cross-over design. Six healthy male volunteers aged 20 to 25 y, weighing 50 to 55 kg, participated in the study. The written informed consent was taken from all volunteers and the experimental protocol was approved by a local Ethical Committee. None of the volunteers received any other drug two weeks prior to study and during the study. The volunteers were abstained from consumption of any xanthine containing food or drinks (chocolates, tea, coffee or coke) for 48 h before administration of the dose and were fasted overnight. Fasting was continued until 4 h post dose, but water intake was not restricted. Each volunteer received a single dose of Formulation T on the day one of the study and after a wash-out period of eight days each of them received Formulation S. Blood samples were withdrawn before administration of the formulation and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h after the administration. The samples were collected in tubes containing an anticoagulant (heparin sodium) and centrifuged (3500 rpm, 20 min) to separate plasma. Separated plasma was then stored at -20°, until analysis and was protected from exposure to light. These samples were then subjected to analysis as described earlier.

Pharmacokinetic parameters (C_{max}, t_{max}, AUC_{0-24}, $AUC_{0-\infty}$ and K_{el}) were calculated using the plasma THP concentration-time data with the help of computer software WinNonlin-Pro™[18] by subjecting the data to

noncompartmental analysis. The AUC_{0-24}, C_{max} and t_{max} were subjected to an analysis of variance (ANOVA) using a general linear model (sequence, subject sequence, period, treatment) to perform bioequivalence comparisons between treatments S and T using S as the reference formulation. The relative bioavailability was determined as the ratio of the AUC_{0-24} for formulation T relative to that for formulation S. Classical and Westlake 95% confidence intervals were estimated for AUC_{0-24}, C_{max} and t_{max} and the two one sided 't' test, proposed by Schuirmann, and Anderson-Hauck power analysis were also performed. A p value less than 0.05 and confidence limits greater than 20% were considered to be statistically significant. All statistical analysis of the data were performed using WinNonlin-Pro™[18].

RESULTS AND DISCUSSION

UV-Vis spectrophotometric analysis of biological samples has the limitation that the endogenous components and related compounds (metabolites) may interfere with the analysis and hamper specificity of the method. These methods also require complicated sample preparation and are less sensitive (sensitivity of about 10 µg/ml of plasma)[4,5,6]. GC methods may require derivatization of THP prior to analysis[7]. HPLC method was selected owing to its attributes like simplicity, versatility, specificity, sensitivity, reliability and reproducibility. It also provides rapid analysis with minimum amount of sample. Various HPLC methods have been reported for the determination of THP from plasma[8-17].

THP shows protein binding of about 60% which depends on temperature, pH and concentration[19]. Efficient extraction procedure is, therefore, critical for obtaining good recoveries. Of the various extraction methods reported, the method reported by Tanaka[11], seemed to be rapid, having minimum sample preparation steps. This extraction procedure was modified for the extraction of drug and 8-CITHP from acidified plasma (pH ≅ 4) with 5+3 ml dichloromethane:2-propanol (9.5:0.5 v/v) mixture. While addition of 2-propanol in the extraction solvent effectively improved the extraction of internal standard, acidification of plasma and double extraction helped in considerable clean-up and efficient extraction of both the drug as well as the internal standard. Addition of an internal standard offered the advantage that any losses during extraction can be compensated. 8-CITHP was selected as an internal standard due to its structural and physicochemical similarity to THP.

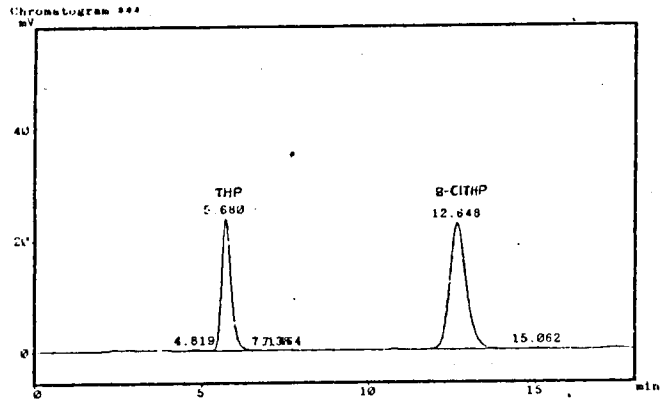

Fig.1 : Chromatogram for plasma sample spiked with THP (+ 8-CITHP)

Chromatogram showing separation of THP and 8-CITHP in the plasma sample spiked with 10µg/ml of THP and 20 µg/ml of 8-CITHP

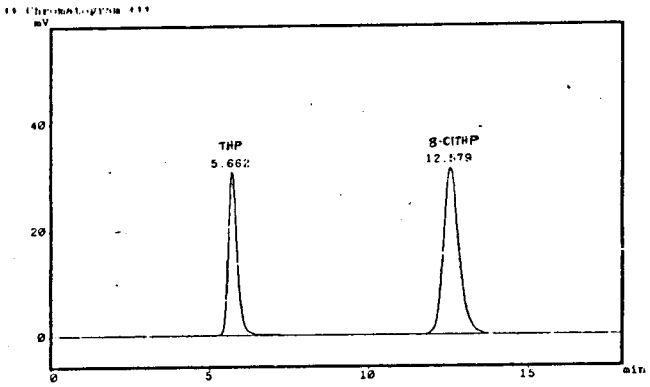

Fig. 2 : Chromatogram for THP along with 8-CITHP in mobile phase

Chromatogram showing separation of THP and 8-CITHP in the mobile phase (concentration - THP:8-CITHP is 20:40 µg/ml)

RP-HPLC on an octadecyl column with 10 µm particle size gave better resolution of THP and 8-CITHP and better peak response to detect THP in lower concentration range. The mobile phases containing methanol:0.05 M potassium dihydrogen phosphate buffer (pH 4 adjusted with 10% v/v o-phosphoric acid) 30:70 v/v[11] and acetonitrile:buffer (pH 3) 15:85 v/v[8], failed to resolve THP from endogenous components present in plasma samples. Based on the eluotropic strength of these mobile phases a ternary mixture of methanol, tetrahydrofuran and 0.05 M potassium dihydrogen phosphate (pH 4) buffer (15:2:83 % v/v) was used [eluotropic strengths-MeOH (5.5); THF (8.4); water (or buffer solutions) (0.0)][20]. Higher salt concentration and lower pH of the buffer in the mobile phase assisted to obtain sharper peaks and hence,

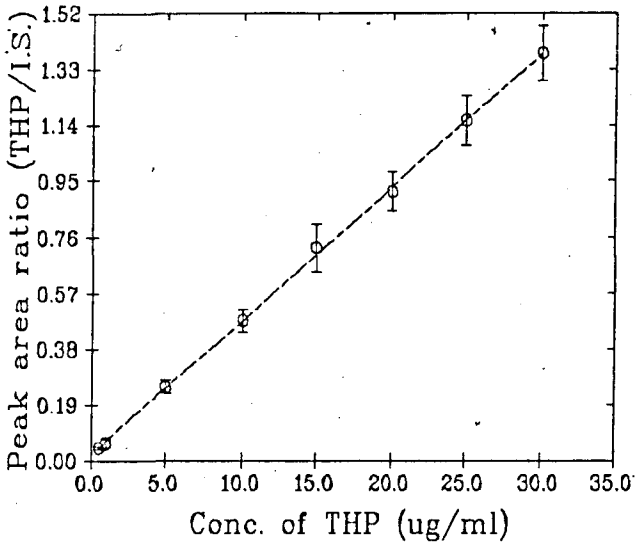

Fig. 3: Calibration curve for THP spiked in plasma

Calibration curve for THP spiked in plasma plotted as peak area ratio (THP: 8-CITHP) v/s concentration of THP (µg/ml) by proposed HPLC method

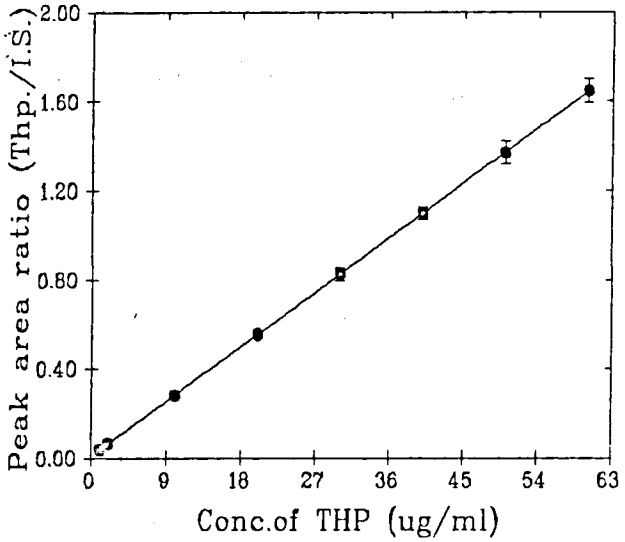

Fig. 4: Calibration curve for THP in mobile phase

Calibration curve for THP in mobile phase plotted as peak area ratio (THP: 8-CITHP) v/s concentration of THP (µg/ml) in the mobile phase by proposed HPLC method

to increase the chromatographic efficiency. This eluent (MeOH:THF:0.05 M potassium dihydrogen phosphate buffer (pH 4 adjusted with 10% v/v phosphoric acid) showed good resolution of THP and 8-CITHP (resolution, R is about 5.3, Fig. 1). It was found that THP and IS were well separated from the interfering endogenous plasma components in this eluent at a flow rate of 1.1 ml/min. The detection was carried out at 272 nm, the wavelength maxima of THP in the mobile phase. Chromatograms of THP (+IS) spiked in plasma and THP (+IS) solution in mobile phase are shown in Fig. 1 and 2. The retention times for THP and 8-CITHP were about 5.75 ± 0.2 min and 12.75 ± 0.2 min, respectively.

Peak area ratios (drug/IS) obtained for different concentrations of THP spiked in plasma (0.5-30 µg/ml) and in the standard solutions prepared in mobile phase (1-60 µg/ml) using 8-CITHP as an internal standard (20 µg/ml) by proposed HPLC method are given in Table 1. The least square linear regression evaluation of the peak area ratio (y) versus concentration (x) obtained by assaying plasma samples spiked with THP (0.5, 1, 5, 10, 15, 20, 25 and 30 µg/ml) and 8-CITHP (20 µg/ml in each sample) gave equation of the straight line "x = (y - 0.02149)/ 0.04542", with a correlation coefficient of 0.9997 (Fig. 3). Similarly solutions containing known quantities

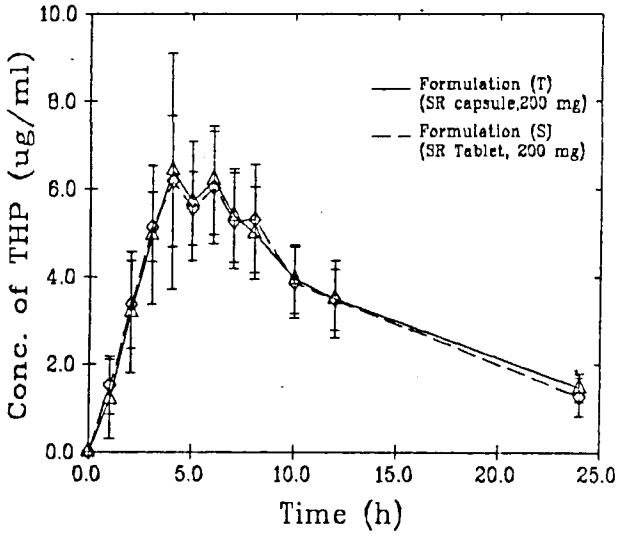

Fig. 5: Plasma levels of theophylline

Average plasma concentration (µg/ml) v/s time (h) profiles after administration of sustained release formulations (200 mg) of theophylline (Formulation T and Formulation S) to six healthy human volunteers (The verticle lines indicate the S.D. from the mean concentration of THP)

of THP and 8-CITHP prepared in mobile phase were assayed. The least square linear regression evaluation of peak area ratio (y) versus concentration (x) in the mobile phase gave equation of the line "x = (y - 0.008882) / 0.027235" and the correlation coefficient was 0.9999

TABLE 1

DETERMINATION OF CALIBRATION RANGE FOR THP SPIKED IN PLASMA AND ITS SOLUTION IN THE MOBILE PHASE

S. No.	THP spiked in plasma (μg/ml)	Area ratio Mean ± S.D. (n = 6)	Conc. of THP in mobile (μg/ml)	Area ratio Mean ± S.D. (n = 6)
1	0.5	0.043 ± 0.005	1	0.038 ± 0.001
2	1	0.059 ± 0.011	2	0.064 ± 0.001
3	5	0.251 ± 0.023	10	0.279 ± 0.007
4	10	0.477 ± 0.039	20	0.555 ± 0.016
5	15	0.725 ± 0.079	30	0.826± 0.030
6	20	0.915 ± 0.066	40	1.097± 0.028
7	25	1.155 ± 0.085	50	1.368 ± 0.051
8	30	1.384 ± 0.094	60	1.646 ± 0.055

Peak area ratios (Drug:IS) obtained for different concentrations of THP in the standard solutions prepared in mobile phase and spiked in drug-free plasma (0.5-30 μg/ml) using 8-CITHP as an Internal Standard (20 μg/ml) by proposed HPLC method

TABLE 2

DETERMINATION OF PRECISION OF THE PROPOSED HPLC METHOD FOR ESTIMATION OF PLASMA LEVELS OF THP

S. No.	Conc. of THP (μg/ml)	THP spiked in plasma % C.V.		Conc. of THP (μg/ml)	THP in mobile phase % C.V.	
		Interday (n = 6)	Intraday (n = 3)		Interday (n = 6)	Intraday (n = 3)
1	0.5	11.071	0.216	1	2.021	1.307
2	1	19.067		2	2.257	
3	5	9.186		10	2.591	
4	10	8.070	0.170	20	2.786	0.154
5	15	10.957		30	3.621	
6	20	7.172		40	2.567	
7	25	7.373		50	3.747	
8	30	6.767	0.170	60	3.336	0.221

Interday and intraday precision for the peak area ratios (THP:8-CITHP) determined by proposed HPLC method for different concentrations of THP spiked in drug-free plasma and also in mobile phase, on six days in a week and for three times on the same day, respectively.

(Fig. 4). The linearity range in this case was 1 to 60 μg/ml for THP. The limit of quantitation and minimum detectable concentration of THP were found to be 0.5 μg/ml and 0.1 μg/ml of plasma respectively.

The method was validated by determining reproducibility and accuracy for six spiked plasma samples (n = 6) with respect to calibration curve. Precision of the proposed method in terms of intraday (intraassay)

TABLE - 3 : RECOVERY OF THP AND INTERNAL STANDARD (8-CITHP) FROM PLASMA

S.No.	Conc of THP (μg/ml)	Mean % Recovery (n=6)
1	0.5	89.535
2	1	88.704
3.	5	79.294
4.	10	73.171
5.	15	77.254
6.	20	72.600
7.	25	77.545
8.	30	83.881
Average % recovery (THP)		79.316
Average % recovery for IS (8-CiTHP)		88.488

Extraction efficiency of the sample preparation method for extraction of THP and 8-CITHP from plasma

coefficients of variation and the day-to-day (interassay) coefficients of variation for analysis of plasma samples in triplicate on the same day and on six days over a period of one week varied from 0.170 % to 0.216 % (n = 3) and 6.7667 % to 19.0674 % (n = 6), respectively. The corresponding coefficients of variation for the drug samples prepared in mobile phase were in the range of 0.22 to 1.307 % and 2.02 to 3.747 % (Table 2).

The overall average recoveries for THP and 8-CITHP were found to be 79.316 % and 88.488 %, respectively (Table 3). It was observed that endogenous plasma components, caffeine and theobromine do not interfere with the drug peak. The peaks of THP and 8-CITHP were well resolved (Resolution, R is about 5.3) (Fig. 1).

The data showing concentration of THP in plasma after oral administration of SR formulations (Formulation T and Formulation S) versus time is given in Table 4. Both the formulations show sustained release as the maximum concentration was reached in about 6 h (t_{max}) for test formulation T and standard formulation S. The observed maximum concentrations (C_{max}) were 5.9757 ± 1.4062 μg/ml and 6.2758 ± 1.2612 μg/ml, respectively. Measurable amount of THP appeared in the plasma till 24 h after single oral administration of both the formulations. Figure 5 shows the average plasma concentration of THP versus time profiles for formulation T and S. The

TABLE - 4 : AVERAGE PLASMA CONCENTRATION - TIME DATA AFTER ADMINISTRATION OF THP SR FORMULATIONS

Time (h)	Average plasma concentration of THP (μg/ml) (± S.D.)	
	FormulationT	Formulation S
0.0	0.000	0.000
1.0	1.0532 ± 0.7461	1.3568 ± 0.5926
2.0	2.7810 ± 0.4788	3.2339 ± 1.1165
3.0	4.6080 ± 0.5244	5.1681 ± 0.8799
4.0	5.9495 ± 2.8999	6.2063 ± 1.6667
5.0	5.1721 ± 1.1712	5.3749 ± 0.7814
6.0	5.9757 ± 1.4062	6.2758 ± 1.2612
7.0	4.8998 ± 0.9528	5.3605 ± 1.1803
8.0	4.5958 ± 0.9410	5.3381 ± 1.3675
10.0	3.5674 ± 0.6554	3.9598 ± 0.8956
12.0	3.0482 ± 0.4926	3.4290 ± 0.7738
24.0	1.3852 ± 0.3285	1.3602 ± 0.4095

Average of the observed plasma concentration data for six healthy human volunteers. Formulation T and Formulation S - THP SR capsule (200 mg) test formulation and THP SR tablet (200 mg) standard formulation, respectively.

in vivo profiles for both these formulations are overlapping, indicating thereby the similar *in vivo* behavior of these formulations. Different model independent pharmacokinetic parameters calculated using the software WinNonlin-Pro™ for both the formulations are given in Table 5. ANOVA of the theophylline data showed that differences between the two formulations (formulation T and S) were not significant when AUC_{0-24}, C_{max} and t_{max} values were compared. The estimates of 95% Classical and Westlake confidence intervals (CI) were within ± 20% for $AUC_{0\,24}$ and C_{max}. However, for T_{max}, it was observed that the CI values were not in the 80- 120% range. This may be because a SR capsule formulation was being compared to a SR tablet dosage form. These differences may not be clinically significant. The relative bioavailability of formulation T with respect to formulation S was found to be 101.9468% when AUC_{0-24} was employed for estimation. The ANOVA data along with the estimates of CI, the two one-sided 't' test and Anderson-Hauck power

TABLE 5

PHARMACOKINETIC PARAMETERS FOR TWO EXTENDED RELEASE ORAL SOLID DOSAGE FORMS OF THP DETERMINED BY NONCOMPARTMENTAL ANALYSIS USING WINNONLIN-PRO™[18]

Pharmacokinetic parameter calculated	Formulation S.	Formulation T.
C_{max} (μg/ml)	6.647 ± 0.591	6.946 ± 0.798
t_{max}(h)	5.667 ± 0.422	5.167 ± 0.543
K_{el}(/h)	0.084 ± 0.007	0.071 ± 0.004
$t_{1/2}$ (h)	8.496 ± 0.630	9.937 ± 0.620
AUC_{0-24} (μg.h/ml)	80.595 ± 5.490	81.440 ± 7.646
$AUC_{0-\infty}$ (μg.h/ml)	96.808 ± 8.110	102.675 ± 9.458
Vd (ml)	25.742 ± 1.726	28.904 ± 2.825
Clearance (h)	2.162 ± 0.231	2.024 ± 0.166
$AUMC_{0-\infty}$ (μg.h/ml)	1364.788 ± 179.162	1616.228 ± 162.443
$MRT_{0-\infty}$(h)	13.810 ± 0.848	15.733 ± 0.673

Note: The values are given as mean ± S.E.M. for data from six healthy volunteers.

TABLE 6

BIOEQUIVALENCE STATISTICS AND PAIRWISE COMPARISONS (FORMULATION S AND FORMULATION T)

	AUC_{0-24}	C_{max}	t_{max}
Classical (95%)*	(90.56, 113.33)	(86.10, 128.19)	(64.37, 118.89)
Westlake (95%)*	(88.03, 111.96)	(75.26, 124.73)	(68.72,131.28)
Two one-sided 't' test (Schuirmann)**			
p<80%	0.0006	0.0078	0.1853
p>120%	0.0023	0.1040	0.213
Anderson-Hauck statistics			
p-value	0.0017	0.0962	0.1640
power	0.9375	0.4583	0.2875
% relative bioavailability	101.9468		

*The values in the bracket indicate (lower, upper confidence intervals) for the ratio of pharmacokinetic parameter of formulation T to formulation S. ** Probability values

analysis (the power value for the ratio of AUC_{0-24} for formulation T to formulation S nearing to 1), suggested that the two formulations had comparable bioavailability (Table 6).

Thus, the proposed simple, sensitive, specific, accurate and precise method can be used in the determination of THP in biological fluids for therapeutic monitoring of THP levels and for bioavailability and bioequivalence studies of various THP formulations.

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