www.sphinxsai.com

PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.3, No.3, pp 1219-1226, July-Sept 2011

Determination of Active Metabolite Desmethyl Trimebutine Levels after a Single Dose of Trimebutine Tablets in Healthy Human Male Volunteers

Imran Khan*

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

Corres. Author: khanimran_75@yahoo.co.in

Abstract: A simple, rapid and selective HPLC method has been developed and validated for the determination of an active metabolite desmethyl trimebutine in human plasma, after a single dose of trimebutine tablet to healthy human volunteers. Following, liquid-liquid extraction, the analyte and internal standard (carbamazepine) were separated from human plasma using an isocratic mobile phase on Rp-18 stationery phase. The analysis was monitored on a UV detector at 265 nm. The method was linear in the concentration range of 20–2000 ng/ml using 1ml of plasma. The lower limit of quantification was 20 ng/ml. The method was found to be precise, accurate and could be successfully used in a bioequivalence study of trimebutine.
Key words: Trimebutine, desmethyl trimebutine, bioequivalence, HPLC-UV.

Introduction:

Trimebutine, 3,4,5-trimethoxybenzoic acid 2-(dimethylamino)-2-phenylbutyl ester maleate **(Fig. 1)** is an antispasmodic compound widely used for functional intestinal diseases since 1969 in France[1]. The efficacy of the compound to relieve abdominal pain has been demonstrated in various clinical studies. The mechanism of action involves the non-specific stimulation of opioid receptors [2-4], reproducing the local stimulating action on enkephalines and dynorphines [5]. After oral administration, the drug exhibits a high hepatic first pass effect so that the main entity in plasma is its main metabolite desmethyl trimebutine[6]. Literature search revealed that few pharmacokinetic studies are published[7-12]. Reported methods though sensitive and accurate are too expensive, time consuming, energy intensive and involve complex sample preparation. This communication describes a simple, sensitive, and economical reverse phase HPLC analytical method and its successful application to a bioequivalence study of trimebutine tablets in healthy human subjects under fasting condition. Author has presented this research in the 16th International Symposium on Pharmaceutical and Biomedical Analysis, 28-31Aug 2005, Baltimore, MD USA.

Material and Methods

Desmethyl trimebutine and carbamazepine were obtained from Macleods Pharmaceuticals Ltd, Mumbai, India and Dr. M.K.R. Drug Testing Laboratory, Mumbai, India, respectively. HPLC grade acetonitrile and methylene chloride were purchased from Qualigens Fine Chemicals, Mumbai. Analytical grades potassium dihydrogen phosphate and ortho phosphoric acid 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.

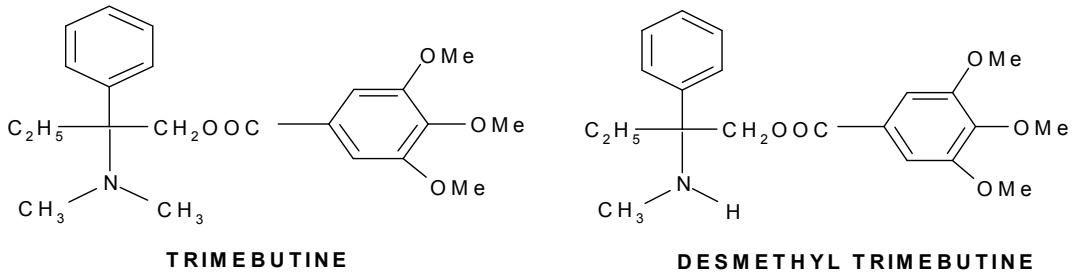


Fig. 1: Chemical structures of trimebutine and desmethyl trimebutine

Chromatographic conditions:
The HPLC system consisted of a Jasco–PU 980 intelligent pump (Jasco Ltd., Japan), manual injector port with 50µl loop (Rheodyne, USA) and Jasco UV–Vis 975 intelligent detector (Jasco Ltd., Japan). The wavelength of the detector was set at 265nm. 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 X 250 mm, using acetonitrile:phosphate buffer (0.1M, pH 3.8) in the proportion of 37:63, respectively, 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.

Preparation of desmethyl trimebutine standard solution:
A standard stock solution of desmethyl trimebutine was prepared in water at a concentration of 1 mg/ml. Working desmethyl trimebutine solutions ranging from 20 to 2000 ng/ml were prepared by suitable dilution of the stock standard solution with water and stored at 4° C.

Preparation of internal standard solution:
A standard stock solution of 1 mg/ml carbamazepine was prepared in methanol. This solution was further diluted with methanol to 10 ml yield 100 µg/ml coumarin solution.

Preparation of calibration standard solutions in plasma:
Calibration standards in plasma were prepared by spiking drug free plasma with 100 µl of 1000 µg/ml desmethyl trimebutine stock solution, diluted to 10 ml with drug free plasma to furnish a 10 µg/ml actarit calibration standard. This solution was further diluted with drug free plasma to furnish the calibration standards of 20, 50, 100, 250, 500, 1000, 1500 and 2000 ng/ml. They were frozen in small portions at –20° C till analyzed.

Preparation of quality control standard solutions in plasma:
Lowest quality control standards (LQC), median quality control standards (MQC) and highest quality control standards (HQC) were prepared by spiking drug free plasma with desmethyl trimebutine to give solutions containing 20, 500 and 1500ng/ml, respectively. These samples were used to validate the method and were stored in small portions at –20° C until analyzed.

Sample preparation for HPLC injection:
To 1ml plasma containing desmethyl trimebutine (calibration standard), internal standard carbamazepine (20µl of 10µg/ml) was added and vortexed for 30 s. Subsequently the drug was extracted with 7ml methylene chloride by vortexing for 30 s followed by centrifugation at 6000 X g for 15 min at 4° C. The organic layer was separated and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 250 µl of the mobile phase, 50 µl of which was injected in the column.

Validation of the analytical method [13]:
The analytical method developed for the determination of desmethyl trimebutine from plasma was validated for its selectivity, Limit of detection and quantitation, precision, accuracy, linearity, recovery, sensitivity, and stability in plasma.

Selectivity:
A quality control sample containing desmethyl trimebutine (1500 ng/ml) and internal standard along with six different samples of drug free blank plasma samples were prepared and checked for the endogenous interference near the Retention time of the analyte and the internal standard.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):
A blank plasma sample was extracted and developed under chromatographic conditions. The noise level was determined. The LOD (signal-to-noise ratio = 3) and LOQ (signal-to-noise ratio = 10) for desmethyl trimebutine was determined.

Precision and accuracy:
Intra–day and inter–day precision and accuracy were determined by analyzing quality control standards of desmethyl trimebutine (20, 500 and 1500 ng/ml). six times a day randomly and once on each of six different days respectively. Six samples of each concentration along with a fixed concentration of internal standard were developed under optimized chromatographic conditions and the response was measured.

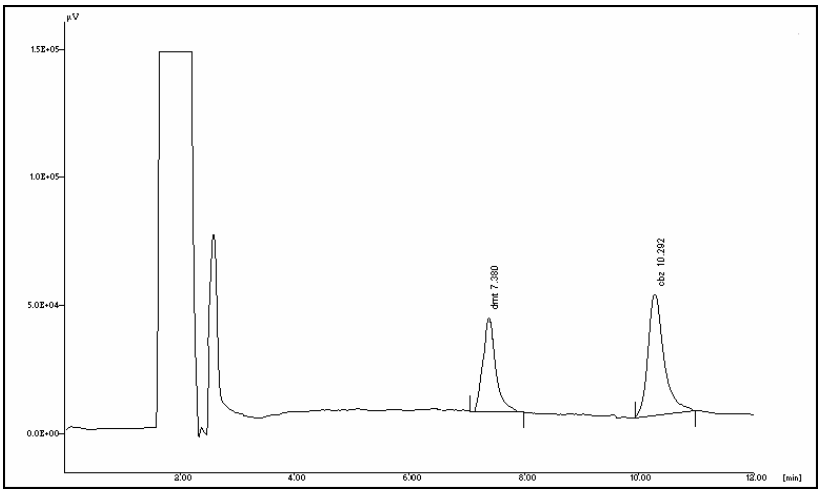
Linearity:
Linearity of the proposed method was determined by spiking various known concentrations of desmethyl trimebutine in plasma 20–2000 ng/ml. For linearity study eight different concentrations of desmethyl trimebutine were analyzed (20, 50, 100, 250, 500, 1000, 1500, 2000 ng/ml). To each of these samples, 20 µl of an internal standard (10 µg/ml) was added. Spiked concentrations were plotted against the peak area ratios of desmethyl trimebutine to internal standard. The best-fit line was obtained by linear regression analysis of the resultant curve. The linearity equation ($y = mx + c$) and the regression coefficient were calculated.

Recovery:
Recovery was performed in triplicates on quality control standards of desmethyl trimebutine (20, 500 and 1500 ng/ml) containing internal standard 200 ng/ml in each of these quality control standard plasma samples. Recovery of desmethyl trimebutine was determined by comparing the peak area ratio of desmethyl trimebutine to internal standard obtained from the extracted quality control standards with that of peak area ratio of desmethyl trimebutine to internal standard obtained from working standards solution of the respective concentration.

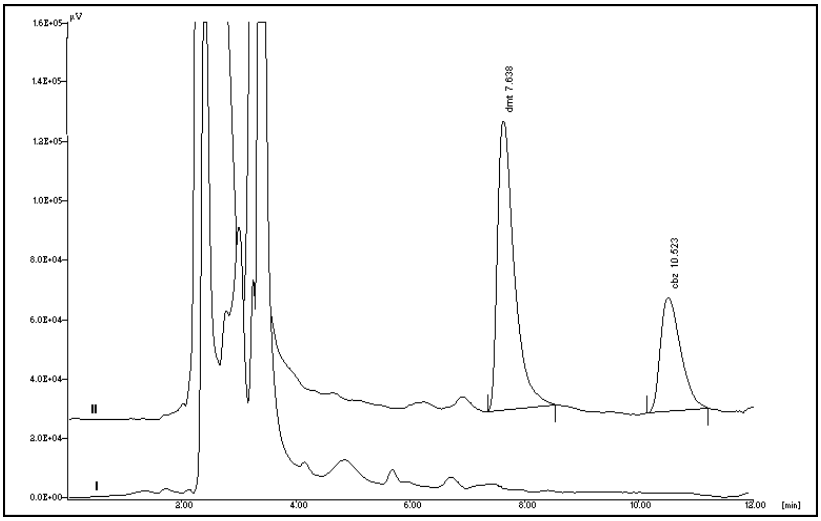
Stability studies:
The stability of desmethyl trimebutine was determined by measuring concentration change in quality control samples over time. The plasma control samples were stored in polypropylene plasma tubes at –20º C. Stability was tested by subjecting the quality controls to three freeze - thaw cycles. The long term stability of desmethyl trimebutine was determined on day 5, day 15 and day 30.

In vivo study:
The method described in this communication was applied to a bioequivalence study of two oral formulations of trimebutine 100 mg tablets from Macleods Pharmaceutical Ltd. (Test formulation) versus that of Debridat tablets (100 mg) manufactured by Pfizer (Reference formulation). Institutional Ethics Committee of Bombay College of Pharmacy approved the study protocol. Twelve healthy male Indian subjects with mean age group 20-30 years and average weight 64.7±5.9 kg were included in the study. Subjects were excluded from the study if one or more of the following criteria were present at time of medical screening, allergic to trimebutine, 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. The study was 12×2 single dose, randomized, open, and crossover design. Subjects were fasted for at least 10 h prior and until 4 h after dosing of drug. The volunteers administered per oral one tablet (test or reference) of trimebutine 100 mg with 240 ml water. Blood sample (5 ml) were collected at 0 (before dosing) and then at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, and 12.0 h post drug administration through an indwelling cannula into heparinised tubes. After drug administration, 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 seven 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 HPLC method developed. Pharmacokinetic parameters (C_{max}, T_{max}, $T_{1/2}$, K_{ele}, AUC_{0-t} and AUC_{0-inf}) were calculated using Basica® (V 1.12).

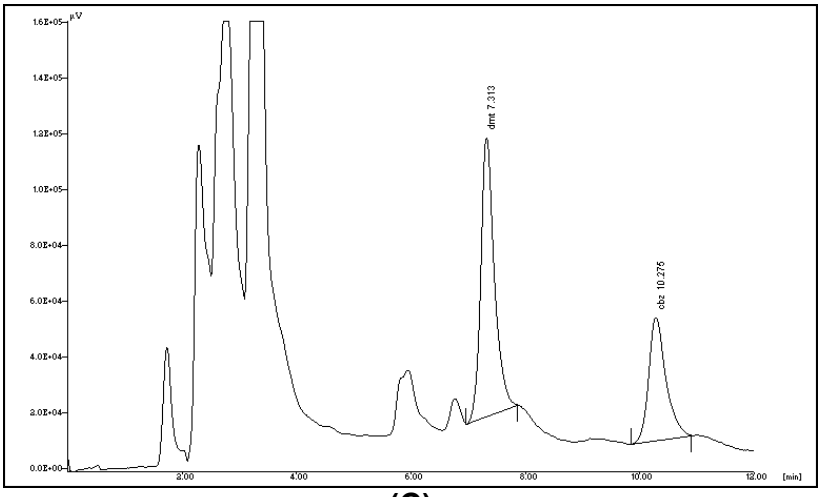
Statistical Analysis [14]:
For the purpose of bioequivalence analysis, untransformed and log transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-inf} were subjected to Analysis of Variance (ANOVA) to test the variance due to subject, period and formulation effects. The 90 % confidence interval for log- transformed data of C_{max} and AUC_{0-t} and AUC_{0-inf} were computed. The products were considered bioequivalent when the difference between two compared parameters was found statistically insignificant ($p \geq 0.05$) and 90 % confidence interval for theses parameters fell within 80 -125 %.



(A)



(B)



(C)

Fig. 2: Representative chromatograms of standard, quality control and volunteers plasma sample (A-C)

Results and Discussion

During the method development for the determination of desmethyl trimebutine from the human plasma, the chromatographic conditions such as columns, mobile phases, flow rate, detection wavelength were studied during optimization. The optimal chromatographic conditions for the separation of desmethyl trimebutine were achieved. The UV scan of pure desmethyl trimebutine in water showed a λmax of 265 nm. Hence UV detector was set at this wavelength for the entire analysis of desmethyl trimebutine.

Based on the literature, a C18 column was the first choice in the development of chromatographic conditions. Chromatograms of three different concentrations of desmethyl trimebutine were studied using Zorbax XDB C18, Inertsil and Hypersil columns. The Zorbax XDB C18 and Inertsil column showed tailing. However, better peak shapes were obtained with Hypersil column.

Beside the column, the composition of the mobile phase is another key factor among the chromatographic conditions. Various mobile phases constituting viz acetonitrile and citric acid (0.1M, 40:60, pH 5.0), acetonitrile and phosphate buffer (0.1M, 37:63, pH 3.8) and (0.05 M, 40:60, pH 5.6) were attempted. These mobile phases were tried at different flow rate, to study the separation of desmethyl trimebutine and internal standard from plasma interfering substance on C18 column. No systematic method of optimization was followed except for trial and error procedure.

Phosphate buffer (0.1 M, pH 3.8) along with acetonitrile gave better peak shapes with good resolution. The molar concentration of phosphate buffer, the proportions of acetonitrile and phosphate buffer were optimized through serial trials to achieve good peaks and resolution of analyte and internal standard. The proportion of acetonitrile in the mobile phase is crucial and was optimized to 37 %, v/v. A slight increase or decrease in concentration of acetonitrile resulted in the drug peak merger with the plasma peak or increased the retention time of drug from 7.3 min to 10.2 min, respectively. A slight decrease in the molar concentration of phosphate buffer (i.e from 0.1M) in the mobile phase and mobile phase consisting of citric acid, resulted in less peak area and poor shape (increasing asymmetry) of desmethyl trimebutine when compared with 0.1M phosphate buffered mobile phase. Thus a mixture of acetonitrile and 0.1 M phosphate buffer adjusted to pH 3.8 with phosphoric acid (37 : 63, v/v), delivered at a flow rate of 1 ml/min with detection at 265 nm could achieve our purpose and was finally adopted as mobile phase.

In the present study, liquid-liquid extraction procedure was adopted to get high recovery. During development of sample preparation different solvents in different proportions such as methylene chloride, n-hexane, chloroform-isopropyl alcohol, diethyl ether, and ethyl acetate were tested for extraction of analyte and internal standard. The selected solvent for extraction was methylene chloride, due to its satisfactory efficiency in extraction with less endogenous impurity compared to that observed with chloroform-isopropyl alcohol, diethyl ether and ethyl acetate. All the solvents used were evaluated for recovery and selectivity of the method. The best recovery and clean chromatograms were obtained when extracted with methylene chloride.

Selectivity of the method described was investigated by screening six different batches of human blank plasma. Under the proposed assay condition desmethyl trimebutine and internal standard had a retention time of 7.3±0.3 min and 10.2±0.3 min, respectively, rest of the peaks were due to the plasma components. Desmethyl trimebutine and internal standard were very well resolved under the proposed chromatographic conditions. None of the drug free plasma samples studied in this assay yielded endogenous interference at the retention times of drug and internal standard.

Fig. 2(A–C) shows the typical chromatograms of a standard desmethyl trimebutine and internal standard, blank plasma sample, blank plasma sample spiked with desmethyl trimebutine and internal standard, and a plasma sample obtained at 1.5 h after an oral administration of 100 mg trimebutine tablet to a healthy human volunteer under fasting condition.

The limit of quantification was 20 ng/ml for determination of desmethyl trimebutine in plasma. The precision and accuracy at the concentration of LOQ is shown in **Table 1**. Under the present LOQ, the desmethyl trimebutine concentration could be determined in plasma samples up to 12 h after a single oral dose of 100 mg of trimebutine tablets, which is sensitive enough to investigate the pharmacokinetic behavior of the drug.

Table 1: Intra-day and inter-day precision and accuracy data

Spiked Conc. (ng/ml)	Intra-day			Inter-day		
	Precision		Accuracy(%)	Precision		Accuracy (%)
	Mean±SD (n=6)	%CV		Mean±SD (n=6)	%CV	
20	17.14±1.90	11.0	85.7	18.30±1.95	10.6	91.5
500	499.71± 25.75	5.15	99.94	501.7±26.41	5.20	100.34
1500	1483.05± 95.1	6.41	98.87	1488.84±98.02	6.58	99.25

The table gives mean and standard deviation (± SD) of concentration found from the quality control samples, calculated from six samples at each of the concentrations mentioned (n=6) along with their coefficient of variation (% CV).

Table 2: Three freeze–thaw stability of desmethyl trimebutine in plasma

Spiked Conc (ng/ml)	Freshly prepared Sample(ng/ml)		Three freeze - thaw cycle(ng/ml)	
	mean±SD (n=3)	%CV	mean±SD(n=3)	%CV
LQC (20)	18.03±2.30	12.7	16.32±2.20	13.4
MQC (500)	523.28±51.01	8.8	518.00±45.62	9.7
HQC (1500)	1515.45±68.85	4.5	1501.00±70.52	4.6

The table gives three freeze thaw stability data of desmethyl trimebutine in human plasma at 3 different concentrations i.e. lowest quality control (LQC), median quality control (MQC) and highest quality control (HQC) standards and standard deviations (± SD) along with their coefficient of variation (% CV). Each sample was analysed in triplicates (n = 3).

Table 3: Long-term stability of desmethyl trimebutine in plasma

Days of storage	LQC (20 ng/ml)	MQC (500 ng/ml)	HQC (1500 ng/ml)
After 5 days	19.18	492.36	1592.00
After 15 days	20.56	512.34	1490.00
After 30 days	23.32	506.68	1502.00
Mean	21.02	498.13	1528.00
SD	2.10	7.56	55.75
% CV	10.02	1.52	3.65

The table gives long term stability (30 days) data of desmethyl trimebutine in human plasma at 3 different concentrations i.e. lowest quality control (LQC), median quality control (MQC) and highest quality control (HQC) standards and standard deviations (± SD) along with their coefficient of variation (% CV).

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range of 20–2000 ng/ml (eight calibration points) in human plasma. A typical equation of the calibration curves was as follows: $y = 0.0014x - 0.01$ ($r^2 = 0.9957$), where y represents the peak area ratio of analyte to IS and x represents the plasma concentration of desmethyl trimebutine.

Intra day and inter day precision and accuracy were tabulated in table1 at the quality control standards of desmethyl trimebutine (20, 500 and 1500 ng/ml).

Mean extraction recoveries of desmethyl trimebutine at 20, 500 and 1500ng/ml were 77.12±4.03 %, 83.32±5.52 % and 85.06±6.89 %, respectively (*n*=3). Mean recovery of the internal standard (200 ng/ml) was 95.00±4.80 % (*n*=3).

Table 4: Pharmacokinetic parameters of desmethyl trimebutine after single oral dose of 100 mg trimebutine tablet in 12 healthy male volunteers

Parameters	Test	Reference	90 %CI$^{\varphi}$ (80-125 %)	P value$^{\Psi}$
C_{max} (ng/ml)	1756.4±570.5	1694.0±377.3	97.85 to 102.3	0.67
T_{max} (h)	1.08±0.44	1.20±0.32		0.52
K_{ele}	0.19±0.15	0.19±0.07		0.99
$T_{1/2}$ (h)	5.3±1.9	4.5±3.1		0.57
AUC_{0-t} (ng.h/ml)	4459.5±1003.9	3932.5±1184.8	101.8 to 105.7	0.07
AUC_{0-inf} (ng.h/ml)	5049.9±1297.0	4447.0±1711.5	101.6 to 105.8	0.23

The table gives mean pharmacokinetic parameters of desmethyl trimebutine tablet after single oral dose of 100mg trimebutine tablet in 12 healthy male volunteers and 90% confidence interval for C_{max}, AUC_{0-t} and AUC_{0-inf}. All the values are given as standard error of mean. **Ψ** indicates non-significant difference at 95% confidence limits. φ indicates statistics were applied on logarithm transformed data (n= 12).

The stabilities of desmethyl trimebutine after three freeze–thaw cycles and after long period of storage at −20° C were investigated. The results are listed in **Table 2 and 3**. The mean concentration of desmethyl trimebutine in quality control samples did not change significantly within the time under the indicated storage conditions. Long-term stability studies indicated that desmethyl trimebutine is stable in plasma matrix at least for 30 days when stored at –20° C.

This validated analytical method has been successfully applied to determine the plasma concentration of desmethyl trimebutine after an oral administration of tablet containing 100 mg trimebutine to 12 healthy subjects in a bioequivalence study.

The pharmacokinetic parameters and statistical values after an oral administration of the both formulations are given in **Table 4**. The relative bioavailability of the test formulation as judged from AUC_{0-12} was found to be 101.51% as compared to the reference formulation for trimebutine. For bioequivalence evaluation, statistical modules were applied to AUC_{0-t}, AUC_{0-inf} and C_{max} as per current guidelines. The 90 % confidence interval for the ratio of the log normal transformed data of C_{max}, AUC_{0-t}, and AUC_{0-inf} of test product and the reference product were found to in the range of 97.85 to 102.3, 101.8 to 105.7 and 101.6 to 105.8 and are within prescribed limits (80 to 125 %). Analysis of variance (ANOVA), after log-transformation of the data, showed no statistically significant ($p>0.05$) difference between the two formulations.

The statistical comparison of AUC_{0-t}, AUC_{0-inf} and C_{max} clearly indicated no significant difference in the two brands of trimebutine 100 mg tablets. Concise decision on bioequivalence was taken based on confidence intervals because these were entirely within acceptability limit. These data demonstrated that the developed method could provide a satisfied sensitivity for bioequivalence studies.

An HPLC-UV method for the determination of desmethyl trimebutine in human plasma was developed and validated. This paper describes a simple, rapid and sensitive method for the determination of desmethyl trimebutine and was found to be sensitive and selective for the analysis of desmethyl trimebutine in comparison to the previously reported analytical methods. The developed and validated method was successfully applied to a bioequivalence study of trimebutine tablets and was found to be reasonably sensitive and reliable.

Acknowledgements

The authors wish to thank M/s Macleods Pharmaceuticals and Shri. Amrut Mody Research Fund (AMRF) for providing gift samples of desmethyl trimebutine and financial support respectively to conduct this project work.

References

1. Fraitag B, Hostein J, Pascaud X, Junien JL. Trimebutine: Pharmacology, recent concepts about its mechanism of action, therapeutic results. Gastroenterol. Clin. Biol 1992;16:413-20.
2. Fioramonti J, Fargeas MJ, Bueno L. The involvement of opiate receptors in the effects of trimebutine on intestinal motility in the conscious dog. J. Pharm. Pharmacol. 1984;36:618-21.
3. Pascaud X, Roman F, Petoux F, Vauche D, Junien JL. Involvement of opiate receptors in the mode of action of trimebutine. Gastroenterol. Clin. Biol. 1987;11(3 Pt 2):77B-81B
4. Allescher HD, Ahmad S, Classen M, Daniel EE. Interaction of trimebutine and Jo-1196 (fedotozine) with opioid receptor in canine ileum**.** J. Phamacol. Exp. Ther. 1991;257:836-42.
5. Roman F, Pascaud X, Taylor JE, Junien JL. Interaction of trimebutine with guinea- pig opioid receptors. J .Pharm. Pharmacol. 1987;39:404-07.
6. Bueno L, Honde C, Pascaud X, Junien JL. Effects of orally vs parenterally administered trimebutine on gastrointestinal and colonic motility in dogs. Gastroenterol. Clin. Biol**.** 1987;11(3 Pt 2):90B-93B.
7. Lavit M, Saivin S, Boudra H, Michel F, Martin A, Cahiez G, *et al*. Determination of Trimebutine and Desmethyl trimebutine in Human Plasma by HPLC. Arzneim.-Forsch/Drug Res. 2000;50:640-44.
8. Saivin S, Lavit M, Michel F, Labaune JP, Martin A, Cahiez G, *et al*. Pharmacokinetic and Bioequivalence of Two Trimebutine Formulations in healthy Volunteers Using and Desmethyl trimebutine levels. Arzneim.-Forsch/Drug Res 2000;50:717-21.
9. Eun-Hee J, Woo-Ik C, Injoon O, Sang-Chul S, Han-Kwang N, Yong-Bok L. High-performance liquid chromatographic determination of trimebutine and its major metabolite, N-monodesmethyl trimebutine, in rat and human plasma. J. Chromatogr. B 1999;723:239-46.
10. Wang H, Zhou H, Horimoto S, Jiang J, Mayumi T, Hu P. Quantitative determination of trimebutine maleate and its three metabolites in human plasma by liquid chromatography-tandem mass spectrometry. J. Chromatogr. B 2002;779:173-87.
11. Astier A, Deutsch AM. Quantitative high-performance liquid chromatographic determination of antispasmodic trimebutine in human plasma: pharmacokinetic studies after intravenous administration in humans. J Chromatogr. 1981;224:149-55.
12. Jiang H, Ding L, Yang J, Huang X, Liu GY, Zhang ZX. Pharmacokinetic and bioequivalence of trimebutine dispersive tablet in healthy subjects. Yao Xue Xue Bao 2004;39:208-11.
13. Guidance for Industry. Bioanalytical Method Validation. U.S. Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research. (CDER), Rockville, Maryland: 2001.
14. FDA Guidelines Statistical Approaches in Establishing Bioequivalence. U.S. Department of Health and Human Services. Food and Drug Administration, Center for Drug Evaluation and Research. (CDER), Rockville, Maryland: 2001.

*****