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A simple sample preparation with HPLC–UV method for estimation of tiropramide from plasma: Application to bioequivalence study

Khan Imran, Loya Punnamchand, Saraf Madhusudan Natvarlal *

Department of Pharmacology, The Bombay College of Pharmacy, Kalina, Santacruz (E), Mumbai 400 098, India

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Abstract

A simple, rapid and selective method was developed for estimation of tiropramide from human plasma. The method involves extracting the tiropramide with *n*-hexane using diphenhydramine hydrochloride as internal standard. Chromatographic separation was carried out on a reversed phase C_{18} column using mixture of water and acetonitrile as mobile phase with UV detection set at 230 nm. The retention time of internal standard and tiropramide were 5.6 ± 0.2 and 8.3 ± 0.3 min, respectively. The method was validated and found to be linear in the range of 10–200 ng/ml. The co-efficient of variation for intra-day and inter-day accuracy and precision was less than 12.8%. The mean recovery was found to be 89%. An open, randomized, two-treatment, two period, single dose crossover, bioequivalence study in 12 fasting, healthy, male, volunteers was conducted. After dosing, serial blood samples were collected for the period of 12 h. Various pharmacokinetic parameters including AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, T_{max}, $T_{1/2}$, and elimination rate constant (K_{el}) were determined from plasma concentration of both formulations. Log transformed values were compared by analysis of variance (ANOVA) followed by classical 90% confidence interval for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ and was found to be within the range. These results indicated that the analytical method was linear, precise and accurate. Test and reference formulation were found to be bioequivalent.

Keywords: Tiropramide; HPLC–UV; Bioequivalence

1. Introduction

Tiropramide is D,L-alpha-benzamide–*p*-[2-(diethylamino)-ethoxy]-*N*,*N*-dipropylhydrocinnamamide (Fig. 1) a synthetic anti-spasmodic agent, derived from tyrosine amino acid, which is devoid of atropine like actions and acts on smooth muscular fibro cells, however avoiding, atony since it does not bind itself to calmodulin. The myorelaxing activity of tiropramide stems from the capacity to increase intracellular cAMP, which activates a protein kinase, which by phosphorylating the LCMK (light chain myosin kinase) decreases its affinity to the calcium activated calmodulin, thus favoring decontraction of muscles.

Tiropramide has been used to treat acute cramping pain such as gallbladder, abdominal, urethral and renal colic. Tiropramide is a broad-spectrum anti-spasmodic agent with direct spasmolytic actions on smooth muscle cells. It is most effective on spastic hypertonic smooth muscle [1].

Literature search revealed that very few pharmacokinetic studies are published and the reported methods are based on liquid chromatography-tandem mass spectrometry [2], gas chromatography/nitrogen phosphorus detector [3,4] semi-micro HPLC using column switching [5] and gas chromatography coupled to mass spectrometry [6]. Although the reported methods are sensitive and accurate but are expensive, and labor intensive. Multistep sample preparation leads to low recovery and is time consuming, thus we have proposed sample preparation method based on liquid–liquid extraction, which is easy as compared reported method. Due to easy and fast sample preparation it was routinely applied for bioequivalence study sample analysis.

2. Experimental

2.1. Reagents and solutions

Tiropramide and diphenhydramine was obtained from Macleods Pharmaceuticals Ltd. and Dr. M.K.R. Drug Testing Laboratory, respectively, Mumbai, India. Acetonitrile (HPLC

* Corresponding author. Tel.: +91 22 26670871/1027; fax: +9122 26670816.
E-mail addresses: khanimiran_75@yahoo.co.in (K. Imran), pcloya@gmail.com (L. Punnamchand), saraf@bcpindia.org (S.M. Natvarlal).

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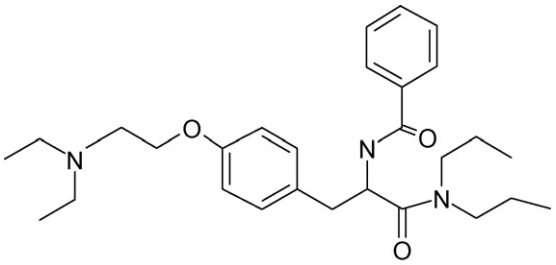

Fig. 1. Chemical structure of tiropramide.

grade), obtained from Qualigens Fine Chemicals, Mumbai and potassium dihydrogen phosphate, ortho phosphoric acid, methanol, perchloric acid, dichloromethane and *n*-hexane (all analytical grade reagent) were purchased from S.D. Fine Chem. Ltd., Mumbai. Freshly prepared double distilled water was used throughout the study. Fresh frozen human plasma used in the method development was obtained from the National Plasma Fractionation Center, K.E.M. Hospital, Mumbai, and was stored at −20 °C until required.

2.2. Instrumentation and chromatography

The HPLC system consisted of a JASCO-PU 980 intelligent pump (JASCO Ltd., Japan), manual injector port with 100 μl loop (Rheodyne, USA) and JASCO UV–Vis 975 intelligent detector (JASCO Ltd., Japan). The wavelength of the detector was set at 230 nm. Detector output was quantified on JASCO–Borwin (Version 1.50) chromatography software with Hercules 2000 chromatography Interface (Version 2.0). Separation was carried out on a HiQ Sil C_{18}W, 4.6 μm × 250 mm, Japan, using acetonitrile:water (pH 2.5 adjusted with dilute ortho-phosphoric acid) in the ratio of 40:60 as a mobile phase, at a flow rate of 1 ml/min. The mobile phase was filtered through nylon membrane filter (0.45 μm pore size, pall, Gelman Laboratories) and ultrasonically degassed prior to use. Total analysis time was 10 min. All analysis was performed at room temperature.

2.3. Preparation of calibration standard

Stock solutions of tiropramide and diphenhydramine hydrochloride (1 mg/ml) were in water and stored at 4 °C. The stock solution of tiropramide was further diluted with water to give series of standard solutions. Calibration standard of tiropramide (10, 25, 50, 100, 200 ng/ml) were prepared by spiking appropriate amount of the standard solution in blank plasma.

2.4. Quality control standards

Lowest quality control standards (LQC), median quality control standards (MQC) & highest quality control standards (HQC) were prepared by spiking drug free plasma with tiropramide to give solutions containing 10, 50 and 200 ng/ml, respectively. They were stored at −20 °C till analysed.

2.5. Sample preparation

To 1 ml plasma sample containing tiropramide (calibration standard), 20 μl of internal standard (10 μg/ml) was added and was vortexed for 1 min. A 0.4 ml of 0.05 M phosphate buffer (pH 7.0) was added and again vortexed for 1 min. The drug was extracted by vortexing with 7 ml of *n*-hexane for 60 s followed by centrifugation at 4000 rpm for 15 min at 4 °C. The organic supernatant was separated and evaporated. The residue was reconstituted with 200 μl of mobile phase and vortexed for 1 min, of which 100 μl was injected.

3. Validation [7]

The selectivity of the method was checked for interference from plasma. The standard curve consisting of five points ranging from 10 to 200 ng/ml was developed. Quality control samples i.e. LQC (10 ng/ml), MQC (50 ng/ml) and HQC (200 ng/ml) were used to determine the intra and inter-day precision and accuracy of the assay. Peak area ratios of tiropramide to internal standard were fit to linear equation ($y = 0.0141x - 0.0383$) and drug concentration in control samples along with the same day standard curve samples were calculated using this equation. For all the curves the correlation coefficients (r^2) were never lower than 0.9996.

3.1. Clinical design

The study protocol was approved by The Institution Ethics Committee. Twelve healthy male Indian subjects with mean age group 20–30 years and average weight 65.8 ± 6.1 kg were included in the study. Subjects were excluded from the study if one of more of following criteria were present at time of medical screening: allergic to tiropramide, history or clinical data of renal or liver disease, positive test for hepatitis B, HIV, history of alcohol, drug addiction or donated blood within 72 days prior to study. Test and reference formulation of tiropramide 100 mg tablet were administered with 240 ml of water. The study was conducted according to the principles outlined in the declaration of Helsinki. The study was conducted as 12 × 2 single dose, randomized, open, and complete crossover design. Volunteers were fasted overnight before and 4 h after drug administration. Blood sample (5 ml) were collected at 0.00 h and 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0 h of post drug administration through an indwelling cannula into heparinised glass vials. After drug administration standard breakfast and lunch were provided at 4 and 6 h post dose. The blood samples were immediately centrifuged, plasma was separated and stored at −20 °C until analysed. After a washout period of 7 days, the study was repeated in the same manner to complete the crossover design. The plasma samples obtained at various time intervals were analysed by the HPLC method developed.

3.2. Pharmacokinetic analysis

The plasma concentration profile obtained was fed into S-inverse (S-INV), computer software on BASICA® Version 1.12

and Microsoft excel®, to determine the pharmacokinetic parameters. The maximum tiropramide concentration C_{max} and the corresponding peak time T_{max} were determined by the inspection of the individual drug plasma concentration–time profiles. The elimination rate constant K_{el} was obtained from the least-square fitted terminal log-linear portion of the plasma concentration–time profile. The elimination half-life $T_{1/2}$ was calculated as $0.693/K_{el}$. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/K_{el}$ where C_t is the last measurable concentration.

3.3. Statistical analysis [8,9]

For the purpose of bioequivalence analysis AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were considered as primary variables. Bioequivalence of two formulation was assessed by means of an analysis of variance (ANOVA) for crossover design and calculating 90% confidence interval of the ratio of test/reference using log-transformed data. The formulation was considered bioequivalent when the difference between two compared parameters was found statistically insignificant ($p > 0.05$) and confidence interval for these parameters fell within 80–125%.

4. Results and discussion

4.1. Method development

Tiropramide is a water-soluble compound [2]. Hence, an attempt has been made to extract the drug from plasma by precipitating the plasma samples with solvents like acetonitrile, perchloric acid and methanol. It was found that the analyte recovery was less (51.23 ± 4.28%) with these precipitating agents, may be due to decrease in the solubility of tiropramide in water after the addition of these solvents.

Therefore liquid–liquid extraction procedure was tried using dichloromethane as extracting solvent. But due to the interference from blank plasma sample when extracted with dichloromethane, a non-polar solvent like *n*-hexane was tried. Being less polar than dichloromethane it was found that there were no interference from plasma samples when extracted with *n*-hexane. This proves the selectivity of the method and also the recovery was satisfactory with *n*-hexane. The extraction was carried out in one simple step in 0.05 M phosphate buffer (pH 7.0), which removed interferences due to plasma and yielded cleaner chromatograms. Thus *n*-hexane was used for sample preparation.

The composition of the mobile phase was chosen to provide concomitantly the best peak resolution and retention times. Thus to get the best resolution various mobile phases, in different proportion, buffered and non-buffered at various pH were attempted. The good resolution of analyte and internal standard was obtained with the mobile phase containing acetonitrile:water (pH 2.5 adjusted with dilute ortho phosphoric acid) in the ratio of 60:40 respectively pumped at 1 ml/min rate. The pH of the mobile phase was found to play an important role in peak resolution. It was first set at pH 5.5, but then lowered to pH 2.5, so that the peaks shape and elution increases.

4.2. Selectivity

Selectivity of the method described was investigated by screening six different batches of human blank plasma. Under the proposed assay condition internal standard and tiropramide had a retention time of 5.6 ± 0.2 and 8.3 ± 0.3 min, respectively, rest of the peaks were due to the plasma components. Tiropramide and internal standard were very well resolved under the proposed chromatographic conditions. None of the drug free plasma samples studied in this assay yield endogenous interference at these retention times. [Fig. 2(I–III)].

4.3. Accuracy

The mean percent accuracy of the proposed method was found to be 94.3 ± 4.23%.

4.4. Precision

Intra day precision for tiropramide was 9.6 ± 1.1, 45.6 ± 4.1 and 188.6 ± 8.5 for the spiked concentration at 10, 50 and 200 ng/ml and the percent coefficient of variation (%CV) was 11.4, 8.9 and 4.5, respectively. Inter day precision for tiropramide was 10.2 ± 1.2, 45.6 ± 5.1 and 193.5 ± 14.1 for the spiked concentration at 10, 50 and 200 ng/ml and the percent coefficient of variation (%CV) was 12.8, 11.1 and 7.3, respectively. (Table 1).

4.5. Linearity

The linearity of each calibration curve was determined by plotting the peak area ratio of tiropramide to internal standard verses nominal concentration of tiropramide. For linearity study five different concentration of tiropramide were analysed (10, 25, 50, 100, and 200 ng/ml). The peak area response was linear over the concentration range studied. Each experiment at all

Table 1
Precision of RP–HPLC method developed for the determination of tiropramide from human plasma

	Added concentration (ng/ml)		
	10	50	200
Intra day ($n = 6$)			
Found (mean ± S.D.)	9.6 ± 1.1	45.6 ± 4.1	188.6 ± 8.5
%CV	11.4	8.9	4.5
%Error	−4	−8.8	−5.7
Inter day ($n = 6$)			
Found (mean ± S.D.)	10.2 ± 1.2	45.6. ± 5.10	193.5 ± 14.1
%CV	12.8	11.1	7.3
%Error	2	−13	−3.25

The table gives mean and standard deviation (±S.D.) of concentration found from the quality control samples, calculated from six samples at each of the concentrations mentioned ($n = 6$) on each day (intra-day) and six samples per day at each of the concentrations mentioned ($n = 6$) on different days (inter-day). The table also gives their coefficient of variation (%CV).

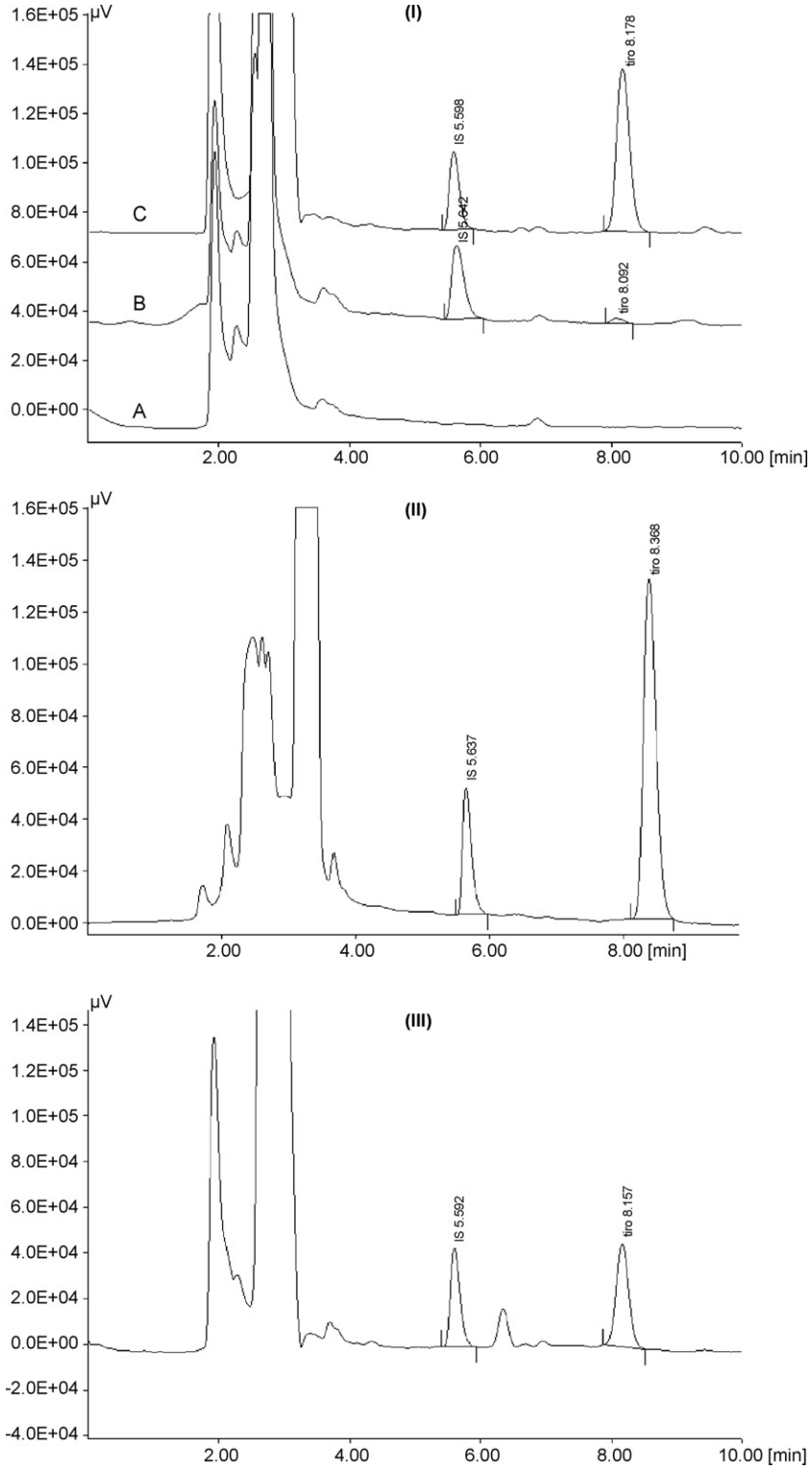

Fig. 2. Representative chromatograms. (I) Selectivity–comparative HPLC chromatogram, chromatogram A is blank plasma chromatogram, chromatogram B is quality control sample of LQC, which shows peaks of internal standard (IS) at Rt −5.642 min and tiropramide (tiro) at Rt −8.092 min. Chromatogram C is plasma spiked with drug (200 ng/ml) and internal standard (200 ng/ml), which shows peaks of internal standard (IS) at Rt −5.598 min and tiropramide (tiro) at Rt −8.178 min. (II) Representative standard chromatogram of internal standard peak (IS) at Rt −5.637 min and tiropramide peak (tiro) at Rt −8.368 min. (III) Human plasma sample collected at 1.75 h after administration of tiropramide formulation, which shows peak of internal standard at Rt −5.592 min and tiropramide at Rt −8.157 min.

Table 2
Recoveries of tiropramide and internal standard from drug free human plasma

Tiropramide	Concentration (ng/ml)		
	10	50	200
Mean % recovery ± S.D. ($n = 6$)	80.12 ± 3.01	86.32 ± 6.11	89.09 ± 6.18
%CV	3.7	7.0	6.9
Internal standard			200 (ng/ml)
Mean % recovery ± S.D. ($n = 6$)			92.06 ± 7.3
%CV			7.92

The table gives mean percent recovery and standard deviation of tiropramide quality control samples and internal standard (200 ng/ml), calculated from six samples at each of the concentrations mentioned ($n = 6$) along with their coefficient of variation (%CV).

concentration was repeated three times on three separate days to obtain the calibration data. The coefficient of correlation 'r^2' was found to be 0.9996. The limit of quantification and limit of detection were 10 and 2 ng/ml, respectively.

4.6. Recovery

The mean extraction recoveries of tiropramide determined over the concentration of 10, 50 and 200 ng/ml were 80.12 ± 3.01, 86.32 ± 6.11 and 89.09 ± 6.18%. For the internal standard (200 ng/ml), the mean extraction recovery was 92.06 ± 7.3% (Table 2).

4.7. Stability study

Short-term and long-term stock solution stability study was evaluated, which proved no significant deviation from normal value when stored at 4 °C. The stability of tiropramide in plasma was determined by measuring concentration change in quality control samples over time. Stability was tested by subjecting the quality controls to three freeze-thaw cycles and compared with freshly prepared quality control samples. As shown in (Table 3), the mean concentration of tiropramide in quality control samples did not change significantly within the time period under the indicated storage conditions. Long-term stability studies results conclude that tiropramide is stable in plasma matrix at least for 30 days when stored at −20 °C.

4.8. System suitability

System suitability test was performed daily before the run of analytical batch to check detector response to the analyte. This method showed a good ruggedness, in fact little change in mobile phase ratio or normal laboratory condition of humidity, light, and air exposure temperature did not influence the retention time of tiropramide and internal standard.

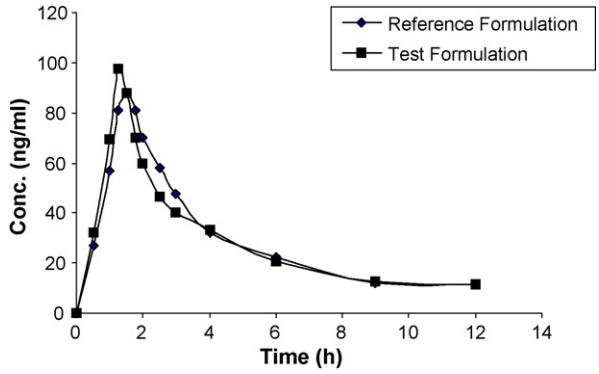

Fig. 3. Mean plasma concentration time profile of tiropramide 100 mg tablet in healthy male volunteers.

Both the formulations were well tolerated by all the volunteers in both the phases of study. No clinical adverse events occurred during the study. The mean concentration–time profile for the two brands of tiropramide 100 mg tablets is shown in Fig. 3. All calculated pharmacokinetic parameter values were in good agreement with the previously reported values. The pharmacokinetic parameters and statistical values for both formulations are given in Table 4.

For bioequivalence evaluation, C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were considered as primary parameters. The mean and standard deviation of these parameters of the two formulations were found to be very close, indicating that the plasma profiles generated by test formulation is comparable to those produce

Table 3
Three freeze thaw stability studies of tiropramide in plasma

Spiked concentration (ng/ml)	QC sample analysed immediately (0 h) (mean ± S.D., ng/ml) ($n = 3$)	%CV	QC sample analysed at 24 h after freeze thaw cycle (mean ± S.D., ng/ml) ($n = 3$)	%CV
10	9.6 ± 0.9	9.3	9.5 ± 1.1	11.5
50	43.9 ± 2.6	5.9	46.7 ± 2.4	5.1
200	197.6 ± 17.5	8.8	197.61 ± 18.2	9.21

The table gives three freeze thaw stability data of tiropramide in human plasma at three different concentrations i.e. Lowest quality control (LQC), median quality control (MQC). Highest quality control (HQC) standards and standard deviations (±S.D.) along with coefficient of variation (%CV). Each sample was analysed in triplicates ($n = 3$).

Table 4
Mean pharmacokinetic parameters of tiropramide tablet after single oral dose in 12 healthy male volunteers

Parameters	Test	Reference	90%CI[a] (80–125%)	*P* value[b]
C_{max} (ng/ml)	106.76 ± 11.9	105.35 ± 15.7	98.65–102.78	0.58
T_{max} (h)	1.29 ± 0.1	1.5 ± 0.2		0.19
$T_{1/2}$ (h)	3.07 ± 0.86	3.3 ± 1.0		0.57
K_{el}	0.24 ± 0.1	0.20 ± 0.1		0.51
AUC_{0-t} (ng/ml h)	320.56 ± 85.3	341.7 ± 74.9	102.28–106.05	0.44
$AUC_{0-\infty}$ (ng/ml h)	352.81 ± 90.8	375.4 ± 76.7	102.18–105.96	0.97

The table gives mean pharmacokinetic parameters of tiropramide tablet after single oral dose in 12 healthy male volunteers and 90% confidence interval for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$. Values are given as standard error of mean.

[a] Statistics were applied on logarithm transformed data, $n = 12$.

[b] Non-significant difference at 95% confidence limits.

by reference formulation. Analysis of variance (ANOVA), after log transformation of the data, showed no statistically significant ($p > 0.05$) difference between the two formulations.

The mean peak plasma concentrations for 100 mg tiropramide tablet were found to be 105.35 ± 15.7 and 106.76 ± 11.9 ng/ml for the reference and test formulations respectively. These values were achieved at 1.5 ± 0.2 and 1.29 ± 0.1 h, respectively for reference and test formulations. AUC_{0-12} were found to be 341.7 ± 74.9 and 320.56 ± 85.3 ng h/ml for the reference and test formulations respectively. $AUC_{0-\infty}$ were found to be 375.4 ± 76.7 and 352.81 ± 90.8 ng h/ml for the reference and test formulations respectively.

The elimination rate constant K_{el} for the reference and test formulation was found to be 0.20 ± 0.1 and 0.24 ± 0.1, respectively while the elimination half for the two formulations was 3.3 ± 1.0 and 3.07 ± 0.86, respectively.

The relative bioavailability of the test formulation as judged from AUC_{0-12} was found to be 98.90% as compared to the reference formulation for tiropramide.

The 90% confidence limits for the ratio of the log normal transformed data of C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ of test and reference formulation were found to in the range of 98.65–102.78, 102.28–106.05 and 102.18–105.96% respectively and are within prescribed limits (80–125%) (Table 4).

5. Conclusion

An HPLC–UV based method has been developed for quantification of tiropramide in human plasma. The sensitivity and simplicity of the method makes it suitable for pharmacokinetic studies. The statistical comparison of AUC and C_{max} clearly indicated no significant difference in the two formulations of 100 mg of tiropramide tablets. Ninety percent confidence interval for the mean (T/R) of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} indicates that the reported values were entirely within the bioequivalence acceptance range of 80–125% (using log transformed data). Based on these results a concise decision on bioequivalence was taken. Hence it was concluded that formulation 'Test' is bioequivalent with formulation 'Reference' and can be interchangeable in clinical practice.

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References

[1] I. Setnikar, R. Cereda, M.A. Pacini, L. Revel, F. Makovec, Arzneimittelforschung 39 (1989) 1114–1119.
[2] H.W. Lee, H.Y. Ji, H.H. Kim, H.Y. Cho, Y.B. Lee, H.S. Lee, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 796 (2003) 395–400.
[3] O.S. Kwon, Y.J. Park, Y.B. Chung, Arzneimittelforschung. 53 (2003) 578–583.
[4] O.S. Kwon, Y.J. Park, J.C. Ryu, Y.B. Chung, Arch. Pharm. Res. 26 (2003) 416–420.
[5] S.K. Baek, S.S. Lee, E.J. Park, D.H. Sohn, H.S. Lee, J. Pharm. Biomed. Anal. 31 (2003) 185–190.
[6] O.H. Jhee, Y.C. Jeon, H.S. Choi, M.H. Lee, A.S. Om, J.W. Lee, J.W. Hong, Y.S. Kim, J.C. Kang, Y.S. Lee, L.M. Shaw, J.S. Kang, Clin. Chim. Acta 366 (2006) 179–184.
[7] Guidance for Industry, Bioanalytical Method Validation. Rockville, Maryland: U.S. Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research, CDER, 2001.
[8] Guidance for Industry. Statistical Approaches in Establishing Bioequivalence. Rockville, Maryland: U.S. Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research, CDER, 2001.
[9] S. Bolton (Ed.), Experimental Design in Clinical trail in: Pharmaceutical Statistics Practical and Clinical Applications, vol. 80, 3rd ed., Marcel Dekker Inc., New York, pp. 384–443.