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The Pharmacokinetic Properties and Bioequivalence of Methyldopa Formulations: Results of an Open-label, Randomized, Two-period, Crossover, Single-dose Study

Alexander Leonidovich Khokhlov[1,2], Leonid Nikolaevich Shitov[1,2], Miroslav Ryska[3], Yuriy Aleksandrovich Dzhurko[2], Vladimír Kubeš[3], Vitaliy Nikolaevich Shabrov[1,2*], Aleksey Evgenyevich Miroshnikov[1], Elena Valeryevna Korneva[4], Anna Vitalyevna Demchinskaya[4], Anastasiya Mikhailovna Shitova[2,5], Alexander Alexandrovich Khokhlov[1], Igor Evgenyevich Shokhin[6] and Elena Georgievna Lileeva[1]

[1]*State Budget-funded Educational Establishment of Higher Professional Education "Yaroslavl State Medical University" of the Ministry of Health of the Russian Federation, Yaroslavl, Russia*
[2]*Quinta-Analytica Yaroslavl LLC, Yaroslavl, Russia*
[3]*Quinta-Analytica s.r.o., Prague, Czech Republic*
[4]*R-Pharm JSC, Moscow, Russia*
[5]*State Educational Establishment of Higher Professional Education "Yaroslavl State University named after P. G. Demidov" of the Ministry of Education and Science of the Russian Federation, Yaroslavl, Russia*
[6]*Federal State Budget-funded Establishment of Science "Research Centre of Biomedical Technologies" of the Federal Medico-biological Agency of the Russian Federation, Svetlye Gory Village, Moscow Region, Russia*

Abstract

Comparative assessment of the pharmacokinetic properties and bioequivalence (BE) of two methyldopa formulations (Methyldopa, 250 mg tablets, R-Pharm CJSC, Russia-investigational medicinal product, and Dopegyt®, 250 mg tablets, EGIS Pharmaceuticals PLC, Hungary-reference product) were investigated in 24 healthy volunteers (13 women and 11 men, caucasian) in an open-label, randomized, crossover, two-period, two-sequence trial with 7-day washout period. A comparative dissolution test was carried out in advance in 3 media, including quantitative determination of methyldopa by UV spectrophotometry. The release patterns of the active ingredient from the test and reference products were equivalent. Methyldopa concentrations in plasma were measured by validated method of high-performance liquid chromatography-tandem mass spectrometry, using the deuterated internal standard. The validation method yields data meeting all acceptance criteria for the plasma methyldopa concentration range of 0.020-3.000 μg/mL. Stabilization of plasma samples was developed that involved addition of ascorbic acid to the plasma during the sampling procedure at the study site. The BE assessment involved calculation of 90% confidence intervals for AUC, C_{max}, and C_{max}/AUC using analysis of variance (ANOVA) of log-transformed data within an range of 80.00-125.00%. No statistically significant differences were observed between the two drugs. The point estimates and 90% confidence interval limits were as follows: AUC_{0-t}-92.93% (80.69-107.03%), C_{max}-94.89% (80.88-111.34%), C_{max}/AUC_{0-t}-102.11% (93.95-110.98%), corresponding to the acceptable ranges (80.00-125.00%). The test and reference drug products are characterized by a high degree of pharmacokinetic similarity and thus are bioequivalent.

Keywords: Pharmacokinetic; UV spectrophotometry; Bioequivalence; LC/MS/MS

Introduction

Methyldopa (3-hydroxy-alpha-methyl-L-tyrosine), being a centrally acting antihypertensive agent, exerts an antihypertensive effect through its active metabolite, α-methylnoradrenaline, produced after the active ingredient crosses the blood-brain barrier; α-methylnoradrenaline stimulates central α2-adrenergic receptors, causing inhibition of sympathetic impulses and blood pressure reduction. Methyldopa is a drug of choice for the treatment of arterial hypertension in pregnancy; use of methyldopa in pregnant women with arterial hypertension is one of the effective and safe treatment options which allow prevention of cardiovascular complications and thus improvement of the short-term and long-term outcomes for the mother and the foetus. In view of the above, development of a generic methyldopa formulation is necessary to ensure improved quality and availability of antihypertensive therapy in pregnancy [1-9].

Methyldopa is poorly absorbed from the gastrointestinal tract; the average bioavailability following oral administration is 25%, and can vary over a broad range (8% to 62%) [7]; the Biopharmaceutics Classification System (BCS) lists methyldopa in Class III, because this substance is characterized by a high solubility and poor crossing potential (only small quantities cross the enterocyte membrane) [9]. The aforementioned specifics of the biopharmaceutical properties of methyldopa necessitate particularly stringent methodology for a bioequivalence study of drugs containing the substance in question. At the same time, there are only a few available publications that describe bioanalytical methods used to measure methyldopa concentrations within comparative pharmacokinetic and bioequivalence studies [1,8].

Subjects and Methods

The study was conducted in accordance with the basic ethical principles laid down in the World Medical Association (WMA) Declaration of Helsinki and reflected in the National Standard of the

***Corresponding author:** Vitaliy Nikolaevich Shabrov, State Budget-funded Educational Establishment of Higher Professional Education "Yaroslavl State Medical University" of the Ministry of Health of the Russian Federation, Yaroslavl, Russia, Tel: 375-296053960; E-mail: v.shabrov@qayar.ru

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Russian Federation GOST R 52379-2005 "Good Clinical Practice requirements", GCP, as well as in accordance with the basic principles of pharmacokinetic and bioequivalence studies [2,3,10,11].

Comparative dissolution kinetics test

A comparative dissolution kinetics test had been conducted for the test and reference drug products beforehand with the aim to demonstrate comparability of the active ingredient release patterns between the test drug and reference drug batches that should serve to select appropriate batches to be studied *in vivo*.

The study was carried out using the Agilent Technologies 708-DS Dissolution Apparatus (USA) and the UV spectrophotometer ПЭ-5400УФ, PROMECOLAB (Russia). Dissolution media: 0.2% sodium chloride solution in 0.1 M hydrochloric acid solution pH 1.2, acetate buffer solution pH 4.5, phosphate buffer solution pH 6.8. The volume of the dissolution medium is 900 mL. Sampling time points: 10 min, 30 min, 50 min, 90 min, 130 min. The test was carried out with the paddle rotation speed set at 50 revolutions per minute, at 37.0 ± 0.5°C.

In dissolution experiments were studied the same batches of test and reference formulation that have been taken to clinical part of the study.

Inclusion and exclusion criteria

The bioequivalence study was conducted at one study site, the State Autonomous Establishment of Health Care of Yaroslavskaya Region "Clinical Hospital No. 2". The study included 24 male and female healthy volunteers residing in the Russian Federation. Inclusion criteria: Age 18-45 years, verified diagnosis "healthy", body mass index in the range of 18.5 kg/m^2 to 30.0 kg/m^2, body weight above 45 kg; ability to follow the requirements of the study protocol, including use of adequate contraception. Exclusion criteria: a history of allergy; hypersensitivity to methyldopa or to any other substance included in the formulation of the medicinal product; depression and/or use of any antidepressants within the preceding 6 months; use of monoamine oxidase inhibitors; cardiovascular, bronchopulmonary, neuroendocrine, immune, gastrointestinal, liver, kidney, or blood diseases; a history of acute infectious disease within the 4 weeks preceding the study; intake of any medicinal products within 2 weeks of the start of the study; use of substances exerting a significant effect on hepatic microsomal enzymatic activity, haemodynamics, and gastrointestinal tract function within the preceding 2 months; abnormalities revealed by clinical instrumental and laboratory investigations at screening; loss of more than 450 mL of blood within 2 months of the start of the study; excessive alcohol consumption; abuse of caffeine-containing products; consumption of grapefruit-containing foods and drinks; special diets and lifestyles; smoking more than 10 cigarettes a day; positive tests for pregnancy, alcohol, drugs or drug substances.

Study design

This was an open-label, randomized, crossover, two-period, comparative study of pharmacokinetics and bioequivalence. In accordance with the randomization plan, the volunteers were divided into two groups based on the drug administration sequence (TR or RT: T-test drug product, Methyldopa, 250 mg tablets, R-Pharm CJSC, Russia; R-reference drug product, Dopegyt®, 250 mg tablets, EGIS Pharmaceuticals PLC, Hungary-reference product). Sample size was determined on the basis of data from previous study [12]. The calculation was performed considering the following values: 1-β=0.8, α=0.05, %CV=15.4, geometric mean ratio 90% to 110% and an equivalence range of 80% to 125%, yielded with a sample size of 24 subjects.

Study volunteers were hospitalized for 36 hours on the evening preceding the drug administration day. Volunteers took the drugs in the fasting state, with 200 mL of water. At 1 hour before the drug administration and within 1 hour post dose, study volunteers did not take any liquids, including water. Standard breakfast was served to subjects at 4 hours, lunch at 6 hours and dinner at 10 hours after the drug administration. No medicinal products except the study drugs could be taken in the course of the study. Use of grapefruit or related citrus fruit; alcohol; caffeine and/or xanthine-containing foods and beverages; fatty and roasted foods were prohibited during the study.

Tolerability assessment

The following procedures were carried out throughout the study with the aim to evaluate the tolerability and safety of the study drugs: Assessment of the general clinical condition; physical examination; blood pressure, heart rate, and body temperature measurements; complete blood count and blood chemistry tests; urinalysis; 12-lead ECG; registration of adverse events and assessment of their frequency.

Sampling procedures

A 6 mL blood sample was collected from each study volunteer into a pre-labeled vacuum centrifuge tubes containing EDTA as an anticoagulant. A permanent catheter was placed in a forearm vein of each study volunteer for 24 hours after the drug administration. After each blood sampling procedure, the catheter was flushed with 0.5 mL of heparinized normal saline (500 IU in 100 mL of 0.9% sodium chloride solution) to avoid clotting. Beginning from the second blood sampling procedure, 1 mL of the blood was evacuated from the catheter in advance with the aim to avoid contamination with heparin of the blood sample drawn into the vacutainer. The catheter was removed after the blood sampling procedure, at 24 hours post dose. Blood samples were collected at the following time points: 0 (prior to drug administration), at 15 min, 30 min, 45 min, 1 h, 1 h 15 min, 1 h 30 min, 1 h 45 min, 2 h, 2 h 15 min, 2 h 30 min, 3 h, 4 h, 6 h, 9 h, 12 h, 18 h, and 24 h after drug administration. After the blood sampling procedure, the test tubes were immediately placed in an ice bath and sent for centrifugation; an interval not exceeding 15 minutes was allowed between the time of blood sampling and the start of centrifugation. Centrifugation was done for 10 minutes at 4°C, at a rate of 3000 revolutions per minute. A 1.0 mL plasma volume was transferred into a cryotube prepared beforehand and containing a stabilizer, 0.2 mL of 50 mg/mL ascorbic acid solution (information on the need to stabilize methyldopa can be found in the "Validation results for the analytical method" section). The plasma was mixed with the stabilizer and immediately frozen on dry ice; afterwards, the frozen plasma samples were stored at a temperature not exceeding -20°C until the analysis.

Development and validation of the bioanalytical method

Methyldopa concentrations were measured by HPLC-MS/MS, using a system containing an LC-20 liquid chromatograph (Shimadzu Corporation, Japan) and an LCMS-8050 triple quadrupole liquid chromatograph-mass spectrometer (Shimadzu Corporation, Japan). The sample preparation was done by precipitating the protein with an internal standard solution (methyldopa-D_3) in acetonitrile with 1% formic acid added (400 µL of the internal standard solution was added to 100 µL of the plasma), with subsequent centrifugation at a speed of 3500 revolutions per minute. A 8 µL portion of the supernatant was injected into the chromatographic system. Chromatographic conditions: pre-

column Luna 5u Phenyl-Hexyl, 50 × 3.0 mm, Phenomenex, column Synergi 4u Fusion–RP 80A, 150 × 3.0 mm, Phenomenex, mobile phase with constant composition (methanol, ultrapure water, 80 mM ammonium formiate). Detection was performed in positive ion mode, using the DUIS-ESI dual ion source that combines electrospray ionization with atmospheric pressure chemical ionization. Deuterated methyldopa (methyldopa-D_3) was used as the internal standard. An MRM transition of 211.95→138.90 m/z was selected for the analyte, and 214.95→169.00 m/z for the internal standard (Figure 1).

Validation was carried out in accordance with the EMEA guidelines «Guideline on bioanalytical method validation» (2011) on the following parameters: selectivity, linearity, lower Limit of Quantification, trueness and precision, matrix effect, recovery, carryover effect, dissolution acceptance test, and stability.

Pharmacokinetic assessment and statistical analyses

The pharmacokinetic values were obtained, and statistical analyses performed, with the Rv application packages 3.2.1, Module Bear (Lee, Hsin-ya and Lee, Yung-jin (2014). bear: Data Analysis Tool for Average Bioequivalence and Bioavailability. Rpackage version 2.6.4) and StatSoft STATISTICA v.12. The following pharmacokinetic parameters were calculated: C_{max}-maximum measured plasma concentration of the drug obtained in study volunteers; T_{max}-time to maximum measured plasma concentration of the drug obtained in study volunteers; AUC_{0-t}-area under the pharmacokinetic "concentration - time" curve from zero to the last blood sampling procedure yielding a drug concentration equal to or greater than the lower Limit of Quantification; $AUC_{0-\infty}$-area under the pharmacokinetic curve from time zero to infinity; $AUC_{0-t}/AUC_{0-\infty}$-ratio of the AUC_{0-t} to the $AUC_{0-\infty}$; C_{max}/AUC-relative absorption rate; λ_z-terminal elimination rate constant; $T_{½}$-elimination half-life of the drug; MRT-mean retention time of the drug in the blood (mean resident time). The pharmacokinetic parameters were calculated, and statistical analyses of obtained data carried out, presuming a log-normal distribution of the AUC, C_{max}, and C_{max}/AUC parameters and a normal distribution pattern of the other parameters except T_{max}. When a log-normal distribution pattern is expected, any mean values obtained for the test drug and the reference drug product are compared using a multiplicative model while confidence intervals are plotted for the ratios of the respective mean values. After logarithmic transformation, these values are analyzed by means of analysis of variance (ANOVA; parametric method). Analysis of variance was used to test the hypothesis of statistical significance of the contributions of various factors (differences between the drugs, difference between study subjects, drug administration sequence, study phases) to the variability observed. The residual variability estimate obtained by analysis of variance is used to calculate the confidence interval for the ratios of the mean values of the respective parameter. The statistical comparison procedure involves calculation of parametric two-sided 90% confidence intervals for the ratios of the respective mean values obtained for the test drug and the reference drug. Drugs are considered bioequivalent if the limits of the estimated confidence interval for AUC_{0-t}, C_{max}, and C_{max}/AUC_{0-t} lie within the range of 80.00% to 125.00%.

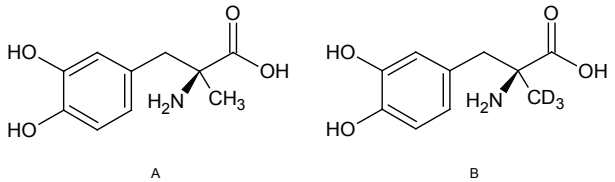

Figure 1: Structural formulas of methyldopa (A) and methyldopa-D_3 (B).

Results

Comparative dissolution kinetics test

The study demonstrated comparable dissolution kinetics of the study drugs in the medium consisting of 0.2% sodium chloride solution in 0.1 M hydrochloric acid solution pH 1.2 and acetate buffer solution pH 4.5, as shown by the calculated convergence factor values, 66.01 and 58.36, respectively. In the phosphate buffer solution pH 6.8 medium, results were disregarded as a result of the partial methyldopa degradation (observed as a changing color of the solution).

Validation results obtained for the analytical method

The characteristics presented below were obtained as a result of the development and validation of the analytical method. The lower Limit of Quantification for methyldopa in plasma was found to be 0.020 μg/mL. The ratio of the analyte response to the internal standard response was in a linear relationship with the concentration of the analyte over the target concentration range: 0.020-3.000 μg/mL of plasma. A preliminary plasma stability test conducted for the analyte demonstrated that methyldopa had undergone considerable degradation. In view of the above, the addition of ascorbic acid, a stabilizer with antioxidant properties, to the plasma was used to prevent a change in the methyldopa concentration after blood sampling. The addition of 0.2 mL of 50 mg/mL ascorbic acid solution to 1 mL of the plasma permitted complete stabilization of the methyldopa level at the time of storage and during the conduct of analytical procedures. All validation tests were carried out on plasma samples containing the stabilizer. The proposed method is highly selective: The chromatograms obtained with blank plasma (6 samples from independent sources, including haemolyzed and hyperlipidaemic plasma) featured no peaks with the retention time characteristic of the analyte and the internal standard. Quality control (QC) samples with concentrations of 0.020 μg/mL (LLOQ); 0.060 μg/mL (L); 0.300 μg/mL; 1.200 μg/mL (M); 2.400 μg/mL (H); and 3.000 (ULOQ) μg/mL were used in the trueness and precision tests. All obtained results met the acceptance criteria (precision at the CV level ≤ 20.00% and trueness 80.00% to 120.00% for LLOQ; precision at the CV level ≤ 15.00 % and trueness 85.00% to 115.00% for other concentrations). The analyte recovery rate was found to be 63.49% and 62.40% at low and high concentration levels, respectively. The use of the deuterated internal standard led to counteraction of the effects of the biological matrix components on the analyte ionization efficacy: the normalized matrix factor (NMF) values were 1.010 and 1.018 at low and high concentration levels, respectively; the coefficients of variation for NMF were 0.40% and 1.24%, respectively, thus meeting the acceptance criterion (CV ≤ 15.00 %). The analyte was demonstrated to be stable (average concentration within the range of 85.00 % to 115.00 % of the nominal value) in solutions (for at least 17 days in the temperature range of 2-8°C), in plasma samples stabilized with ascorbic acid as described above (at least 24 hours at room temperature, at least 29 days when frozen at temperatures not exceeding minus 20°C, as well as after three freeze-thaw cycles at an interval of at least 12 hours), in whole blood (20 minutes on ice bath, with subsequent 40-minute storage at room temperature), in prepared samples located in the sampler (at least 48 hours).

Characteristics of the study population

The pharmacokinetic analysis population included 24 healthy volunteers; the mean age of the volunteers was 25.8 ± 7.1 years, height

171.5 ± 9.4 cm, weight 68.1 ± 10.5 kg, and BMI 23.3 ± 2.8 kg/m^2. This group consisted of 13 women and 11 men; all volunteers were Caucasian. All volunteers completed the study according to protocol. In the TR treatment group, the mean age of subjects was 25.8 ± 7.2 years, height 170.1 ±5.9 cm, weight 67.1 ± 6.8 kg, and mean BMI 23.6 ± 2.7 kg/m^2. There were 6 women and 6 men in this group. In the RT treatment group, the mean age of participants was 24.9 ± 7.3 years, height 170.6 ± 11.9 cm, weight 64.3 ± 7.5 kg, and mean BMI 22.1 ± 1.9 kg/m^2. There were 7 women and 6 men in this group.

Tolerability assessment

The study drugs were well tolerated. The majorities of detected adverse events was caused by the pharmacodynamic effects of the study drugs, and are described in the Prescribing Information. Adverse events were registered strictly as required by the international guidelines [4,5]. Comparable numbers of adverse events were detected after the administration of the study drug and after the use of the reference product; their spectra were comparable.

Twenty-three adverse events were registered in the course of the study; 22 of them were due to abnormal values of the vital parameters: Blood pressure and heart rate. Twelve adverse events developed after the administration of Methyldopa, and 11 adverse events after the administration of Dopegyt®. A certain relationship with the administration of the drug was established for 63.6% of subjects administered Methyldopa and 75.0% of healthy volunteers given Dopegyt®.

Analysis of plasma samples obtained in the clinical part of the study

The analytical part of the study was conducted within 15 days after the completion of the clinical part; the validation results demonstrated that methyldopa remained stable over the specified time interval. The analysis of plasma samples obtained from study volunteers in the course of the clinical part was done on 24 analytical series that included a blank sample, 9 calibration samples, 6 quality control samples (low, medium, and high concentrations, two replicates of each), and samples obtained from one volunteer in each of the phases. The results obtained for all analytical series met the acceptance criteria. Incurred Sample Reanalysis (ISR test) of previously analyzed samples was also carried out to evaluate the reproducibility and stability of the analytical method used for real samples. A total of 96 samples were selected for the reanalysis, which amounted to 11.1% of the total number of samples. The obtained results demonstrated a 100% reproducibility of the analytical method.

Pharmacokinetic assessment and statistical analyses

The results presented in the tables indicate a high similarity of the pharmacokinetic results obtained for the test drug product and reference drug product (Tables 1 and 2). The average pharmacokinetic curve profiles presented in Figure 2 demonstrate similar post-dose concentrations for the test drug product and reference drug product.

The analysis of variance results demonstrated that the only factor with a considerable contribution to the AUC_{0-t} and C_{max} variability observed were the subjects (the respective p-values for this comparison were 0.002 and 0.027). The contributions of the other factors (drug, phase, sequence) did not reach statistical significance (p>0.05).

Table 3 presents the point estimates of individual ratios and 90 % confidence interval limits obtained for the principal pharmacokinetic parameters characterizing the bioavailability of methyldopa. The confidence interval limits obtained for all pharmacokinetic parameters meet the normative requirements, thus indicating the bioequivalence of the compared products.

Discussion

As mentioned above, methyldopa is a medicinal product necessitating particular requirements for pharmacokinetic study methods. This fact is due to the specific physico-chemical and biopharmaceutical properties of methyldopa. The presence of two phenolic hydroxyl groups in the methyldopa molecule renders it susceptible to oxidative processes. The low bioavailability resulting from the low permeability of the enterocyte membrane for methyldopa molecules is one of the factors that complicate creation of generic products pharmacokinetically equivalent to the original product. The reported study demonstrates the high value of the organizational aspects of bioequivalence studies, in particular the inter-relationship of the clinical and bioanalytical stages: the methyldopa stabilization technique used for plasma samples in this trial had been developed and validated at the preliminary stage and was subsequently used successfully during the sampling in the clinical part of the study.

There are rather few publications on the pharmacokinetic properties of methyldopa. The pharmacokinetic values obtained for methyldopa in the reported study are similar to the respective results obtained for Dopegyt® by Róna et al. In the study published by Valizadeh et al., administration of an equivalent methyldopa dose produced results different from those yielded by this study and the data reported by Róna et al.-there were lower C_{max} and AUC and higher $T_{½}$ values. The mentioned differences may be due to differences in the characteristics of the dosage forms administered to volunteers in the discussed studies, specific features of the study populations, and the employed bioanalytical methodology.

Other factors that should be mentioned include differences in the intra-subject variability of the methyldopa pharmacokinetics obtained in the presented study and in the publications by other authors. The present study demonstrates a rather high level of intra-subject variability for the principal pharmacokinetic parameters of methyldopa: The coefficients of variation were found to be 33.01%, 29.08%, and 16.92% for AUC_{0-t}, C_{max}, and C_{max}/AUC_{0-t}, respectively; the coefficient of variation for C_{max} exceeded 30%, being at a level of a highly variable medicinal product. Furthermore, the study by Valizadeh et al. produced data corresponding to a lower level of intra-subject variability

Parameters	T	R
C_{max}, µg/mL	1.227 ± 0.601	1.233 ± 0.419
AUC_{0-t}, µg·h/mL	6.219 ± 3.080	6.385 ± 2.153
$AUC_{0-\infty}$, µg·h/mL	6.436 ± 3.181	6.529 ± 2.187
C_{max}/AUC_{0-t}, h^{-1}	0.2019 ± 0.0377	0.1978 ± 0.0372
λ_z, h^{-1}	0.22943 ± 0.10984	0.20651 ± 0.10709
$T_{½}$, h	3.89 ± 2.13	4.45 ± 2.37
MRT, h	4.97 ± 0.77	4.98 ± 0.76

Table 1: Comparative pharmacokinetic results obtained for the test drug product and reference drug product (M ± SD).

Parameter	Median		p-value*
	T	R	
T_{max}	3.00	3.00	0.467

*Wilcoxon signed-rank test

Table 2: Non-parametric test results for T_{max}.

(15.37% and 12.54% for C_{max} and AUC_{0-t}, respectively), whereas the study reported by Róna et al. yielded a significantly higher intra-subject variability (40.06% and 34.85% for C_{max} and AUC_{0-t}, respectively). It also should be mentioned that the intra-subject variability observed in the reported study apparently was not associated with the drugs or the bioanalytical methodology utilized; this is evidenced by the ANOVA results demonstrating that the only factor with a significant contribution to the observed AUC_{0-t} and C_{max} variability were the study subjects; the contributions of the factors Drug, Phase, and Sequence were statistically insignificant ($p>0.05$); the accuracy, reproducibility, and stability of results obtained with the analytical method had been demonstrated at the validation stage and by the ISR test.

Conclusions

The reported study permits a conclusion that the test drug, Methyldopa 250 mg tablets (R-Pharm CJSC, Russia), and the reference drug, Dopegyt® 250 mg tablets (EGIS Pharmaceuticals PLC, Hungary), have highly similar pharmacokinetic properties and are thus bioequivalent. The presented results demonstrate a successful solution of the methodological problems associated with the specific properties

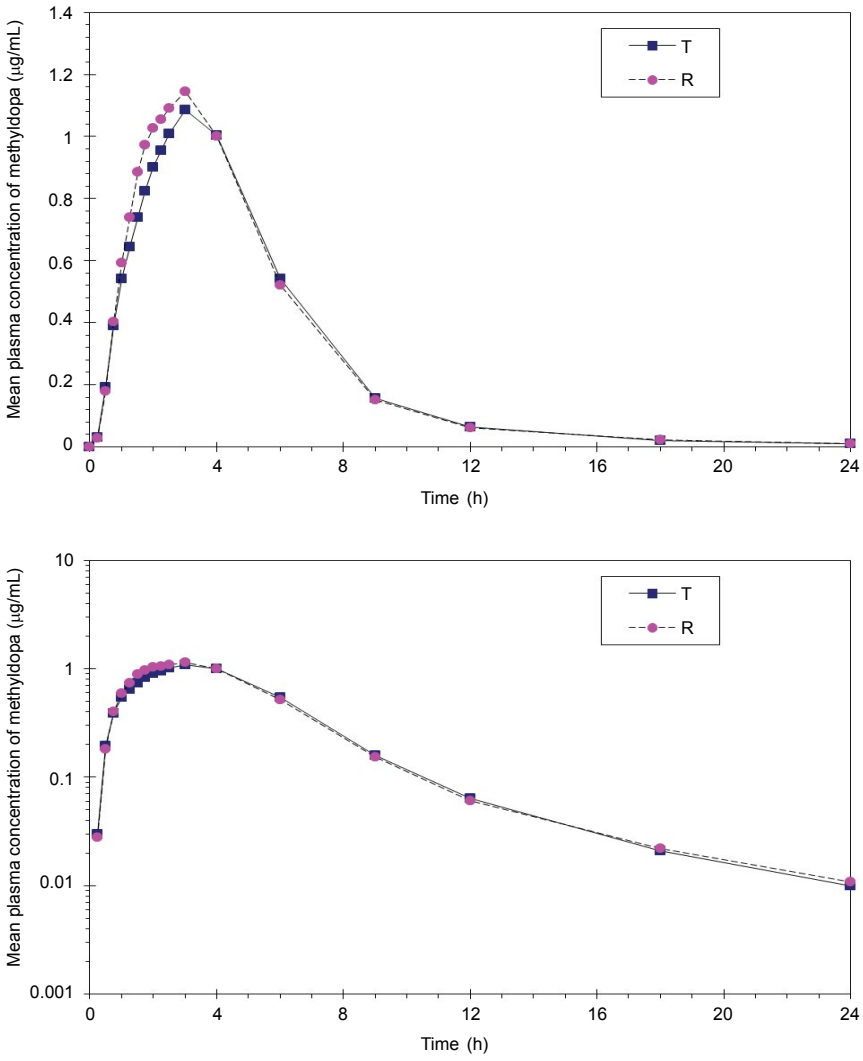

Figure 2: Averaged pharmacokinetic profiles of plasma methyldopa concentrations measured in study volunteers (M ± SD) after a single dose of the test (T) drug, Methyldopa 250 mg tablets (R-Pharm CJSC, Russia), and the reference (R) drug, Dopegyt® 250 mg tablets (EGIS Pharmaceuticals PLC, Hungary), in linear and semi-logarithmic coordinates.

Parameter	Ratio of mean values	90% confidence intervals		Bioequivalence	Intra-individual variability coefficient
		Lower limit	Upper limit		
AUC_{0-t}	92.93%	80.69%	107.03%	Yes	29.08%
C_{max}	94.89%	80.88%	111.34%	Yes	33.10%
C_{max}/AUC_{0-t}	102.11%	93.95%	110.98%	Yes	16.92%

Table 3: 90% confidence intervals of the ratios of the mean values (%) of the pharmacokinetic parameters characterizing the bioavailability of methyldopa.

of methyldopa; the proposed approaches may also be used successfully to study other medicinal products with similar physico-chemical and biopharmaceutical characteristics.

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