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Quantification of methyldopa in human plasma by high-performance liquid chromatography–electrospray tandem mass spectrometry Application to a bioequivalence study

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Abstract

A method based on LC–MS–MS is described for the determination of methyldopa in human plasma using dopa-phenyl-D3 as the internal standard. The method has a chromatographic run time of 5.5 min and was linear in the range of 20–5000 ng/ml. The limit of quantitation was 20 ng/ml, the intra-day precisions were 7.3, 5.4 and 4.3% and the intra-day accuracies were −8.0, −1.3 and −2.0% for 30, 600 and 3000 ng/ml, respectively. The inter-day precisions were 7.7, 0.5 and 0.7% and the inter-day accuracies were 0.2, −1.1 and −2.3%, respectively, for the above concentrations. This method was employed in a bioequivalence study of two tablet formulations of methyldopa.

Keywords: Bioequivalence studies; Methyldopa

1. Introduction

Methyldopa, 3-hydroxy-α-methyl-L-tyrosine, is an antihypertensive agent that primarily acts within the central nervous system as an α-adrenergic agonist [1]. Methyldopa is taken up by adrenergic neurons, where it is decarboxylated and hydroxylated to form the false transmitter, α-methylnoradrenaline, which is less active than noradrenaline on α_1-receptors and thus less effective in causing vasoconstriction.

In this paper, we describe a fast, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the quantitation of methyldopa using dopa-phenyl-D3 as internal standard (I.S.). The method was applied to a bioequivalence study of two oral formulations of methyldopa (500 mg tablet; Legrand Metildopa from EMS Indústria Farmacêutica, Brazil, as test formulation and Aldomet from Prodome Química e Farmacêutica, Brazil as reference formulation).

2. Experimental

2.1. Chemicals and reagents

Methyldopa was provided by EMS, Brazil, lot No. 00110806. Dopa-phenyl-D3 was purchased from

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CDN Isotopes, Canada, lot No. V166P7. Acetonitrile, methanol, and dichloromethane (HPLC grade), perchloric acid and formic acid (analytical grade) were purchased from Mallinckrodt (USA). Fuming hydrochloric acid was purchased from Merck, Brazil. Ultra pure water was obtained from an Elga UHQ system (Elga, UK). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −20 °C until needed.

2.2. Calibration standards and quality control

Stock solutions of methyldopa and I.S. were prepared in methanol–water (50:50, v/v) containing 1 m*M* hydrochloric acid at concentration of 1 mg/ml. The solutions were protected from light by wrapping the container with aluminum foil. Calibration standards of methyldopa were prepared in blank plasma at concentrations of 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml and were assayed in duplicate in each batch. Calibration standards were prepared in bulk and dispensed in 1.5-ml aliquots into properly labeled Eppendorf tubes and stored at −20 °C until required for assay. The quality control samples were prepared in blank plasma at concentrations of 30, 600 and 3000 ng/ml (QCA, QCB, and QCC, respectively).

2.3. Sample preparation

Prior to assay, frozen human plasma samples were thawed at ambient temperature and centrifuged at 2000 *g* for 5 min at 4 °C to precipitate solids. In the following order, 200 μl of ultra pure water, 50 μl of the internal standard solution (10 μg/ml dopa-phenyl-D3 in methanol–water, 50:50, v/v, solution), and 50 μl of 10% (v/v) perchloric acid were added to each disposable glass tube (non-siliconised; 15 ml) containing a 200-μl aliquot of plasma sample. The tubes were briefly vortex-mixed and then lipophilic impurities were extracted with 4 ml of dichloromethane. The mixture was vortex-mixed for approximately 40 s. The tubes were centrifuged at 2000 *g* for 10 min and 200 μl of the aqueous (upper) phase was then transferred to the HPLC auto-injector microvials.

2.4. Chromatographic conditions

An aliquot (40 μl) of each plasma extract was injected into a Genesis C_{18} 4 μm analytical column (100 mm×2.1 mm I.D.) preceded by a pre-column of the same material (10 mm×2.1 mm I.D.) at a flow-rate of 0.15 ml/min of the mobile phase [CH_3CN–water (10:90)+10 m*M* formic acid]. The column operated at room temperature (22–27 °C). Under these conditions, typical standard retention times were 3.3 min for both methyldopa and I.S., and back-pressure values of approximately 30–40 bar were observed.

A split of the column eluent of approximately 1:10 was included so that only 15 μl/min entered the mass spectrometer. The temperature of the auto-sampler was kept at 5 °C and the total run time was 5.5 min.

2.5. Mass spectrometric conditions

The mass spectrometer (Micromass Model Quattro Ultima) equipped with an electrospray source using a crossflow counter electrode was run in positive mode (ES+) with multiple reaction monitoring (MRM). Full-scan positive-ion mass spectra of methyldopa and dopa-phenyl-D3 showed the protonated molecular species $[M+H]^+$ at *m*/*z* 212.1 and 201.2, respectively. The most abundant ion in the product ion spectra was at *m*/*z* 166.1 for methyldopa and 154.2 for I.S. From these results, the mass spectrometer was set as follows: *m*/*z* 212.1 for methyldopa and *m*/*z* 201.2 for I.S. as the precursor ions and *m*/*z* 166.1 and 154.2 as the respective product ions (Fig. 1A–D). The proposed fragmentation route for methyldopa and I.S. is shown in Scheme 1.

For both methyldopa and I.S. the dwell time and the capillary were 0.8 s and 3.8 kV. The optimum values for both cone voltage and the collision energy were 25 V and 15 eV for methyldopa and 15 V and 16 eV for I.S., respectively. Data acquisition and analysis were performed using the software MassLynx (v 3.5) running under Windows NT (v 4.0) on a Pentium personal computer.

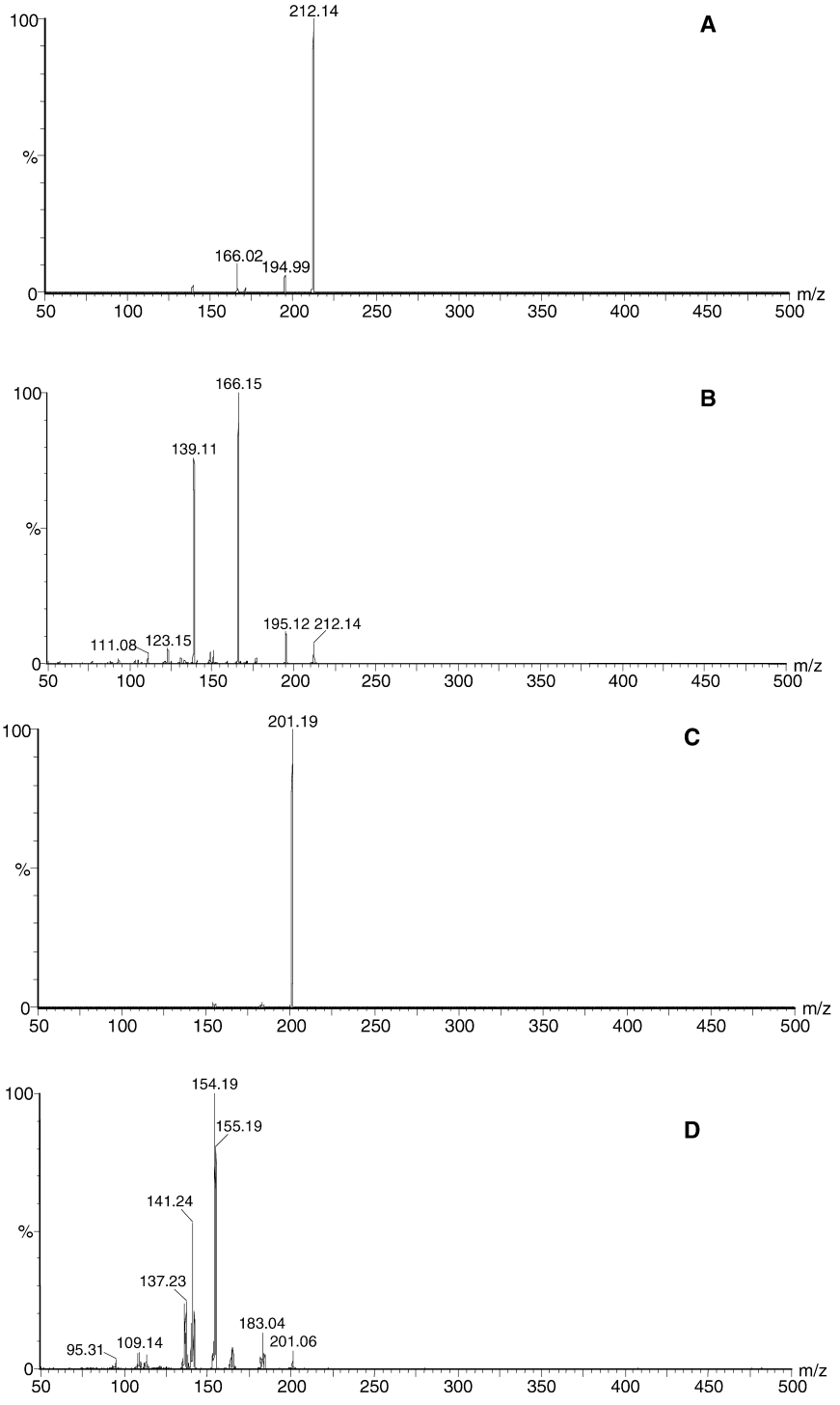
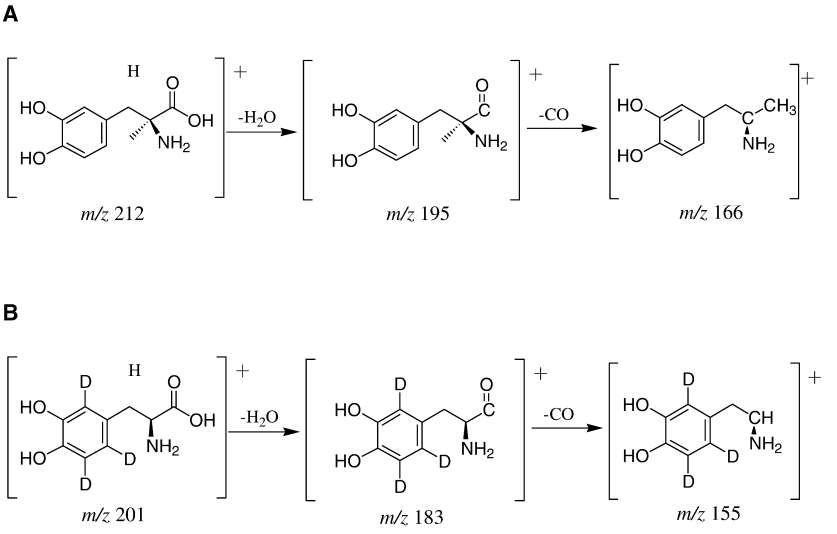


Fig. 1. Full scan mass spectra of (A) methyldopa and (C) I.S. Product ion spectra of (B) methyldopa and (D) I.S.



Scheme 1. Fragmentation route proposed for (A) methyldopa and (B) I.S.

2.6. Stability

Quality control plasma samples (500 and 3000 ng/ml; $n=5$ for each concentration) were subjected to short term (6 h) room temperature, three freeze–thaw (−20 to 25 °C) cycles and 24-h autosampler (5 °C) stability stability tests. Subsequently the methyldopa concentrations were measured comparing with fresh prepared samples and the significance of the obtained results was analyzed by the Student's *t*-test ($P>0.05$).

2.7. Bioequivalence study

The analytical method was applied to evaluate the bioequivalence of two tablet formulations of methyldopa in healthy volunteers: Legrand Metildopa (test formulation from EMS Indústria Farmacêutica; lot No. 003352, expiration date July 2002) and Aldomet (standard reference formulation from Prodome Química e Farmacêutica; lot No. BB089, expiration date February 2003).

Twenty-five healthy volunteers of both sexes (12 male and 13 female), aged between 18 and 50 years and within 15% of the ideal body mass, were selected for the study after assessment of their health status by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, γ-gluthamil transferase (γ-GT), total bilirubin, albumin and total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, and routine urinalysis. All subjects were negative for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV), except for serological scar.

The volunteers had the following clinical characteristics (divided by gender and expressed as mean±SD [range]): male: age: 34.1±7.0 years [27–45], height: 174.8±8.6 cm [160.5–190.0], body mass: 81.1±9.9 kg [65.0–94.5]; female: age: 30.9±5.2 years [25–39], height: 155.6±5.2 cm [150.0–165.0], body mass: 57.8±7.1 kg [45.5–69.4].

The study was a single dose, two-way randomized crossover design with a 5-day washout period between the doses. During each period, the volunteers were hospitalized at 21:00 h having already had a normal evening meal, and after an overnight fast they received (at 06:00 h) a single dose of methyldopa (500 mg of either tablet formulation). Water (200 ml) was given immediately after the drug administration and the volunteers were then fasted for 4 h, after which period, a standard lunch was served; an evening meal was provided 10 h after dosing. No other food was permitted during the

''in-house'' period and liquid consumption was allowed ad libitum after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and cola). Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and hourly after the administration of each methyldopa dose.

Blood samples (6 ml) from a suitable antecubital vein were collected by indwelling catheter into EDTA containing tubes before and 20, 40, 60, 80, 100 min, 2, 2.5, 3, 4, 5, 6, 8, and 12 h post-dosing. The blood samples were centrifuged at 2000 *g* for 10 min at room temperature and the plasma stored at −20 °C until assayed for methyldopa content.

Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under curve (AUC) of plasma concentration until the last concentration observed (AUC_{last}), and the area under curve between the first sample (pre-dosage) and infinite ($AUC_{0-\infty}$). The C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the methyldopa plasma concentration vs. time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity ($AUC_{0-\infty}$) was done by adding the value C_{last}/ke to the calculated AUC_{last} (where C_{last} = the last detectable concentration, and ke = the first-order terminal elimination rate constant which was estimated by linear regression from the points describing the elimination phase on a log-linear plot). The AUC and C_{max} data for the two formulations were analyzed by analysis of variance (ANOVA) to establish whether the 90% confidence interval (CI) of the ratios was within the 80–125% interval indicating bioequivalence as proposed by the US Food and Drug Administration.

Parametric and non-parametric analyses of ln-transformed arithmetic means and individual T_{max} differences between test and reference formulations were performed.

3. Results

Under the LC–MS–MS conditions previously described, and as shown in Fig. 2A–C, no endogenous peak was observed in the mass chromatogram of blank plasma. The mass chromatograms of a sample are shown in Fig. 3, in which both the retention times of methyldopa and I.S. were 3.4 min. Mean absolute recoveries were calculated as the ratio of the mean response areas for extracted and unextracted (spiked blank plasma extract) samples at the same concentration expressed as a percentage. The recoveries observed were (means±SD) 93±5, 89±7 and 83±11% (25, 250 and 2500 ng/ml, respectively) for methyldopa, and 80±11% for I.S. (2000 ng/ml).

Stability analysis was performed with plasma

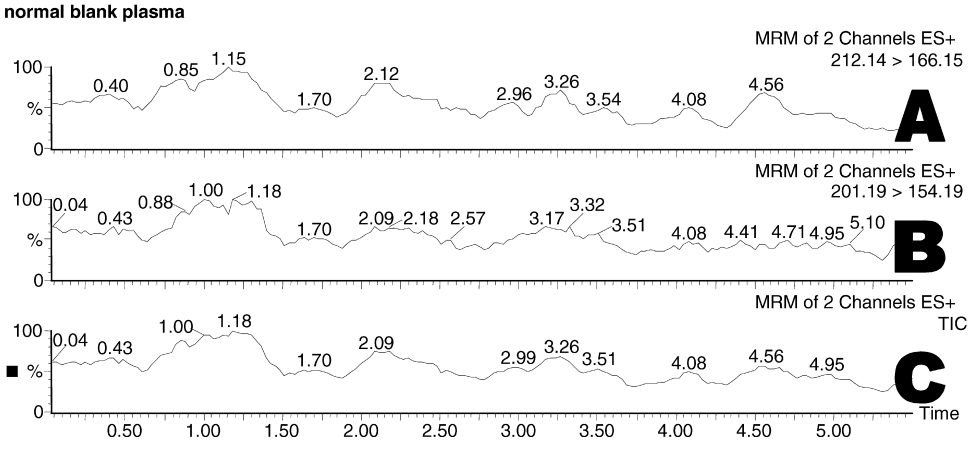


Fig. 2. MRM chromatograms of blank human plasma: (A) methyldopa, (B) I.S., and (C) total ion chromatogram (TIC).

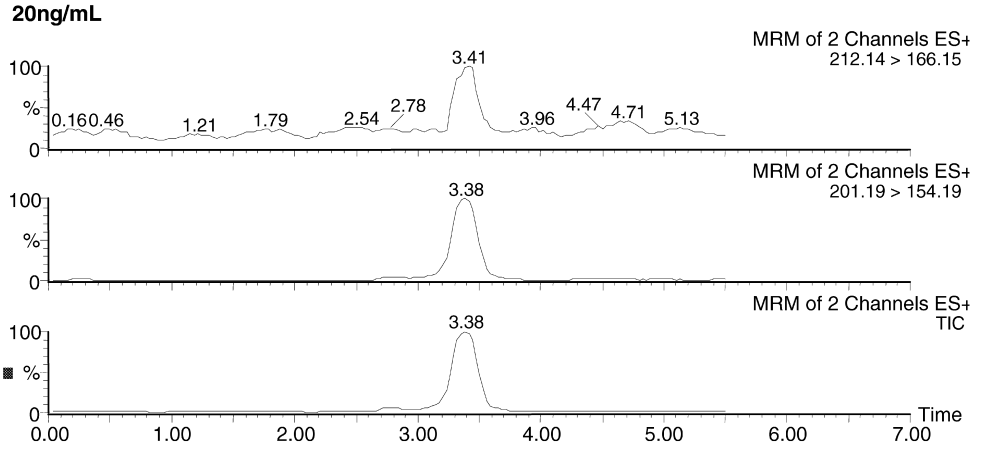


Fig. 3. MRM chromatograms of methyldopa, I.S. and TIC.

quality control samples (500 and 3000 ng/ml). All samples showed no significance degradation under the conditions previously described in the Experimental section.

3.1. Assay performance

A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of methyldopa and I.S. vs. methyldopa concentrations of the eight human plasma standards (in duplicate) to generate a calibration curve. Calibration curves showed linear relationship between peak area ratios (methyldopa/internal standard) and α-methyl DOPA concentrations ($y = ax + b$, where y = peak area ratio and x = methyldopa concentration in ng/ml). Mean (SD) values for coefficients a and b obtained along the study were 0.00058 (0.00008) and 0.036 (0.007), respectively; regression coefficient (r) values varied from 0.992 and 0.997 (mean: 0.995, SD: 0.001). The calibration curve was linear over the range 20 to 5000 ng/ml. The mean coefficient of correlation (r) was greater than 0.997.

The lower limit of quantification (LOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 20 ng/ml.

The precision and accuracy of quality control samples are shown in Table 1. Precision was determined as the percent relative standard deviation, RSD (%) = $100 \cdot (SD/M)$ and accuracy as the percent relative error, RE (%) = $(E - T) \cdot (100/T)$, where M is the mean, SD is the standard deviation, T is theoretical concentration and E is the experimentally determined concentration.

Two volunteers dropped out the study (one female because difficult in getting and keeping a venous access, and one male because right foot cellulitis which required medication). Six volunteers (four female) reported headache that was considered mild and of short duration; one male volunteer needed to take medication (paracetamol). Five volunteers (four female) reported drowsiness that was also considered mild. Additionally, three volunteers (two female) reported mild dizziness and one female volunteer reported mild nausea.

Table 1
Intra- and inter-batch variation of analytical data

	QL1	QCA	QCB	QCC
Intra-batch				
Nominal concentration (ng/ml)	20	30	600	3000
Mean	19	28	592	2941
SD ($n = 8$)	1.6	2.0	31.9	127.9
Accuracy (%)	−7.1	−8.0	−1.3	−2.0
Precision (%)	8.6	7.3	5.4	4.3
Inter-batch				
Nominal concentration (ng/ml)	20	30	600	3000
Mean	20	30	593	2930
SD ($n = 3$)	1.5	2.0	3.2	19.0
Accuracy (%)	1.1	0.2	−1.1	−2.3
Precision (%)	8.9	7.7	0.5	0.7

Table 2
Mean pharmacokinetic parameters obtained from 23 volunteers after administration of 500 mg methyldopa tablet

Parameter	Aldomet	Metildopa
AUC_{last} ([ng h]/ml)		
Geometric mean	6365	6651
SD	2917	3587
$AUC_{0-12\ h}$ ([ng h]/ml)		
Geometric mean	6545	6862
SD	2904	3643
C_{max} (ng/ml)		
Geometric mean	1303	1358
SD	480	621
$T_{1/2}$ (h)		
Median	2.6	2.2
Range	1.0–7.6	0.6–10.6
T_{max} (h)		
Median	3.0	3.0
Range	1.7–6.0	1.7–6.0

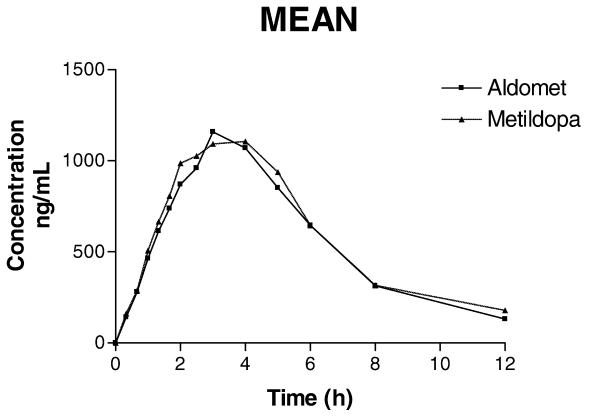


Fig. 4. Mean plasma concentrations vs. time curve for both methyldopa formulations.

Table 2 shows the mean pharmacokinetic parameters obtained after the administration of 500 mg of methyldopa to 23 healthy volunteers. The mean plasma methyldopa concentrations vs. time curves for both preparations are shown in Fig. 4, and the statistical analysis of the bioequivalence parameters and their confidence intervals are recorded in Table 3.

The geometric mean and respective 90% CIs of Legrand Metildopa/Aldomet percent ratios were 98.7% (88.9–103.7%) for C_{max}, 97.0 (85.0–110.7%) for AUC_{last}, and 100.5% (87.9–114.8%) for $AUC_{0-\infty}$ (Table 3). T_{max} was also statistically analyzed and the point estimate for individual differences (Legrand Metildopa/Aldomet) was 0.0 h (90% CI of −0.7 to 0.5).

4. Discussion

Although methyldopa plasma concentrations have been measured through different analytical methods, the use of HPLC seems the more frequently used [2–7]. Róna et al. [3] obtained an LOQ of 10 ng/ml using solid-phase extraction and HPLC–fluorescence detection but the retention time and the total run time were very long (11.4 and 35 min, respectively). Lucarelli et al. [4], using dual working electrode coulometric detection, obtained an LOQ of 1.6 ng/ml, but the total run time was also long (>20 min) and the extraction was more complex. Other electrochemical detection have been reported [5–7] in which the retention times were approximately 10 min and the extraction methods used were more complex than our method. The LOQ observed ranged between 50 to 200 ng/ml.

In the present study, the retention time and the

Table 3
Geometric means of the individual AUC_{last}, $AUC_{0-\infty}$, and C_{max} ratios (test/reference formulation) and the respective 90% confidence intervals (CIs)

Metildopa/Aldomet	Statistical analysis			
	Parametric		Non-parametric	
	Geometric mean	90% CI	Geometric mean	90% CI
AUC_{last} % ratio	97.0	85.0–110.7	99.8	88.7–114.4
$AUC_{0-\infty}$ % ratio	100.5	87.9–114.8	104.4	91.1–118.3
C_{max} % ratio	98.7	88.9–103.7	100.4	88.8–112.5

total run time for methyldopa were shorter (5.5 and 3.3 min, respectively) than those previously reported assays. We decided to use deuterium labeled L-dopa for avoiding possible endogenous interference providing from the plasma, although some authors have reported the use of unlabeled dopa [3]. On the other hand, the extraction procedure in our case is simple and rapid and do not include a concentration step. The LOQ observed in the present study (20 ng/ml) was higher than reported in other studies but it was considered sufficient for the bioequivalence study.

After oral administration of the methyldopa tablets to the volunteers, the observed methyldopa peak plasma concentration (C_{max}) values and the time values taken to be achieved (T_{max}) were similar to those reported in the literature and equivalent between the formulations. In addition, the calculated 90% CIs for mean $C_{max,}$ AUC_{last} and $AUC_{0-\infty}$ Legrand Metildopa/Aldomet individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [8].

5. Conclusion

An LC–MS–MS method for the quantification of methyldopa in human plasma was developed and validated. The method satisfied the requirements of high sensitivity, specificity and rapid sample throughput that are necessary for pharmacokinetic studies.

The pharmacokinetic data demonstrated that the methyldopa test formulation (Legrand Metildopa) is bioequivalent in terms of both rate and extent of absorption to reference formulation (Aldomet).

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