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Development of a sensitive high-performance thin-layer chromatography method for estimation of ranitidine in urine and its application for bioequivalence decision for ranitidine tablet formulations

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Abstract

A sensitive and simple HPTLC method was developed for estimation of ranitidine in human urine. The drug was extracted from urine after basification using dichloromethane. Dichloromethane extract was spotted on silica gel 60 F_{254} TLC plate and was developed in a mixture of ethyl acetate–methanol–ammonia (35:10:5 v/v) as the mobile phase and scanned at 320 nm. The R_F value obtained for the drug was 0.67 ± 0.03. The method was validated in terms of linearity (50–400 ng/spot), precision and accuracy. The average recovery of ranitidine from urine was 89.35%. The proposed method was applied to evaluate bioequivalence of two marketed ranitidine tablet formulations (150 mg, Formulation 1 and Formulation 2) using a crossover design by comparing urinary excretion data for unchanged ranitidine in six healthy volunteers. Various pharmacokinetic parameters like peak excretion rate $[(dAU/dt)_{max}]$, time for peak excretion rate (t_{max}), $AUC_{0\text{-}24}$, $AUC_{0\text{-}\infty}$, cumulative amount excreted were calculated for both formulations and subjected to statistical analysis. The relative bioavailability of Formulation 2 with respect to Formulation 1 was 93.76 and 95.31% on the basis of $AUC_{0\text{-}24}$ and cumulative amount excreted, respectively. Statistical comparison of various pharmacokinetic parameters indicated that the two ranitidine tablet formulations are bioequivalent.

Keywords: Ranitidine; Urinary excretion; Bioequivalence

1. Introduction

Ranitidine is a H_2 receptor antagonist. It inhibits gastric acid secretion. The drug has been widely used in the treatment of duodenal and gastric ulceration with a recommended dose of 150 mg twice daily and 300 mg once-a-day [1].

USP 24 states that in bioequivalence studies, samples of an accessible biological fluid (blood or urine) are analyzed for drug and/or metabolite concentrations [2]. Various methods employed to evaluate bioequivalence of ranitidine formulations involve estimation of ranitidine levels in plasma [3–5], which proves to be complex and time consuming. Several reports indicate that urinary excretion data of drugs can be successfully used to compare the bioavailability of different formulations [6–8]. Urine provides a non-invasive sample collec-

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tion method and determination of drug levels in urine is comparatively less complex. Since a considerable amount of ranitidine (15–30%) gets excreted unchanged in urine [9–11], it was decided to utilize urinary excretion data of ranitidine to evaluate bioequivalence of two marketed ranitidine tablet formulations in healthy human volunteers.

The present study describes the development, validation and application of a simple and specific HPTLC method for estimation of ranitidine in urine. The urinary excretion data thus obtained was successfully used to compare bioavailability of ranitidine after administration of two marketed ranitidine tablet formulations in healthy volunteers for bioequivalence decision.

2. Experimental

2.1. Instruments

A HPTLC system consisting of Camag Linomat IV semiautomatic spotting device; Camag glass twin-trough chamber (10×10 cm); Camag TLC Scanner 3; Camag CATS 4 software (Camag Sonnenmattstr., Muttenz, Switzerland) and a 100-μl HPTLC Syringe (Hamilton Company, Reno, NV, USA), was used for chromatographic studies.

2.2. Reagents

Analytically pure ranitidine hydrochloride was gifted by Cadila Pharmaceuticals Ltd., Ahmedabad, India. Sodium hydroxide (Samir Tech. Chemical Pvt. Ltd., Vadodara, India), dichloromethane, methanol (Ranbaxy Laboratories, S.A.S. Nagar, India), ethyl acetate, ammonia (25%) (JC's Chemicals, Vadodara, India) were of laboratory reagent grade. TLC aluminum sheets precoated with silica gel 60 F_{254} (20×20 cm; layer thickness, 0.2 mm) (E. Merck, Darmstadt, Germany) were used as stationary phase.

2.3. Preparation of standard solutions

2.3.1. Methanolic solution of ranitidine

A stock solution containing 1 mg/ml of ranitidine hydrochloride was prepared in methanol and further diluted with methanol to a concentration of 10 μg/ml (MS1).

2.3.2. Aqueous solutions of ranitidine

Two separate solutions of 1 mg/ml (WS1) and 0.1 mg/ml (WS2) of ranitidine hydrochloride were prepared in water and used for urine spiking studies.

2.4. Chromatographic conditions

Chromatographic studies were performed using the following conditions:

Stationary phase: Silica gel 60 F_{254} precoated TLC plates (10×5 cm; layer thickness, 0.2 mm),

Activation: Prewashing with methanol, drying in oven (60±1°C, 5 min),

Mobile phase: Ethyl acetate/methanol/ammonia (35:10:5 v/v),

Volume of mobile phase: 5 ml,

Chamber saturation time: 45 min,

Temperature: 25±1°C, relative humidity: 35–40%,

Migration distance: 35 mm,

Wavelength of detection: 320 nm,

Band width: 4 mm, space between two bands: 4 mm,

Spraying rate: 10 s/μl.

2.5. Extraction of ranitidine from urine

One milliliter of urine (drug free or drug spiked or volunteer urine sample) was transferred to a tappered-bottom glass centrifuge tube (15-ml capacity). The tube was cooled in an ice bath for 1 min, NaOH (1 *M*, 100 μl) was added and mixed by vortexing for 30 s. The content was extracted with dichloromethane (2×1 ml) by vortexing for 1 min every time at high speed, followed by centrifugation for 10 min (350 *g*); 0.6 ml of dichloromethane layer was collected each time and combined (total of 1.2 ml).

2.6. Chromatographic separation

Twenty microliters of the combined extract or standard ranitidine solution was spotted on the TLC plate 10 mm from the bottom edge using a Camag Linomat IV semiautomatic spotting device. The TLC plate was developed in ascending mode in a twin-

trough chamber previously saturated for 45 min with mobile phase, ethyl acetate/methanol/ammonia (35:10:5 v/v, 5 ml). The plate was removed from the chamber, dried in air and scanned in absorbance/reflectance mode of Camag TLC Scanner 3 at 320 nm. Peak area data were recorded using Camag CATS 4 software.

2.7. Preparation of calibration curve

2.7.1. Calibration curve of standard ranitidine

Aliquots of 5, 10, 20, 30 and 40 μl of MS1 were spotted on the TLC plate. The plate was developed, dried and scanned as described under Section 2.6. A plot of peak area versus corresponding ranitidine concentration was constructed.

2.7.2. Calibration curve of ranitidine spiked in urine

Aliquots of 1.25 ml of WS2 and 0.25, 0.5, 0.75, and 1 ml of WS1 were pipetted out into separate 25-ml volumetric flasks. The solutions were diluted up to the mark with drug free (blank) urine. One milliliter of this solution was extracted and analyzed as described under Sections 2.5 and 2.6.

Quantitative determination was performed by fitting areas of the peaks corresponding to ranitidine from the chromatograms into corresponding calibration curve equations.

2.8. Validation of the method

2.8.1. Linearity

The linearity of response for ranitidine was assessed in the range of 50-400 ng/spot for standard ranitidine and ranitidine spiked in urine after extraction.

2.8.2. Sensitivity

It was determined in terms of limit of quantitation which was taken as the lowest concentration in the calibration range. For limit of detection, concentrations of ranitidine lower than the limit of quantitation were spotted and the minimum concentration detected under given chromatographic conditions was considered as the limit of detection.

2.8.3. Precision

Precision of the proposed method in terms of intra-day variation (%CV) was determined by analyzing urine samples spiked with ranitidine at three different concentrations (50, 200 and 400 ng/spot) for three times on the same day and inter-day precision (%CV) was assessed by analyzing urine samples spiked with different concentrations of ranitidine (50 to 400 ng/spot) on five different days over a period of 1 week.

2.8.3.1. Repeatability of measurement of peak area. Ten microliters of MS1 (10 μg/ml) was spotted on a TLC plate, developed, dried and the spot was scanned seven times without changing the plate position and %CV for measurement of peak area was estimated.

2.8.3.2. Repeatability of sample application. Ten microliters of MS1 (10 μg/ml) was applied seven times on a TLC plate by a semiautomatic spotting device. The plate was developed and analyzed as described under Section 2.6 and %CV for peak area for different peaks was estimated.

2.8.4. Accuracy

The accuracy of an analysis was determined by calculating the systematic error involved. It was determined by standard addition method at different concentration levels of ranitidine. Different amounts of ranitidine were added to the urine samples spiked with ranitidine, extracted with dichloromethane and analyzed as described under Section 2.6. The peak area for total ranitidine in urine was compared with the peak area for corresponding standard ranitidine.

2.8.5. Specificity

The specificity of the method was ascertained by analyzing standard ranitidine, drug free urine and urine spiked with ranitidine. The spot for ranitidine spiked in urine was confirmed by comparing the R_F and spectra of the spot with that of standard ranitidine. The peak purity of spiked ranitidine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot.

2.8.6. Recovery studies

Recovery of ranitidine from urine was calculated as the ratio of area of ranitidine peak after extraction from urine to the area of standard ranitidine corresponding to respective concentrations followed by application of a correction factor.

2.9. Bioavailability study

2.9.1. Study protocol

Two ranitidine tablet formulations, Formulation 1 (Reference) and Formulation 2 (Test), containing 150 mg of ranitidine were studied for bioequivalence. The study was performed in a single dose, two-treatment, two-period, randomized crossover fashion with a wash-out period of minimum 8 days between the treatments in six healthy male volunteers (20–23 years, 55–70 kg). Written informed consent was obtained from all the volunteers and the study protocol was approved by the local Ethical Committee. None of the volunteers received any other drug 2 weeks prior to day one of the study and during the study. The volunteers abstained from consumption of xanthine containing foods and beverages (chocolates, tea, coffee or coke) for 24 h before administration of the dose and were fasted overnight. A standard breakfast and standard lunch were provided after 4 and 6 h sampling, respectively. Each volunteer received a formulation (Reference or Test) along with 150 ml of potable water. Water consumption was restricted up to 4 h after administration and was allowed ad libitum thereafter.

Urine samples were collected before administration and at 1, 2, 3, 4, 6, 8, 10, 12 and 24 h after administration of the formulation. The volume of urine collected at each sampling time from each volunteer was measured. Representative samples of urine were stored at −20°C in test tubes sealed with aluminum foil until analysis.

The urine samples, after bringing to room temperature, were analyzed for ranitidine content by the proposed HPTLC method and the urinary excretion profiles were used to determine various pharmacokinetic parameters.

2.9.2. Pharmacokinetic analysis

The peak excretion rate ($(dAU/dt)_{max}$) and peak excretion time (t_{max}) values are obtained from the urinary excretion rate (dAU/dt) versus time curves obtained for each volunteer after administration of Formulation 1 and Formulation 2. Various other pharmacokinetic parameters such as overall elimination rate constant (K), terminal elimination rate constant (k_{el}), elimination half-life ($t_{1/2}$) were obtained from log-transformed data of urinary excretion ($\log(dAU/dt)$, mg/h) versus mid-point of time (h) curves. Both $AUC_{0\text{-}24}$ and $AUC_{0\text{-}\infty}$, were calculated using untransformed as well as log-transformed (dAU/dt) data. $AUC_{0\text{-}24}$ was calculated using linear trapezoidal rule and was extrapolated to infinite time, $AUC_{0\text{-}\infty}$. k_{el} was calculated from the slope of terminal linear portion of $\log(dAU/dt)$ versus the mid-point of time curve. 'K' was obtained by quotient of intercept of terminal linear line extrapolated to the y-axis and dose (mg). The elimination half life ($t_{1/2}$) was calculated using the formula $t_{1/2}=0.693/k_{el}$.

2.9.3. Statistical analysis

The pharmacokinetic parameters ($AUC_{0\text{-}24}$, $AUC_{0\text{-}\infty}$, $(dAU/dt)_{max}$ and cumulative drug excreted in 24 h) were subjected to an analysis of variance (ANOVA) using a general linear model (sequence, subject-sequence, period, treatment) to perform bioequivalence assessment between Formulation 1 (Reference) and Formulation 2 (Test). The relative bioavailability was determined in terms of $AUC_{0\text{-}24}$ and cumulative amount excreted from Formulation 2 relative to Formulation 1. Classical 90% confidence intervals were estimated for $AUC_{0\text{-}24}$, $AUC_{0\text{-}\infty}$, $(dAU/dt)_{max}$ and cumulative drug excreted. Two one-sided t-tests were also performed for these parameters.

3. Results and discussion

3.1. HPTLC method development and validation

Due to its versatility and speed of analysis, the HPTLC technique was considered to be suitable, for the analysis of ranitidine concentrations in urine.

Various solvents were tried for extraction of ranitidine from urine. Solvents like benzene and chloroform yield very poor efficiency. Ethyl acetate or a mixture of ethyl acetate or dichloromethane with 2-propanol extracted unwanted polar components

from urine. Use of dichloromethane provided better clean up and recovery of ranitidine. Basification of urine (pH ≅ 12) followed by two times extraction with dichloromethane could improve extraction efficiency to more than 80% which was satisfactory.

Different compositions of ethyl acetate and methanol were tried to obtain optimum R_F and separation of ranitidine from urine components on the TLC plate. Various modifiers like triethylamine, diethylamine, ammonia solution were used to achieve a sharp band for ranitidine. A mixture of ethyl acetate/methanol/ammonia (35:10:5 v/v) could provide a sharp peak for ranitidine, well resolved from other urine components, at R_F of 0.67±0.03 (Fig. 1).

It was observed that prewashing of TLC plates with methanol (followed by drying and activation) and pre-saturation of TLC chamber with mobile phase for 45 min ensured good reproducibility and peak shape of ranitidine.

Densitometric evaluation was performed at 320 nm, wavelength of maximum absorbance (λ_{max}), in absorbance/reflectance mode.

3.1.1. Validation

Using the optimized extraction method and chromatographic conditions, the HPTLC method developed was validated in terms of linearity, limit of detection, limit of quantitation, precision, accuracy and specificity.

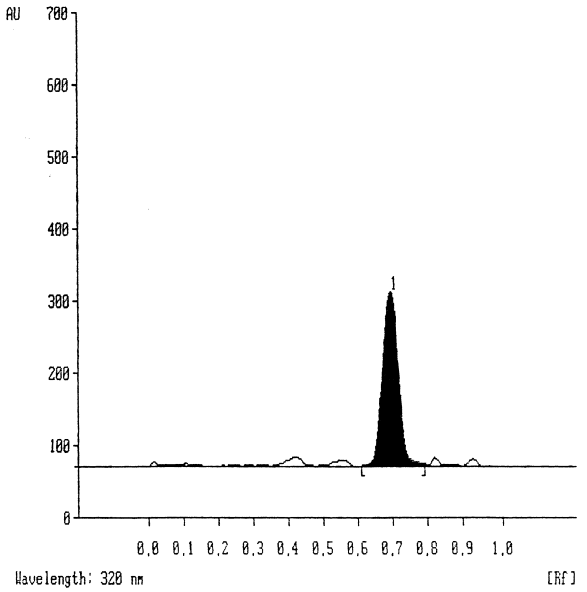

Fig. 1. Chromatogram showing the peak of ranitidine (R_F = 0.67±0.03) extracted from urine.

3.1.1.1. Linearity. Peak areas of standard and spiked ranitidine were found to be linear in the range of 50–400 ng/spot (i.e. 5–40 μg/ml, n=5) with correlations coefficients of 0.9935 and 0.9933, respectively. The average linear regressed equations for the corresponding curves were y=24.923x+1669.8 and y=21.738x+1568.7.

3.1.1.2. Limit of detection and limit of quantitation. The minimum detectable quantity was found to be 10 ng/spot, while the limit of quantitation was 50 ng/spot for ranitidine spiked in urine.

3.1.1.3. Precision. Repeatability of sample application seven times and repeatability of measurement of peak area based on seven repeat measurements of the same spot showed very low RSD (%CV of 1.27 and 0.07, respectively) which, in turn, ensured reproducible performance of the instrument. The intra-day variation for determination of ranitidine in urine was in the range of 2.09–4.56%, while inter-day variation was ranging from 1.58 to 5.55% (Table 1).

3.1.1.4. Accuracy. The percentage accuracy for analysis of ranitidine in urine, determined using the standard addition method, was found to be between 101.43 and 110.81 (Table 2) over the range studied.

3.1.1.5. Specificity. Comparison of chromatograms of blank (drug-free) urine, standard ranitidine and ranitidine spiked in urine, showed no interference

Table 1
Precision of HPTLC method for estimation of standard ranitidine and ranitidine spiked in human urine

Conc. of ranitidine (ng/spot)	Intra-day (n=3) %CV		Inter-day (n=5) %CV	
	Standard	Spiked	Standard	Spiked
50	1.91	4.56	2.58	5.55
100	–	–	1.65	3.14
200	1.36	2.09	2.21	3.70
300	–	–	1.94	1.58
400	2.01	3.13	2.93	3.01

Table 2
Accuracy of determination of ranitidine in urine

Conc. of ranitidine (ng/spot)		Amount of ranitidine found[a] (ng/spot; mean±SD) (n=4)	% Accuracy (mean±SD) (n=4)
Initial quantity spiked	Quantity of standard added		
50	50	106.78±8.98	106.78±8.98
50	150	221.61±6.02	110.81±2.72
50	250	306.81±4.49	102.29±1.48
50	350	402.22±16.34	101.43±2.87

[a] Values after correction for recovery.

from the urine components in the separation of ranitidine. Peak purity check showed a high degree of correlation between spectra scanned at peak start, peak apex and peak end positions (r=0.9992) of ranitidine peak which confirmed that the peak represents a pure single component, i.e. ranitidine (Fig. 2a). This was further supported by an equally good correlation (r=0.9989) between the spectrum of standard ranitidine and the spectrum of ranitidine spiked in urine (Fig. 2b).

3.1.1.6. Recovery. Average recovery of ranitidine, from urine over the range of spiked concentrations of 50–400 ng/spot was found to be 89.35%. Ranitidine in urine was found to be stable over a period of 7 days at −20°C.

Thus, the proposed method is simple, sensitive, specific, precise and accurate and can be used for estimation of ranitidine levels in human urine.

3.2. Bioavailability study

Urinary excretion levels of ranitidine after administration of Formulation 1 and Formulation 2 (both containing 150 mg of ranitidine) in six volunteers were estimated using the proposed HPTLC method. A typical chromatogram showing ranitidine excreted in urine during different time intervals is shown in Fig. 3.

The average values (±SD) for % cumulative amount of ranitidine excreted, rate of excretion and log-transformed rate of excretion for both the formulations with respect to mid-point time are given in Table 3. Plots of average percentage cumulative amount of ranitidine excreted (±SD) over a period of 24 h versus mid-point time and average log

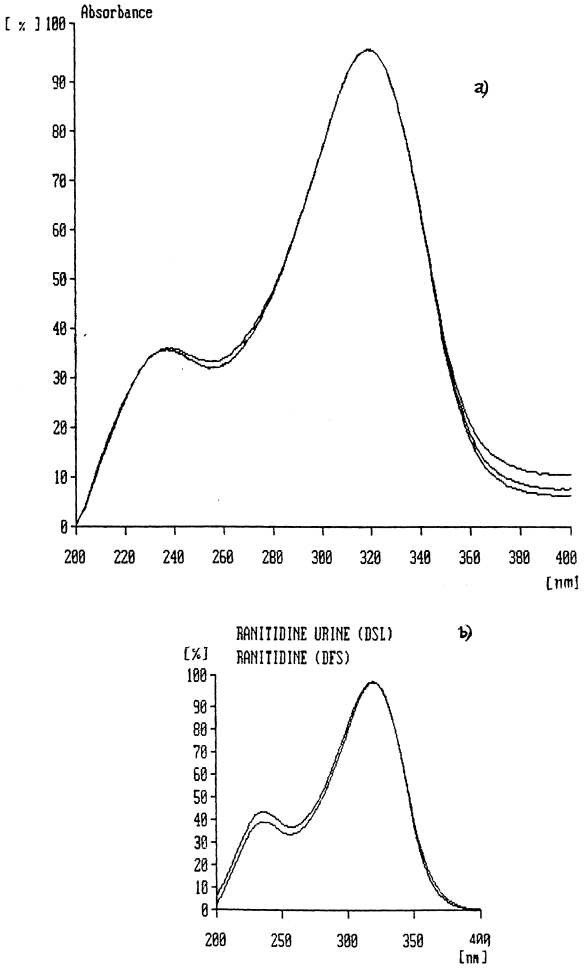

Fig. 2. (a) Peak purity spectra for ranitidine, extracted from urine sample, scanned at the peak start, peak apex and peak end positions of the spot (correlation=0.9992); (b) Comparison of spectra of ranitidine extracted from urine with that of standard ranitidine (correlation=0.9989).

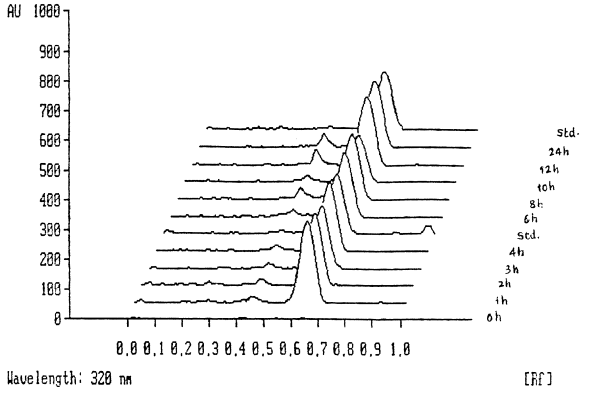

Fig. 3. Typical chromatogram showing levels of ranitidine from the urine samples of a volunteer collected over a period of 24 h after administration of ranitidine tablet formulation.

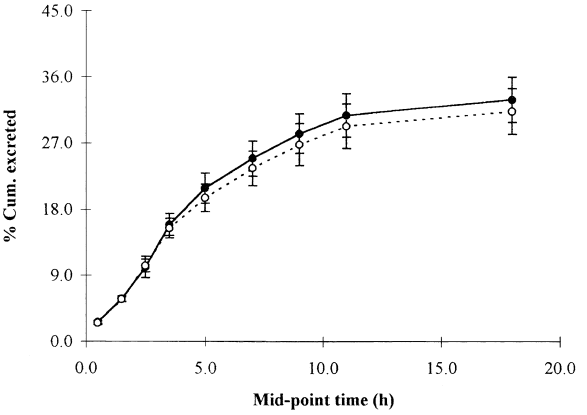

Fig. 4. Average % cumulative excreted, with respect to ranitidine dose, versus mid-point time plots after administration of ranitidine tablet formulations to six healthy male volunteers (note: the vertical lines indicate SD in % cumulative excreted at corresponding mid-point times; —, Formulation 1, ..., Formulation 2).

excretion rate (log (dAU/dt)±SD) versus mid-point time, are shown in Figs. 4 and 5, respectively. From these figures it is evident that both the formulations show similar excretion behavior, which in turn, indicates the similarity in their bioavailability. About 49.24±4.62 mg (32.83±3.09% of dose) and 46.93±4.69 mg (31.29±3.13% of dose) of ranitidine is excreted in 24 h after oral administration of Formulation 1 and Formulation 2, respectively, which is comparable with the values reported for the same dose [11]. It was observed that both the formulations showed maximum excretion rates in the interval of 3–4 h (t_{max}) (Fig. 5).

Average values (±SD) of various pharmacokinetic parameters are reported in Table 4. AUC_{0-24} value for Formulation 1 was found to be 88.30±9.50 mg and that for Formulation 2 was 82.79±7.32 mg, which are comparable. Maximum amounts excreted in corresponding time intervals $((dAU/dt)_{max})$ from Formulation 1 and Formulation 2 were 8.08±0.90 and 7.70±0.80 mg/h, respectively (Table 4). Values of other pharmacokinetic parameters of Formulation 2 were also comparable with that of the reference formulation (Formulation 1). Comparison of all these parameters indicated similar bioavailability of ranitidine from Formulation 2 compared to Formulation 1. The relative bioavailability of ranitidine from Formulation 2, in terms of cumulative amount excreted and AUC_{0-24}, was found

Table 3
Average % cumulative excreted, rate of excretion and log-transformed rate of excretion of ranitidine after administration of Formulation 1 and Formulation 2 (n=6)

Time (h) (mid-point)	Percentage cumulative excreted Mean±SD		Rate of excretion (dAU/dt) Mean±SD (mg/h)		log (dAU/dt) Mean±SD (mg/h)	
	Formulation 1	Formulation 2	Formulation 1	Formulation 2	Formulation 1	Formulation 2
0.5	2.62±0.20	2.53±0.24	3.93±0.31	3.80±0.36	0.59±0.04	0.58±0.04
1.5	5.80±0.39	5.73±0.36	4.77±0.45	4.79±0.26	0.68±0.04	0.68±0.02
2.5	10.09±1.45	10.28±0.87	7.09±0.83	6.38±0.85	0.85±0.05	0.83±0.06
3.5	15.91±1.49	15.41±1.34	8.08±0.90	7.70±0.80	0.91±0.05	0.88±0.04
5.0	20.89±2.05	19.87±1.89	3.71±0.43	3.34±0.48	0.57±0.05	0.52±0.06
7.0	24.94±2.39	23.59±2.35	3.05±0.29	2.79±0.36	0.48±0.05	0.44±0.05
9.0	28.32±2.69	26.83±2.86	2.54±0.28	2.37±0.33	0.40±0.05	0.37±0.06
11.0	30.79±2.96	29.36±3.05	1.89±0.28	1.89±0.25	0.27±0.07	0.27±0.06
18.0	32.83±3.09	31.29±3.13	0.24±0.03	0.25±0.07	−0.61±0.05	−0.61±0.12

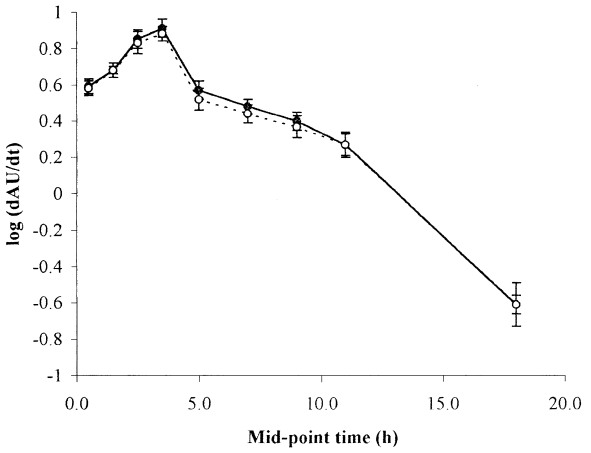

Fig. 5. Average log excretion rate (log (dAU/dt)) versus mid-point time plots for ranitidine after administration of ranitidine tablet formulations to six healthy male volunteers (note: the vertical lines indicate SD in log (dAU/dt) at corresponding mid-point times; —, Formulation 1, ..., Formulation 2).

to be 95.31 and 93.76%, respectively (Table 4). For bioequivalence decision, peak excretion rate ((dAU/dt)$_{max}$), $AUC_{0\text{-}24}$, $AUC_{0\text{-}\infty}$ and cumulative amount of ranitidine excreted values were subjected to statistical analysis. ANOVA revealed that there is no significant difference between Formulation 2 and Formulation 1, since the calculated F-values were found to be less than the corresponding critical values at given degrees of freedom. The estimates of 90% confidence interval ($P=0.05$) for the ratio of these four parameters were found to be within the specified limits of 80–120% for untransformed data as per US FDA requirement for bioequivalence. Thus, it was observed that Formulation 2 is bioequivalent to Formulation 1.

Table 4
Pharmacokinetic parameters of ranitidine

Pharmacokinetic parameters	Formulation 1[a]	Formulation 2[a]
t_{max} (h)	3.50±0.00	3.50±0.00
(dAU/dt)$_{max}$ (mg/h)	8.08±0.90	7.70±0.80
$AUC_{0\text{-}24}$ (log (dAU/dt), mg)	7.53±0.74	6.97±0.64
$AUC_{0\text{-}\infty}$ (log (dAU/dt), mg)	9.68±0.71	9.10±0.44
$AUC_{0\text{-}24}$ (mg)	88.30±9.50	82.79±7.32
$AUC_{0\text{-}\infty}$ (mg)	102.78±11.89	95.99±7.58
$t_{1/2}$ (h)	3.15±0.15	3.24±0.27
Cum. amount excreted (mg)	49.24±4.62	46.93±4.69
% Cum. amount excreted	32.83±3.09	31.29±3.13
K (1/h)	0.22±0.01	0.22±0.02
k_{el} (1/h)	0.10±0.01	0.09±0.02
Relative bioavailability (%)		
Cumulative amount excreted		95.31
$AUC_{0\text{-}24}$		93.76

[a] Values for each pharmacokinetic parameter indicate mean±SD for $n=6$.

4. Conclusion

A simple, sensitive, specific, accurate and precise HPTLC method was developed for estimation of ranitidine excreted in urine. This method was validated and used successfully to obtain urinary excretion data after administration of tablet formulations containing ranitidine in six healthy human volunteers. Statistical analysis of various pharmacokinetic parameters obtained using urinary excretion data of ranitidine revealed that Formulation 2 is bioequivalent to Formulation 1.

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