Validation and Optimization of a Simple RP-HPLC Method for Determination of Trimetazidine in Human Serum and its Application in a Pharmacokinetic Study with Healthy Bangladeshi Male Volunteers

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Abstract: Trimetazidine is an effective and well-tolerated antianginal drug. In the present study, a simple, sensitive and specific liquid chromatography (HPLC) method with UV detection was developed and validated for the quantification of trimetazidine in human serum samples using caffeine as internal standard. Protein precipitation method with methanol was employed in the extraction of trimetazidine and caffeine from biological matrix. The chromatographic separation was accomplished on Xterra C_{18} Column with a mobile phase consisting 0.01 M potassium dihydrogen phosphate buffer (pH 4.16 ± 0.01 adjusted with orthophosphoric acid, with a solvent system of triethanolamine and acetonitrile (90:10) at a flow rate of 1.0 ml/min. The chromatogram was monitored at a wavelength of 207 nm. The method was validated over a linear concentration range of 5-200 ng/ml and limit of quantification (LOQ) was 5.0 ng/ml with a *coefficient of correlation* (r^2) ≥ 0.996. The intra-day and inter-day precision expressed as relative standard deviation was 3.40%-11.63% and 1.30%-10.21%, respectively. The average recovery of trimetazidine from serum was 97.44%. The method was successfully applied to a pharmacokinetic study after oral administration of modified release trimetazidine hydrochloride tablet (35 mg) in healthy Bangladeshi volunteers.

Key words: Trimetazidine; Antianginal drug; Method development and validation; Pharmacokinetics; Bangladeshi volunteer

Introduction

Trimetazidine [1-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride] (Figure 1A) is an antianginal drug having no hemodynamic effect and does not affect cardiac myocyte oxygen demand or supply as other antianginal drugs.[1] It has been used in the prophylaxis and management of angina pectoris, ischemia, and Meniere's disease.[2] Recent studies indicate that this effect is due to the selective blockage of long chain 3-ketoacyl coenzyme-A thiolase activity, the enzyme involved in beta-oxidation.[3] This inhibition decreases the utilization of free fatty acids as a source of energy for the myocardium, resulting in an increase in glucose oxidation.[3] Moreover, trimetazidine reduces intracellular acidosis and protects the toxicity induced by oxygen free radicals. The drug therefore directly protects myocyte structure and function, and increases cell resistance to hypoxic stress.[4-5] For these clinical successes, trimetazidine (TMZ) has become unique among the antianginal agents, and it has been clinically used throughout many countries worldwide.[6-7]

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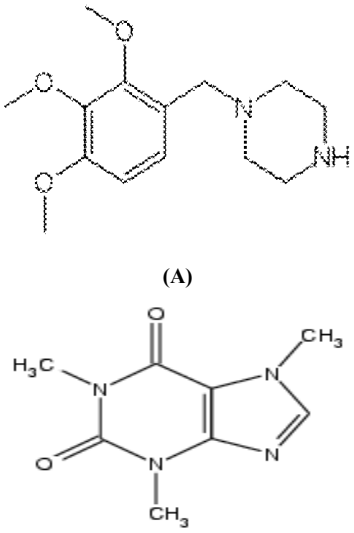
Because of its high solubility, short half-life and therapeutic use in chronic diseases, Trimetazidine is considered as oral modified release dosage forms[8] because of its high solubility, short half-life and therapeutic use in chronic diseases. Modified-release (MR) formulation are aimed to maintain the therapeutic serum concentration with less fluctuation. This drug gives a higher C_{min} compared to immediate release form (20 mg×3) and only twice-daily application (35 mg×2) to maintain antianginal efficacy beyond the trough (12 h after last drug intake) through increased minimal serum concentrations, which is bioequivalent to the immediate-release formulation.[8-11]



(A)

(B)

Figure 1. Chemical structures of (A) trimetazidine and (B) caffeine.

Analytical methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetics.[12] Several methods are described in the literature for the quantitative analysis of Trimetazidine in serum.[13-15] Some authors reported the use of fluorescence detector[7]; while others reported the use of mass spectroscopy[13,14] which is not available in all laboratories for routine analysis. Hence, the aims of the present study were to develop and validate a simple and rapid RP-HPLC method for the determination of trimetazidine in human serum samples with good resolution having the desired sensitivity when applying to a pharmacokinetic study of trimetazidine in healthy Bangladeshi male volunteers.

Experimental

Materials and reagents

Trimetazidine and caffeine (internal standard) (Figure 1B) were provided by Aristopharma Bangladesh Ltd. Modified release film coated tablets were obtained from Les Laboratoires Servier, France. HPLC grade acetonitrile, methanol and triethyl amine were obtained from Fisher Scientific, UK. Potassium dihydrogen phosphate, orthophosphoric acid and other reagents used were of analytical grade and were used without further purification. A Milli-Q® (Millipore, France) water purification system was used to obtain the purified water for the HPLC analysis.

Instrumentation

(a) HPLC systems. A Shimadzu (Kyoto, Japan) HPLC system was used consisting of a SCL-10Avp system controller equipped with two LC-8A pumps, an UV detector (SPD-10Avp) (Shimadzu Corporation, Kyoto, Japan) and a manual injector with a 20 µl loop. The detector was set at 207 nm at a sensitivity of 0.0001 AUFS.

(b) *Columns*. Chromatographic separation was achieved with a XTerra C_{18} column (5µ, 4.6×250 mm, Waters, Massachusetts, USA) at room temperature.

(c) *Software*. The data were acquired and processed using LC solution (Version 1.03 SP3, Shimadzu Corporation, Kyoto, Japan) software running under Windows XP on a Pentium PC.

Chromatographic conditions

Quantification of trimetazidine was done by plotting trimetazidine to internal standard (caffeine) peak area ratio as a function of trimetazidine concentration.

(a) *Separation conditions*. Chromatographic separation was achieved with a XTerra C_{18} column at room temperature (25^0C). The mobile phase consisted of 0.01 M potassium dihydrogen phosphate buffer (pH 4.16 ± 0.01 adjusted with orthophosphoric acid, in a solvent system of triethanolamine and acetonitrile (90:10) at a flow rate of 1.0 ml/min. The samples were kept at room temperature (25^0C) and a volume of 20 µl was injected for analysis. Ultraviolet detection was achieved with a SPD-10Avp UV-VIS detector at 207 nm at a sensitivity of 0.0001 AUFS. The total run time was 13 min.

(b) *Preparation of sample and Internal standards*. Trimetazidine solution was prepared by dissolving appropriate amount of powdered trimetazidine hydrochloride in diluent (buffer: acetonitrile = 90:10) to have a concentration of 400 µg/ml. The solution was further diluted to have a stock solution of the concentration of 10 µg/ml. Similarly a stock solution of caffeine (internal standard) was prepared in diluent having a concentration of 10 µg/ml. Calibration standards were obtained by adding required amount of trimetazidine stock solution, 100 µl of drug free serum (protein precipitated) and 20 µl of caffeine (internal standard) solution (10 µg/ml) to the diluent to achieve the trimetazidine concentrations of 200, 100, 50, 20, 10 and 5 ng/ml. These samples were analyzed by the HPLC for the construction of calibration curves and method validation. A series of control serum samples were prepared by spiking blank serum with required amount of trimetazidine to yield the final serum concentration of 200, 100, 50, 20, 10 and 5 ng/mL of trimetazidine. These samples were then vortexed for 15 seconds and centrifuged at 12,000 rpm for 10 minutes and were run in HPLC after every 10 analytical run to verify its performance.

(c) *Sample preparation.* To 150 µl of serum sample, 230 µl of methanol and 20 µl of caffeine (1 µg/ml solution) were added and vortexed for 1 minute. Then it was centrifuged for 10 minutes and the supernatant was collected in coded eppendorfs and stored at −80ºC until further analysis. 20 µl of the supernatant was injected into the column for HPLC analysis.

Stability analysis

The stability of analyte in the three samples was evaluated by analyzing three replicates under the following conditions: bench top (6 hr at 25^0 C), short term stability (48 hr at −80ºC), long term (30 days at −80ºC) and three freeze thaw cycles. Samples were considered stable if the assay values were within the acceptable limit of accuracy (±15%) and precision (15%).

Bioanalytical method validation

A variety of mobile phases were investigated for the analysis of trimetazidine and caffeine in serum. The suitability of mobile phase was decided based on selectivity and sensitivity of the assay, stability studies, separation and sharp peak among the methods during stability studies.

(a) *Specificity*. The specificity of the method was ascertained by comparing chromatograms of treated blank serum sample, calibrator sample spiked with trimetazidine and internal standard and processed volunteer sample after oral administration of the drug spiked with internal standard.

(b) *Linearity and range*. The linearity of the assay was performed with six points calibration curve in serum (5 to 200 ng/ml). The slope and the intercept of the calibration graphs were calculated through least square method using internal standard peak-area ratio versus drug concentration. Intercept, slope and *coefficient of correlation (r^2)* were evaluated for 5 calibration curves.

(c) *Limit of quantification (LOQ)*. The LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions [12, 16]. The LOQ was estimated by analyzing samples with known amounts of trimetazidine, at progressively lower concentrations.[12,16]

(d) *Limit of detection (LOD)*. LOD is a parameter that provides the lowest concentration of analyte in a sample that can be detected, but cannot be precisely quantified, under the stated experimental conditions. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. The analyte having the concentration lower than the LOQ

was analyzed with progressively lower concentrations to determine the LOD.[12, 16]

(e) *Precision.* The intra-assay (intra-day) (n=3) and inter-assay (inter-day) (n=3) variability of the method were assessed by analyzing control samples. The precision was expressed as relative standard deviation (% RSD) or coefficient of variation (% CV). The RSD to be determined at each concentration level should not exceed 15% for the method to be precise.[12, 16]

(f) *Accuracy.* The accuracy is a measure of the systematic error or bias and is defined as the agreement between the measured concentration and nominal value. It is calculated as percentage of drug recovered.[12,16] The accuracy was determined by standard addition method at different drug levels of trimetazidine. Amount of trimetazidine in the samples were determined by fitting the corresponding peak area into the calibration curve equation.

(g) *Extraction efficiency (Recovery).* The recovery of trimetazidine was determined at concentrations of 5, 50, 200 ng/ml. The extraction efficiency was determined by comparing peak areas of directly injected standards in the mobile phase and those from drug-free serum spiked with standards (n = 6). The recovery of caffeine was also determined at concentrations of 50 ng/ml using the similar process.

Application in pharmacokinetic study

(a) *Volunteers.* Eight healthy male Bangladeshi volunteers was enrolled in this study with mean age, 20.50 (0.53) years (range 20 - 21 years); mean (SD) body weight, 69.13 (7.61) kg (range 62 - 85 kg); mean (SD) height, 1.71 (0.07) m (range 1.60 - 1.80 m) and mean (SD) body mass index (BMI), 23.50 (1.44) kg/m^2 (range 21.60 - 26.23 kg/m^2). All the volunteers completed the study without any adverse effects.

(b) *Inclusion and exclusion criteria.* All volunteers were examined to verify their health status. The examinations included medical history, vital sign measurements, electrocardiography (ECG), blood sample analysis (basic profile, complete blood cell count, bleeding time, clotting time, prothrombin time, viral serology), and urinalysis (sediment, drugs). Volunteers with relevant clinical, analytical, or ECG abnormalities were excluded from the trial. Additional exclusion criteria were smoking, history of alcohol or other drug abuse, consumption of any medication within one month prior to study commencement, participation in a clinical trial in the 4 months before enrollment, history of clinically important illness or major surgery in the 6 months before enrollment, inability to relate to and/or cooperate with the investigators; medication allergy, illnesses or disorders that could affect the absorption, distribution, metabolism, and/or excretion of drugs, blood loss or donation in the 3 months before enrolment; blood or blood-derivative transfusion in the 6 months before enrolment; and exhausting physical exercise in the 72 hours before enrollment. All eligible volunteers provided written informed consent to participate and were free to withdraw from the study at any time without any obligation.

(c) *Study design.* The study was conducted in the Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka in association with a well-equipped private hospital in Dhaka, Bangladesh in accordance with the International Conference of Harmonization (ICH) guidelines for Good Clinical Practice (GCP) and in compliance with the Declaration of Helsinki and its amendments.[17-18] Ethical approval for this study was taken from the Bangladesh Medical Research Council (BMRC). It was a single-dose, randomized, open-label, one-period study. A single dose of 35 mg of trimetazidine modified release tablet formulation (Vastarel® MR, Les Laboratoires Servier, France) was administered with 250 ml of water after an overnight fasting. A standardized breakfast and lunch were given at 4 and 8 hours after drug administration, respectively. During the study period, the volunteers were under medical surveillance to report any adverse events.

(d) *Blood sampling.* A 20-G x 1.25-inch catheter (Vasofix® Braunüle®, B.Braun Melsungen AG, Melsungen, Germany) was inserted into a suitable forearm vein and 3 mL of blood was withdrawn in each time of collection. Blood samples (3 ml each)

were collected prior to dosing (0 h) and at 0.5, 1.0, 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 9.0 and 12.0 h after dosing. The blood samples were kept for 30 minutes at ambient temperature in a dark place and then centrifuged at 1500 rpm for 15 minutes at room temperature to separate serum and stored at −80ºC until assayed for trimetazidine content. [19]

(e) *Pharmacokinetics and statistical analysis.* Pharmacokinetic properties of trimetazidine were calculated by a non-compartmental approach from serum concentrations of trimetazidine using software Kinetica (Version 4.4.1, Thermo Electron Corporation, UK). C_{max} was estimated directly from observed concentrations, and T_{max} as the corresponding time point at which C_{max} occurred. AUC_{0-t} was calculated by the linear trapezoidal method until the last measurable serum drug concentration, and $AUC_{0-\infty}$ was calculated as $AUC_{0-\infty} = AUC_{0-t} + C_{last}/K_{el}$. k_{el} was the terminal elimination rate constant calculated by linear least square regression of the last three to four time points in the log concentration time profile and the terminal half-life was calculated by the following equation [19]

$$t_{1/2} = 0.693/k_{el}$$

RESULTS AND DISCUSSION

Method development

HPLC method is one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility.[12] The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of trimetazidine from serum samples. Protein precipitation technique was utilized in the extraction of trimetazidine and caffeine from the serum samples. Chromatographic conditions, especially the composition and nature of the mobile phase, were optimized through several trials to achieve best resolution and increase the signal of trimetazidine and caffeine. Using the optimized extraction method and chromatographic conditions, the HPLC method was evaluated in terms of specificity, linearity, limit of detection, limit of quantification, precision, accuracy, and recovery. A good separation and elution were achieved using buffer of potassium dihydrogen phosphate: acetonitrile: triethylamine (90 : 10 : 0.04, v/v) at a flow-rate of 1.0 ml /min.

Stability Study

The stability profile of trimetazidine in serum at −80°C for 1 month, after three freeze–thaw cycles and at room temperature for 6 h as well as at −80°C for 48 h after preparation are presented in Table 1. It was clear that trimetazidine was stable at both high and low concentrations under all tested storage conditions and time.

Table 1. Stability profile of trimetazidine in human serum.

Declared conc. (ng/ml)	Conditions	Conc. in serum (ng/ml)	Recovery in serum (%)
5	After 6 hr at ambient temp.	4.73	94.60
	48 hr storage at –80^0 C	4.81	96.20
	Three freeze–thaw cycles	4.93	98.60
	1 month storage at –80^0 C	4.78	95.60
50	After 6 hr at ambient temp.	46.43	92.86
	48 hr storage at –80^0 C	49.05	98.10
	Three freeze–thaw cycles	49.72	99.44
	1 month storage at –80^0 C	49.34	98.68
200	After 6 hr at ambient temp.	199.06	99.53
	48 hr storage at –80^0 C	196.14	98.07
	Three freeze–thaw cycles	197.60	98.80
	1 month storage at –80^0 C	196.72	98.36

Method validation

(a) *Selectivity and specificity.* The method exhibited good specificity and selectivity. The selectivity of the method assessed by comparing chromatograms of a blank serum (Figure 2A), serum spiked with Trimetazidine and internal standard (Figure 2B), treated volunteer's serum sample spiked with internal standard (Figure 2C). The retention times were 9.87 and 11.5 min for trimetazidine and caffeine respectively (Figure 2C). As shown in the figures, there were no interfering peaks from endogenous substances at the elution time of trimetazidine and caffeine.

(b) *Linearity and range.* All calibration curves were linear over the concentration range of 5 - 200 ng/ml, with an average *coefficient of correlation* (r^2) ≥ 0.996. The mean ± S.D. of the slope and intercept of

the serum standards were 0.01695 ± 0.00092 and 0.035 ± 0.0169, respectively.

(c) *Limit of quantification (LOQ) and Limit of detection (LOD).* The LOQ was found as the lowest concentration on the calibration curve (5 ng/ml) for which an acceptable accuracy of 98.6% and a precision 4.94% were obtained, while the minimum detectable quantity of trimetazidine was found to be 2.0 ng/ml.

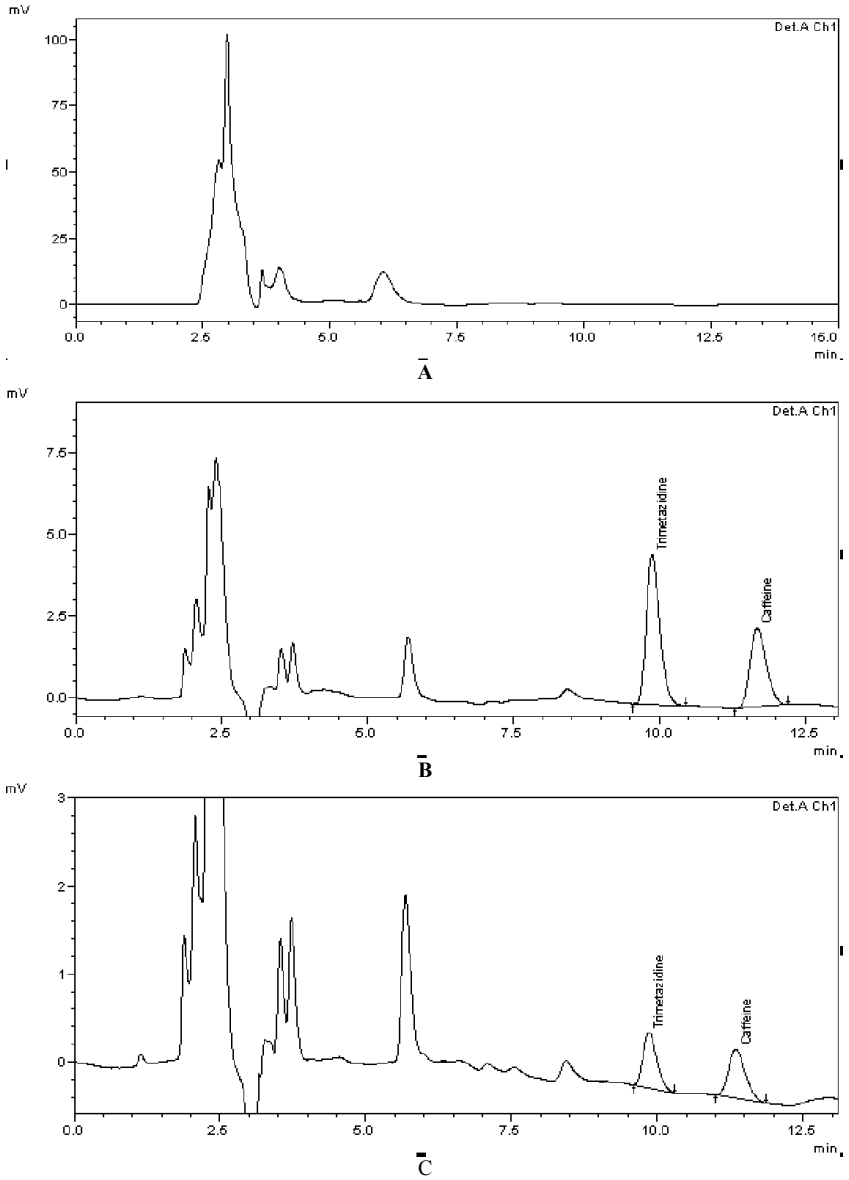


Figure 2. Representative chromatograms, (A) Blank Serum, (B) Serum spiked with Trimetazidine and internal standard (Caffeine) and (C) Serum sample collected from a volunteer after oral administration of trimetazidine (35 mg).

(d) *Precision and accuracy.* The accuracy and precision of the method are presented in Table 2. The intra-day accuracy was found to be in the range of 88.72% - 104.87%, while the inter-day accuracy was in the range of 88.32% - 102.96%. The intra-day precision was in the range of 3.4% - 11.63% and the inter-day precision was in the range of 1.30% - 10.21%. Since all the values of accuracy and % CV are well within the acceptance range, the results indicate that the method is reliable, reproducible and accurate.

Table 2. Precision and accuracy of trimetazidine in human serum.

Drug conc. (ng/ml)	Accuracy and precision			
	Intra-assay (n=3)		Inter-assay (n=3)	
	Accuracy	Precision (% RSD)	Accuracy	Precision (% RSD)
5	88.72%	11.63%	88.32%	10.21%
100	104.87%	4.0%	102.96%	3.60%
200	100.78%	3.4%	99.66%	1.30%

(e) *Extraction efficiency (recovery).* The extraction recoveries of trimetazidine determined at three different concentrations (5, 50 and 200 ng/ml) are presented in Table 3. The method showed good efficiency in terms of recovery as the average recovery for trimetazidine was 97.3% and that of caffeine was 98.1%. Extraction using methanol was simpler and faster than other methods reported previously. [20-21]

Table 3. Trimetazidine recovery from serum samples (n = 6).

Drug conc. (ng/ml)	Conc. found (ng/ml)	Recovery (%)	Average recovery (%)
5	4.94	98.75	
50	46.73	93.46	97.44
200	200.19	100.10	

Pharmacokinetic properties of trimetazidine

The mean serum concentration–time profile of trimetazidine has been shown in Figure 3 and relevant pharmacokinetic parameters have been listed in Table 4. The mean C_{max} was found to be 87.77 ± 43.17 ng/ml occurring at T_{max} of 3±1.49 hr.

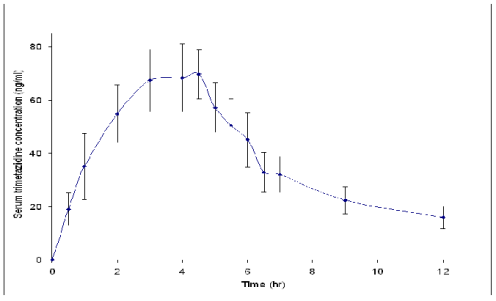


Figure 3. Mean (SEM) trimetazidine concentration-versus-time curve over 12 hours in adult healthy Bangladeshi volunteers (N = 8).

The half life, AUC_{0-12} and $AUC_{0-\infty}$ values were 3.42 ± 1.13 hr, 457.8±294.4 hr-ng/ml and 489.0 ±386.1 hr-ng/ml, respectively. These values are in accor-dance with the previously reported article.[9,11,14, 22]

Table 4. Pharmacokinetic parameters after administration of 35 mg single oral dose of trimetazidine in healthy Bangladeshi male volunteers (N=8).

Parameters	Mean ± SD
C_{max} (ng/ml)	87.77 ± 43.17
T_{max} (hr)	3.0 ± 1.49
AUC_{0-12} (hr-ng/ml)	457.8 ± 294.4
$AUC_{0-\infty}$ (hr-ng/ml)	489 ± 386.1
K_{el} (hr^{-1})	0.20 ± 0.06
$t_{1/2}$ (hr)	3.42 ± 1.13

Conclusion

The present HPLC method was simple, rapid, accurate, precise, reproducible, and selective enough to allow the analysis of trimetazidine in human serum. The extraction process used was very easy and rapid. The method was successfully applied for the determination of pharmacokinetic parameters of trimetazidine in healthy Bangladeshi male volunteers.

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