Levothyroxine Bioequivalence Study: Determination in Healthy Volunteers by Microparticle Enzyme Immunoassay

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NLMC GJMR Classification
QW525.5.I3

***Abstract*-The study was performed to compare the bioavailability of two Levothyroxine 200 mcg mg tablet formulation in 36 volunteers of both sexes. The study was conducted open with randomized two period crossover designs and a two weeks wash out period. Plasma samples were obtained over a 48 hour interval. Levothyroxine concentrations were analyzed by a validated microparticle enzyme immunoassay with optical detection by fluorescence. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% I.C) for the ratio of $AUC_{0\text{-}48h}$ and C_{max} values for the test and reference products, using logarithmic transformed data. The 90% confidence intervals were 93.60% – 103.92%, and 96.14% – 105.95%, respectively. Since the 90% confidence intervals for C_{max} and $AUC_{0\text{-}48h}$ were within the 80 – 125% interval proposed by Food and Drug Administration, it was concluded that the two Levothyroxine formulations are bioequivalent in their rate and extent of absorption.**

I. Introduction

Levothyroxine sodium is the sodium salt of the levo isomer of the thyroid hormone thyroxine. Levothyroxine sodium is a drug with a narrow therapeutic index (NTI), defined as a drug that is subject to therapeutic drug concentration or pharmacodynamic monitoring, and/or where product labeling indicates an NTI designation.[1] To understand the underlying physiologic milieu that dictates this NTI, it is essential to understand the control of the production of endogenous thyroid hormone.Thyroxine (T_4) is the endogenous hormone, synthesized in and released from the thyroid gland.

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It is a pro-hormone" in that it is converted in the body to the short-lived, more biologically potent triiodothyronine (T_3). Both T_4 and T_3 affect protein, Lipid, and carbohydrate metabolism, growth and development. They stimulate the oxygen consumption of Most cells of the body, resulting in increased energy expenditure and heat production. T_4 and T_3 also possess a cardiac stimulatory effect that may result from direct action on the heart.The thyroid hormone system is highly regulated through a tight feedback system via the hypothalamic-pituitary-thyroid gland axis. When thyroid hormone levels are low, the hypothalamus secretes thyroid stimulating hormone-releasing hormone (TRH), which stimulates the pituitary gland to produce thyroid-stimulating hormone (TSH). TSH, in turn, stimulates the thyroid gland to produce both T_4 (the major component) and some T_3. When thyroid hormone levels are high, the synthesis and release of TRH and TSH are inhibited, thus resulting in decreased thyroid hormone production and release from the thyroid gland.Orally administered synthetic levothyroxine is approved for use in the treatment of hypothyroidism, as a TSH suppressant for various types of goiters, and as an adjunct to surgery and radioiodine therapy in the management of thyroid cancer.[3] Levothyroxine is prescribed for patients as young as newborns and is widely used by geriatric patients, patients with underlying coronary heart disease, and in pregnant and nursing women.As outlined by the Food and Drug Administration (FDA), levothyroxine must be precisely and consistently dosed for it to be safe and effective. —fI a drug product of lesser potency or bioavailability is substituted in the regimen of a patient who has been controlled on one product, a suboptimal response and hypothyroidism could result. Conversely, substitution of a drug product of greater potency or bioavailability could result in toxic manifestations of hyperthyroidism such a cardiac pain, palpitations, or cardiac arrhythmias. In patients with coronary heart disease, even a small increase in the dose of levothyroxine may be hazardous.[1] Multiple dosage strengths are available for precise titration to meet an individual patient's specific needs. The bioavailability and bioequivalence between the different marketed levothyroxine formulations have been recognized as important issues in current guidelines. [4,5] To measure the bioavailability of levothyroxine formulations, the US Food and Drug Administration (FDA) [1] recommends administering a single dose (600 mcg), several times above

the typical therapeutic dose, to healthy volunteers with sample collection to 48 hours. The theory is to raise plasma concentrations of the hormone high enough above the endogenous baseline levels of levothyroxine to achieve meaningful pharmacokinetic measurements. Bioavailability studies in healthy subjects have found that, even with such doses, endogenous levothyroxine contributes to the total AUC.The narrow therapeutic index requires precise dosing of the drug and knowing the bioavailability of the preparation used is a prerequisite for the precise dosing required. The aim of this study was to compare in healthy volunteers, the pharmacokinetics profiles and evaluate the bioequivalence of one test formulation of 200 mcg tablet of Levothyroxine, elaborated by Merck S/A, Brazil (test formulation). The test formulation was The aim of this study was to compare in healthy volunteers, the pharmacokinetics profiles and evaluate the bioequivalence of one test formulation of 200 mcg tablet of Levothyroxine, elaborated by Merck S/A, Brazil (test formulation). The test formulation was compared to one commercial formulation of 200 mcg of Levothyroxine (Puran T4®) by Sanofi-Synthlabo, Brazil (reference formulation).

II. Results

1) *demography and safety*

Thirty six healthy volunteers of both sexes aged between 18 and 45 years and within the 15% of the ideal body weight were selected for the study. Demographic data, including age, height, weight and BMI are listed in Table 1.
Levothyroxine was well tolerated at the administered dose. All the biochemical parameters did not any clinical relevant alterations. No adverse effects were either reported or observed

2) *Pharmacokinetic And Statistical Analysis*

The mean (± SD) plasma concentration time profile of the 2 formulations, shown in Figure 01, was similar and superimposable. Central and dispersion measures for all pharmacokinetic parameters for both formulations are shown in Table 2 and Table 3. From this, the mean values of C_{max} were found to be 11.53 (± 2.40 standard deviations [SD]) µg/dL for the reference product and 11.58 (± 2.10) µg/dL for the locally manufactured (test) product. For T_{max} (h), the mean values were found to be similar for both the reference and local product and the value was 2.0 (2.5) h. The mean values of $AUC_{0\text{-}48h}$ were found to be 443.7 (± 75.00) µg.h/dL for reference and 445.08 (± 100.82) µg.h/dL for local product. The mean $AUC_{0\text{-}\infty}$ were found to be 10679.17 (± 17326.22) µg.h/dL and 14447.11 (± 36474.98) µg.h/dL for the reference and locally manufactured product, respectively.Table 4 presents the ratios and the respective confidence intervals for bioequivalence analysis.

III. Discussion

Levothyroxine sodium is a compound with a narrow therapeutic range, its well tolerated and effective use requires careful titration and close clinical follow-up. If treatment is not carefully monitored, a patient might be at risk for iatrogenic hyperthyroidism or hypothyroidism.[6-8] Even in the presence of mild (subclinical) hypothyroidism, there is a potential for cardiovascular adverse events such as hypercholesterolemia,[9] increased fibrinolytic activity,[10] systolic and diastolic dysfunction,[11] atherosclerosis,[12] arrhythmias,[13] and myocardial infarction.[14] Guidelines from endocrinology societies advise careful titration of the dose with monitoring and retitration should the dose or brand of drug change, and emphasize the assessment of the bioequivalence to ensure generics also provide therapeutic equivalence for their patients. The bioavailability and bioequivalence between the different marketed levothyroxine formulations have been recognized as important issues in current guidelines.[6,5] It is a challenge to determine the bioavailability of levothyroxine sodium products because levothyroxine is naturally present in minute quantities in the blood, with the total levels reaching 5.0-12.0 mg/dl and free (or unbound) levels reaching 0.8-2.7 ng/dl in a healthy adult. To assess the bioavailability of levothyroxine sodium after a single dose, several times the normal dose should be given to raise the levels of the drug significantly above baseline to allow measurement. Furthermore, levothyroxine has a long half-life of 6 to 9 days, and therefore, a long washout period is necessary between treatments.[1] To measure the bioavailability of levothyroxine formulations, the US Food and Drug Administration (FDA) recommends administering a single dose (600 mcg), several times above the typical therapeutic dose, to healthy volunteers with sample collection to 48 hours. The theory is to raise serum concentrations of the hormone high enough above the endogenous baseline levels of levothyroxine to achieve meaningful pharmacokinetic measurements. Bioavailability studies in healthy subjects have found that, even with such doses, endogenous levothyroxine contributes to the total AUC. Nonetheless, the FDA recommended that pharmacokinetic profiles to assess bioequivalence should be presented without adjustment by baseline levels because endogenous levothyroxine concentrations are unpredictable during the course of the study. In this study two formulations of levothyroxine had been evaluated without adjustment for baseline hormone levels in accordance with US Food and Drug Administration (FDA).[1] The mean ratio of parameters C_{max} and $AUC_{0\text{-}48}$ and 90% confidence intervals of correspondents were calculated to determine the bioequivalence. The means $AUC_{0\text{-}48}$ for test and reference formulation were 445.08.86 µg.h/dL and 443.47 µg.h/dL, for $AUC_{0\text{-}\ inf}$ were 14447.11 µg.h/dL and 10679.17 µg.h/dL and, for C_{max} 11.58 µg/dL and 11.53 µg/dL, respectively. The ratios were 98.63 % for $AUC_{0\text{-}48}$, 101.87% for $AUC_{0\text{-}inf}$ and 100.93% for C_{max}. The 90% confidence intervals were 93.60 – 103.92% for $AUC_{0\text{-}48}$, 66.09 – 157.03% for $AUC_{0\text{-}inf}$ and 96.14 – 105.95% for C_{max}. The present study showed that 90% CI of mean $AUC_{0\text{-}t}$ and C_{max} were included into the bioequivalence range (80-125%), consequently, the two formulations of levothyroxine are equivalent for the extend of absorption.The statistical comparison of C_{max}, and $AUC_{0\text{-}t}$ clearly indicated no significant difference in the two formulations of

levothyroxine 200 mcg tablet. 90% confidence intervals for the mean ratio (T/R) of C_{max} and AUC_{0-t} were entirely within the US Food and Drug Administration acceptance range. Based on the pharmacokinetic and statistical results of this study, we can conclude that levothyroxine 200 mcg tablet (Merck S/A, Brazil) is bioequivalent to Puran T4® 200 mcg tablet (Sanofi-Synthlabo, Brazil), and that then the test product can be considered consistent in potency and bioavailability and interchangeable in medical practice.

IV. Methods

a) *Study subjects*

Thirty six healthy volunteers were selected for the study. All volunteers were healthy as assessed by physical examination, electrocardiogram (ECG), and the following laboratory tests: blood glucose, urea, creatinine, uric acid, alanine and aspartate aminotransferases (ALT and AST), thyroid-stimulating hormone (TSH), gamma-gluthamil transferase (γ-GT), alkaline phosphatase, total billirubin, albumin and total protein, trygliceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, red blood cell counts, platelet counts and routine urinalysis. All subjects were negative for human immunodeficiency virus, and B (except for serological scar) and C hepatitis virus. All female volunteers were negative for β-HCG (pregnancy test).

b) *Study procedures*

This study was performed according to the resolution 196/96 (Ministério da Saúde, 1996) and the revised Declaration of Helsinki for biomedical research involving human beings. The study protocol was approved by M.M. Assert Serviços Médicos S/A Ltda (Escola Assertiva) ethics committee. All subjects gave written informed consent prior to their inclusion. It was an open-label, randomized, two-period, two-sequence crossover study with 53 days washout period. During each period, the volunteers were hospitalized at 7:00 p.m. They had the usual evening meal until 9:00 p.m., and an overnight fast (minimum of 10 hours) At 7:00 a.m. a single oral dose of 600 mcg (three tablets containing 200 mcg) of each levothyroxine formulation. Water (200 mL) was given immediately after oral drug administration. All volunteers were then fasted for 4 h following drug administration; afterwards a standard lunch was consumed. Standard snack and evening meal were provided 7-8 and 10-12 h after dosing, respectively. No other food was permitted during the confinement period. Liquid consumption was allowed *ad libitum* 2 h after drug administration. However, xanthine-containing drinks including tea, coffee, and cola were avoided.Blood samples (08 mL) from a suitable antecubital vein were collected into EDTA containing tubes at 30 and 15 minutes prior to administration, and 0 (baseline), 0.5, 1, 1.50, 2, 2.50, 3, 3.50, 4, 4.50, 5, 6, 8, 12, 18, 24 and 48 hours after administration of a single oral dose of levothyroxine 600 mcg (three 200 mcg tablets).
The blood samples were centrifuged at 3.000 rpm for 10 min. at 4°C and the plasma decanted and storage at – 20°C until assay for their levothyroxine content. All samples from a single volunteer were analyzed on the same day in order to avoid interassay variation.Arterial pressure(measurednon-
The blood samples were centrifuged at 3.000 rpm for 10 min. at 4°C and the plasma decanted and storage at – 20°C until assay for their levothyroxine content. All samples from a single volunteer were analyzed on the same day in order to avoid interassay variation.Arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and after drug administration at each full-hour sample collection.

c) *Chemicals, Reagents And Instrumentation*

Levothyroxine reference standard was acquired from the United States Pharmacopea (Rockville, MD, USA). Levothyroxine 200 mcg (test formulation, Merck S/A, Brazil) and Puran T4® 200 mcg (reference formulation, Sanofi-Synthlabo, Brazil). HPLC-grade methanol and water was purifield using a MilliQ®. Measurement of levothyroxine in plasma was carried out by an AxSYM® Thyroxine T4 assay (Abbott Laboratories, U.S.A.), a microparticle enzyme immunoassay (MEIA), with optical detection by fluorescence. Samples were stored at -70°C freezer until analysis.

d) *Analytical Procedures*

The microparticle enzyme immunoassay (MEIA) was linear up to a levothyroxine plasma concentration of 24.0 µg/mL. The coefficients of variation between and within runs were less than 10% for concentrations of 8.9, 12.4 and 19.4 µg/mL of levothyroxine. All samples were analyzed and the results interpreted according to the instructions provided by the manufacturer.The samples were analyzed with standards and quality control samples. Standard curves were constructed and levothyroxine concentration 100 µg/mL. The analysis was carried out in duplicate for each concentration. The quality control samples were prepared in blank plasma at concentrations of 8.9 (low), 12.4 (medium) and 19.4 (high) µg/mL. The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.Linearity was determined to assess the performance of the method. A linear least-squares regression was applied to the peak area ratios of levothyroxine versus the concentrations of the 6 plasma standards of levothyroxine (0.0, 3.0, 6.0, 12.0, 18.0 and 24.0 µg/mL) in triplicate to generate a calibration curve. Within and between-run precision was determined as the relative standard deviation and the accuracy as the percentage relative error.To assess stability, quality control plasma samples (8.9, 12.4 and 19.4 µg/mL) were subjected to short-term (6h) incubation at room temperature, 3 freeze/thaw cycles and a long-term incubation (92 days). After each one of these processes, the levothyroxine concentrations were measured and compared with freshly prepared samples.The method was validated according to ANVISA (National Health Surveillance Agency of Brazilian Government) criteria and used to determine the concentration of levothyroxine in volunteers plasma.

e) *Formulations*

The test formulation employed was Levothyroxine 200 mcg tablet (lot number B69237) and the reference formulation was Puran T4® 200 mcg tablet (lot number 5083073).

f) *Pharmacokinetics and Statistical analysis*

Levothyroxine plasma profiles and pharmacokinetic measures were analyzed without adjustment by baseline levels of endogenous T4. The first-order terminal elimination rate constant (Ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot, using the software SAS® Institute (Version 9.1.3). Elimination half-life ($T_{1/2}$) was derived from this rate constant ($T_{1/2}$ = ln (2)/Ke). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the levothyroxine plasma concentration versus time curves from 0 to 48 hours (AUC_{0-48h}) were calculated by applying the linear trapezoidal rule. Extrapolation of these areas to infinity (AUC_{0-inf}) was done by adding the value C48/Ke to the calculated AUC_{0-48h} (where C48=plasma concentration calculated from the log-linear regression equation obtained for the estimation of Ke 48 hours after dose).The bioequivalence between both formulations was assessed by calculating individual C_{max}, AUC_{0-48h}, AUC_{0-inf} and C_{max}/AUC_{0-48h} ratios (test/reference) together with their mean and 90% confidence intervals (CI) after log transformation of the data. The inclusion of the 90% CI for the ratio in the 80% to 125% range was analyzed by nonparametric (SAS® Institute Version 9.1.3) and parametric (ANOVA) methods.

g) Acknowledgments

This research work is financially supported by the Scentryphar Clinical Research, Brazil.

h) Conflict of Interest

The authors declared no conflict of interest.

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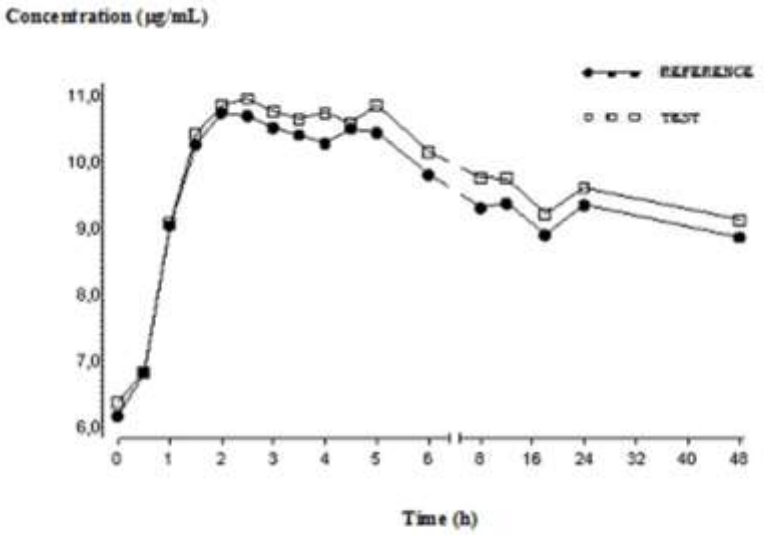

Figure 1 Mean (SD) thyroxine concentration-time profiles after single-dose administration of a test and reference formulation of levothyroxine 600mcg, uncorrected for endogenous thyroxine baseline concentrations.

Table 1 Summary of demographic characteristics for the safety population for study (mean ± SD)

Category	Volunteers
n	36
Age (years)	30.20 ± 7.00
Height (cm)	1.70 ± 0.10
Weight (Kg)	64.70 ± 7.60
BMI (Kg/m^2)	23.50 ± 2.20

Table 2 Mean pharmacokinetic parameters of levothyroxine of test and reference formulation

	TEST		REFERENCE	
Parameter (unit)	**Means (Median)**	**Standard Deviation (Amplitude)**	**Means (Median)**	**Standard Deviation (Amplitude)**
AUC_{0-t} (µg.h/dL)	445.08	100.82	443.47	75.00
AUC_{0-inf} (µg.h/dL)	14447.11	36474.98	10679.17	17326.22
C_{max} (µg/dL)	11.58	2.10	11.53	2.40
T_{max} (median/amp) (h)	2.50	4.50	2.00	23.02
Kel (1/h)	0.00	0.00	0.00	0.00
T½ (median/amp) (h)	403.84	16850.26	348.30	6309.87

Table 3 Geometric mean pharmacokinetic parameters of levothyroxine of test and reference formulation

	TEST	REFERENCE
Parameter (unit)	**Geometric Mean**	**Geometric Mean**
AUC_{0-t} (µg.h/dL)	430.95	436.95
AUC_{0-inf}(µg.h/dL)	6173.17	6059.67
C_{max} (µg/dL)	11.38	11.28

Table 4 Ratios means and the 90% geometric confidence interval of test and reference formulation

Parameter	Ratio T/R (%)	Lower Limit (%)	Upper Limit (%)	Power (%)	Coefficient of Variation (%)
AUC_{0-t}	98.63	93.60	103.92	99.99	12.75
AUC_{0-inf}	101.87	66.09	157.03	0.00	140.36
C_{max}	100.93	96.14	105.95	99.99	11.85
T_{max} (df) (h)	0.25	0.00	1.00		