International Journal of Pharmaceutics 366 (2009) 149–153

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Bioequivalence assay between orally disintegrating and conventional tablet formulations in healthy volunteers

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ARTICLE INFO

Article history:
Received 22 June 2008
Received in revised form 5 September 2008
Accepted 5 September 2008
Available online 20 September 2008

Keywords:
Bioequivalence
Ondansetron
LC–MS
Plasma

ABSTRACT

The purpose of this study was to evaluate bioequivalence of two commercial 8 mg tablet formulations of ondansetrona available in the Brazilian market. In this study, a simple, rapid, sensitive and selective liquid chromatography–tandem mass spectrometry method is described for the determination of ondansetron in human plasma samples. The method was validated over a concentration range of 2.5–60 ng/ml and used in a bioequivalence trial between orally disintegrating and conventional tablet ondansetron formulations, to assess its usefulness in this kind of study. Vonau flash® (Biolab Sanus Farmacêutica, Brazil, as test formulations) and Zofran® (GlaxoSmithKline, Brazil, as reference formulation) were evaluated following a single 8 mg dose to 23 healthy volunteers of both genders. The dose was administered after an overnight fast according to a two-way crossover design. Bioequivalence between the products was determinated by calculating 90% confidence interval (90% CI) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmically transformed data. The 90% confidence interval for the ratio of C_{max} (87.5–103.8%), AUC_{0-t} (89.3–107.2%) and $AUC_{0-\infty}$ (89.7–106.0%) values for the test and reference products is within the 80–125% interval, proposed by FDA, EMEA and ANVISA. It was concluded that two ondansetron formulations are bioequivalent in their rate and extent of absorption.

1. Introduction

The orally disintegrating tablet is a formulation that dissolves in the patient's mouth within a minute and water or chewing is not necessary. Orally disintegrating tablets are useful for patients with difficulties in swallowing conventional tablets, for example pediatric patients and patients under chemotherapy treatment (Habib et al., 2000). Patients on chemotherapy treatment may have nausea so intense that complicates the administration of conventional tablets, usually it has been made with water, especially those with tumors in the mouth and esophagus (Biradar et al., 2006).

Nausea and emesis, continue to cause significant problems for patients with cancer receiving highly or moderately emetogenic chemotherapy. Vomiting and nausea were the two most distressing side effects perceived by patients receiving chemotherapy because they have an impact on quality of life and compliance with treatment. The development of 5-hydroxytryptamine ($5\text{-}HT_3$) antagonists has been a major step forward in the prevention and treatment of chemotherapy-induced nausea and vomiting (Annemas et al., 2008). Ondansetron, {1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-ylmethyl]-4H-carbazol-4-one} is the first of several selective $5\text{-}HT_3$ antagonists available as an antiemetic (Currow et al., 1997). Oral ondansetron is well absorbed, with a bioavailability of approximately 60–70%. Nevertheless the first-pass metabolism removes 30–40% of the drug (Simpson and Hicks, 1996). There are some chromatographic techniques published for the determination of ondansetron in human plasma. Expensive solid-phase procedures have been reported by some authors (Xu et al., 2000; Liu and Stewart, 1997). Others used liquid–liquid extraction with large volumes of solvent (Chandrasekar et al., 2004; Bauer et al., 2002; Dépôt et al., 1997). Only one article reported ondansetron extraction with a small volume (600 μl) of solvent, in this work it was used the semi-automated process extration (Dotsikas et al., 2006).

Most studies used HPLC–UV (Chandrasekar et al., 2004; Bauer et al., 2002; Liu and Stewart, 1997; Dépôt et al., 1997) wavelength of 305 nm and others used liquid chromatography coupled with mass spectrometry (Dotsikas et al., 2006; Chandrasekar et al., 2004; Bauer et al., 2002; Xu et al., 2000; Liu and Stewart, 1997; Dépôt et al., 1997). All studies showed extensive run times and used protocols with multiple steps for the extraction of ondansetron from biological samples, except one that reported the use of a 5-mm long

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doi:10.1016/j.ijpharm.2008.09.021

column and a semi-automated process of extraction (Dotsikas et al., 2006).

Ondansetron quantification in plasma samples is required for pharmacokinetics studies and bioequivalence assays. One of the criteria for the determination of drugs in bioequivalence assays is the time of analysis, since a very large number of samples are generated in this kind of study (Porta et al., 2008).

This paper describes the development and validation of a sensitive, specific, rapid and simple HPLC method with mass spectrometer detection for ondansetron quantification in human plasma, and its application in a bioequivalence study between two pharmaceutical products available in the Brazilian market.

2. Materials and methods

2.1. Materials

Ondansetron hydrochloride reference standard (98.4% pure) was obtained from United States Pharmacopeia (USP). Propranolol, employed as the internal standard (IS), was obtained from FUNED – "Fundação Ezequiel Dias" (Minas Gerais, Brazil). Ethyl acetate, methanol and acetonitrile HPLC grade were obtained from J.T. Baker (São Paulo, Brazil). Formic acid analytical grade was bought from J.T. Baker (São Paulo, Brazil) and ammonium acetate analytical grade was bought from Sigma (São Paulo, Brazil). Water (18.2 MΩ) used to prepare a buffer solution of the mobile phase was freshly prepared from Milli-Q Academic (Millipore) (Belford, USA).

Pharmaceutical products used in the bioequivalence study were Vonau® *flash* (orally disintegrating tablet containing 8 mg of ondansetron, lot no. 604718, produced by Biolab Sanus Farmacêutica, Brazil), as the test product, and Zofran (conventional tablet containing 8 mg of ondansetron, lot no. R207186 V, produced by GlaxoSmithKline, Brazil), as the reference product.

2.2. Instrumentation

Analyses were performed on a Shimadzu Scientific Instruments (Kyoto, Japan) liquid chromatographic system composed of a LC-10ADVP pump accompanied with an SIL-10ADVP auto-sampler fitted with a 50-μl loop and a SCL-10AVP controller unit. This HPLC system was coupled with a Micromass Quattro triple quadrupole mass spectrometer (Milford, MA, USA) equipped with an electrospray ion source and operating under MassLynx 4.0 software. The analytical column was a Gemini® C18 (150 mm × 4.6 mm i.d., 5 μm particle size) protected with a Phenomenex® AJO-4287 C18 guard cartridge (5 mm × 4.6 mm i.d., 5 μm particle size) (Torrance, CA, USA).

2.3. Chromatographic and mass spectrometric conditions

The isocratic HPLC mobile phase was composed of acetonitrile and 10 mM ammonium acetate (55:45, v/v) with 0.1% formic acid; it was prepared daily and degassed before use. Flow rate was 0.4 ml/min. The column was maintained at room temperature (22 °C). The injection volume was 25 μl and the total run time was set for 6.0 min.

The HPLC system was connected to the mass spectrometer through an ESI interface and was operated in the positive ion detection mode. Tuning parameters were optimized for both analytes by infusing a solution containing 500 ng/ml of ondansetron and the IS (propranolol) at a flow rate of 20 μl/min through an external syringe pump directly connected with the mass spectrometer. The source temperature was set at 100 °C, desolvation temperature was 300 °C. The optimized cone voltage values were 35 and 30 V for ondansetron and IS, respectively. Capillary voltage was set at 3.0 kV. The multiplier was set at 650 V and argon was used as the collision gas. Quantification was performed using multiple reaction monitoring (MRM) of the transitions m/z 294.21 > 170.02 for ondansetron and m/z 260.20 > 116.05 for propranolol, with a dwell time of 0.5 s per transition. The optimized collision energy of 28 eV was used for the ondansetron and 20 eV was used for the IS. Data were acquired using MassLynx 4.0 software.

2.4. Calibration standards and quality control plasma samples

Preparation of calibration standard plasma samples (2.5, 6, 10, 15, 20, 35, 45 and 60 ng/ml) was accomplished daily by introducing known amounts (30–62.5 μl) of ondansetron stock solutions (10, 50, 125 and 250 ng/ml) and 50 μl of internal standard stock solution in 8 ml glass tubes, evaporating it to dryness at 40 °C under a nitrogen stream and adding 250 μl of drug free plasma. Quality control plasma samples (7.5, 30 and 50 ng/ml) were prepared in 50 ml volumetric ballons by spiking drug free plasma with known amounts (0.75–1.5 ml) of ondansetron stock solutions (0.25 and 2.0 μg/ml), aliquoted and stored at −80 °C.

2.5. Extraction procedure of ondansetron from plasma

All frozen human plasma samples (calibration standards, quality control plasma samples and volunteers plasma samples) were thawed at room temperature. A 50-μl of IS solution in methanol (500 ng/ml) were introduced in 8 ml glass tubes and evaporated to dryness at 40 °C under a nitrogen stream. Then, 250 μl of plasma was added and vortexed for 30 s. Ethyl acetate was added (2.0 ml) to all tubes and extraction was performed by vortex mixing for 60 s. After this procedure the tubes were centrifuged for 10 min at 3500 rpm. After freezing, upper organic phases were transferred to clean 8 ml conical glass tubes and evaporated to dryness at 40 °C under a nitrogen stream. The extracts were reconstituted with 250 μl of mobile phase and 25 μl were injected into the chromatography system.

2.6. Bioanalytical method validation

Calibration standards and quality control plasma samples were prepared by spiking blank plasma with standard solutions of ondansetron.

The specificity of the method for interference of endogenous compounds was investigated by analyzing six drug free plasma samples obtained from healthy human volunteers who did not take ondansetron (four normal plasma samples, one hemolised plasma sample and one lipemic plasma sample).

The standard curve was obtained through analysis of calibration standard plasma samples and plot of peak area ratios of ondansetron and propranolol versus the corresponding ondansetron concentrations (2.5, 6, 10, 15, 20, 35, 45 and 60 ng/ml). The linearity of the standard curve was evaluated using $1/x$ linear regression analysis. The lower limit of quantification (LLOQ) was defined as the lowest ondansetron concentration that could be determined with mean value deviation and coefficient of variation less than 20%.

The analytical recovery was determined by comparing the response of pre-treated quality control plasma samples (7.5, 30 and 50 ng/ml) with the response of identical standards prepared in the mobile phase which did not undergo sample pre-treatment.

Inter- and intra-assay precision and accuracy were determined by repeated analysis of quality control plasma samples (7.5, 30 and 50 ng/ml) on the same day and on different days. Inter-assay and intra-assay precision were expressed as relative standard deviation (R.S.D.). The accuracy was expressed as the percent ratio between

the experimental concentration and the nominal concentration for each sample

Stability of ondansetron in spiked quality control plasma samples was determined in triplicate after three freezing–thaw cycles. Additionally, stability of spiked processed plasma samples during storage in the auto sampler for 24 and 48 h at room temperature was determined.

2.7. Bioequivalence study

This study was performed according to the rules of Good Clinical Practice. The protocol of this study was approved by Ethical Committee of College of Pharmaceutical Sciences of University of São Paulo. A total of 23 healthy volunteers, 11 females and 12 males, participated in the study after signing a consent form. Subjects had mean age of 30 years, mean body weight of 64 kg, and mean height of 1.66 m. Subjects with history of drug allergies, renal or hepatic impairment, history of any illness of cardiovascular system, or alcohol and drug abuse were excluded. Subjects were selected after a clinical screening procedure including a physical examination and laboratory tests. All subjects avoided using other drugs for at least 1 week prior to the study and until after its completion. They also abstained from alcoholic beverages, and xanthine-containing foods and beverages 48 h prior to each dosing and until the collection of the last blood sample.

The study was an open, randomized, two-period crossover trial with a 1-week washout period.

Subjects were admitted into hospital at 7:00 p.m. the day before the study and fasted 10 h before each drug administration. A single dose (8 mg) consisting of one Vonau® *flash* or Zofran® tablet according to the randomization plan was given to each subject in a fasting state for each treatment period. Fasting continued for a further 4 h after drug administration. The drug was administered with 240 ml of water. Subjects were provide with standard meals 4 h (lunch), 7 h (snack) and 10 h (supper) after drug administration in each treatment.

Heparinized venous blood samples, 8 ml, were collected by means of an indwelling venous canula of the cubital vein on profile days according to the time schedule, which included a blank before drug sample just prior to dosing and then at 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0 and 24.0 h after drug administration. Any deviation from the stated sampling times was recorded. Plasma was immediately separated by centrifugation at 3500 rpm for 15 min, then was transferred to properly labeled tubes and stored at −20 °C until the high-performance liquid chromatographic analysis.

2.8. Pharmacokinetics and statistical analyses

The following pharmacokinetics parameters were calculated using non-compartmental methods: area under the plasma concentration–time curve from zero to the last measurable ondansetron concentration sample time (AUC_{0-t}), area under the plasma concentration–time from zero extrapolated to infinite time ($AUC_{0-\infty}$), maximum plasmatic drug concentration (C_{max}) and time to reach C_{max} (t_{max}), terminal rate constant (K_{el}) and terminal half-life ($t_{1/2}$). C_{max} and t_{max} were obtained directly from the concentration–time curve. AUC_{0-t} was calculated using the linear trapezoidal method. K_{el} was calculated by applying a log-linear regression analysis to at least the last three quantifiable concentrations of ondansetron. $t_{1/2}$ was calculated as $0.693/K_{el}$ (Ritschel, 1992).

For the purpose of bioequivalence analysis AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were considered as primary variables. Bioequivalence between the products was determined by calculating 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data. Analysis of variance (ANOVA) was used to assess product, group and period effects. The products were considered bioequivalent if the 90% CI for AUC_{0-t} and C_{max} fell within 80–125%.

3. Results

3.1. Bioanalytical method validation

Retention time for ondansetron was 4.38 min and it was well resolved from propranolol (5.07 min) (Fig. 1).

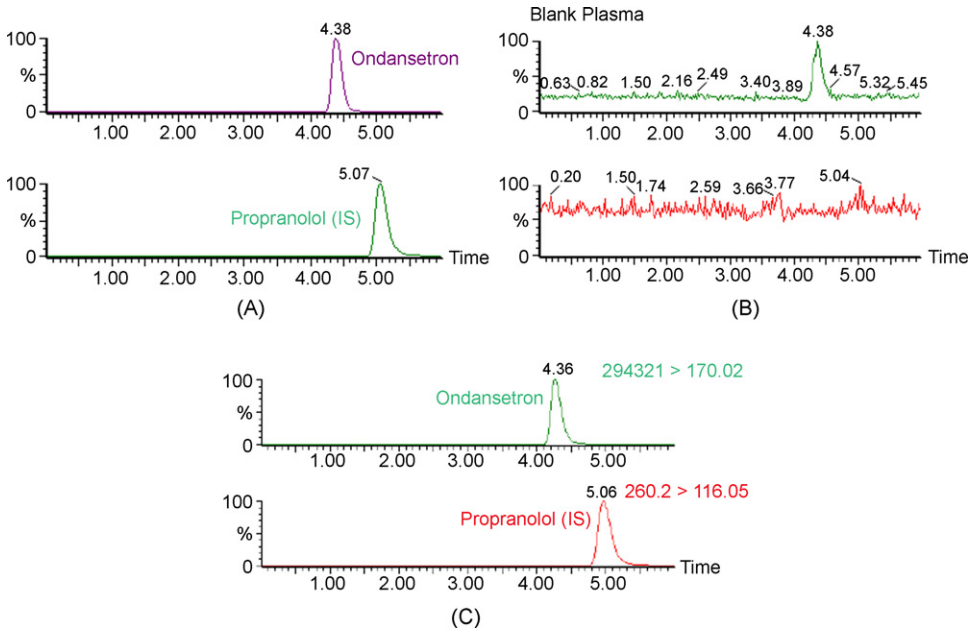

Fig. 1. Representative chromatogram of (A) solution of ondansetron and internal standard; (B) blank plasma; (C) volunteer plasma after 1 h oral 8 mg ondansetron administration.

Table 1
Recovery of ondansetron and propranolol (IS) after extraction procedure ($n = 6$). C.V. = coefficient of variation; S.D. = standard deviation.

Ondansetron concentration (ng/ml)	Recovery (%)	
	Ondansetron	Internal standard (IS)
7.5	95.87	102.69
30	87.46	92.15
50	106.46	107.17
Mean	96.60	100.67
S.D.	9.52	7.71
C.V. (%)	9.86	7.66

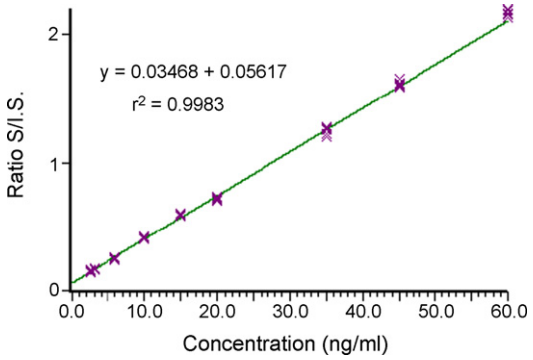

Fig. 2. Calibration curve for ondansetron quantification in human plasma by LC–MS–MS.

The mean recovery of ondansetron from human plasma matrix was 96.60% for ondansetron and 100.67% for propranolol (Table 1).

The method was linear over the range 2.5–60 ng/ml and the calibration curve could be described by the equation $y = 0.03468x + 0.05617$ ($r^2 = 0.9983$) (Fig. 2). The lower limit of quantification was 2.5 ng/ml with relative standard deviation of 9.60%.

The intra-assay accuracy ranged between 90.04 and 96.57% with precision of 1.50–10.89%. The inter-assay accuracy ranged between 92.57 and 100.16% with precision of 6.91–7.90%.

Organic extracts were stable at room temperature for at least 48 h. Plasma samples were stable for at least 6 months at −80 °C and also after three freeze–thaw cycles. The result indicated that the analyte was stable under any of the storage conditions described above and that no stability-related problems would be expected during the samples routine analysis for the pharmacokinetics, bioequivalence and bioavailability studies.

3.2. Bioequivalence evaluation

Average concentration versus time curves after administration of reference and test products to 23 healthy volunteers are shown in Fig. 3.

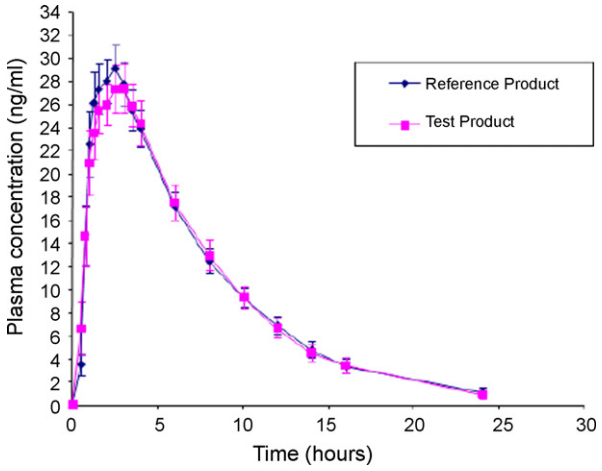

Fig. 3. Average ondansetron plasma concentration–time profiles after test and reference products administration to 23 healthy human volunteers. Bars indicate mean standard error.

Table 2 shows the average values of pharmacokinetic parameters after administration of reference and test products to 23 healthy volunteers.

The results of the analysis of variance for the assessment of product, group and period effects and the 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data, are shown in Table 3.

Power of statistical test was 98% for AUC_{0-t} and 99% for $AUC_{0-\infty}$ and C_{max}.

4. Discussion

The proposed method is suitable for ondansetron quantification in plasma samples. It showed specificity, since propranolol (IS) and ondansetron were well resolved and no interfering peaks from endogenous components of normal, hemolised and lipemic plasma were observed.

In this paper, we introduced a method for determination of ondansetron in human plasma combining a simple liquid–liquid extraction procedure, with short run time (6 min) when compared to Xu et al. (2000), Liu and Stewart (1997), Dépôt (1997), Bauer et al. (2002) and Chandrasekar et al. (2004). Although less sensitivity was obtained comparing to previously published (Dotsikas et al., 2006; Bauer et al., 2002; Dépôt et al., 1997) LC methods, the resulted LLOQ (2.5 ng/ml) was sufficient for human pharmacokinetic and bioequivalence studies.

All calculated pharmacokinetic parameter values for ondansetron in the present study were in agreement with previously reported values (Bozigian et al., 1994; GlaxoSmithKline, 2006). The K_{el} 0.16 h^{-1} to reference and test product was similar to that found Lam et al. (2004).

Table 2
Mean pharmacokinetic parameters of ondansetron after administration of test and reference products to 23 healthy volunteers. C.V. = coefficient of variation; S.D. = standard deviation.

	C_{max} (ng/ml)	t_{max} (h)	AUC_{0-t} (ng h/ml)	$AUC_{0-\infty}$ (ng h/ml)	$t_{(1/2)el}$ (h)	K_{el} (h^{-1})
Reference	31.88	1.99	227.66	252.76	4.81	0.1573
S.D.	11.50	0.79	102.47	110.15	1.67	0.04
C.V. (%)	36.07	39.65	45.01	43.58	34.66	26.26
Test	30.42	2.15	223.68	248.22	4.49	0.1630
S.D.	11.72	0.92	117.49	127.97	1.20	0.04
C.V. (%)	38.53	42.57	52.53	51.55	26.81	21.58

Table 3
Analyses of variance (ANOVA) for the assessment of the product, period and group effects and 90% confidence intervals (90% CI) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data, after administration of reference (Zofran®) and test (Vonau® *flash*) products to 23 healthy volunteers.

Pharmacokinetic parameters	ANOVA (p-value) variation source			90% CI
	Product	Period	Group	
C_{max}	0.3394	0.8289	0.0682	87.5–103.8%
AUC_{0-t}	0.6787	0.9664	0.2821	89.3–107.2%
$AUC_{0-\infty}$	0.6129	0.8105	0.2997	89.7–106.0%

The mean plasmatic decay curves obtained for the test and reference formulations in the present study were not found to be significantly different. The mean AUC_{0-t} (reference, 227.66 ng h/ml; test, 223.68 ng h/ml), $AUC_{0-\infty}$ (reference, 252.76 ng h/ml; test, 248.22 ng h/ml), C_{max} (reference, 31.88 ng/ml; test, 30.42 ng/ml) and t_{max} (reference, 1.99 h; test, 2.15 h) were similar. The values obtained for plasmatic decay $t_{1/2}$ (reference, 4.8 h; test, 4.5 h) were similar to other author's reports (Hsyu et al., 1994; Lam et al., 2004).

The multivariate analysis accomplished through analysis of variance revealed the absence of period, group and product effects for AUC_{0-t} and $AUC_{0-\infty}$, but revealed the presence of group effect for C_{max}.

The group effect measures the differences between the groups of subjects defined by their sequence. In itself it is a nuisance parameter and has little importance in interpreting data. This study used 2×2 crossover design; administration of single dose; only healthy volunteers; the drug is not an endogenous substance; washout period was adequate; the volunteers plasma samples did not show any quantifiable concentration of drug at 0 h; and there has been no deviation of critical protocol. Thus, the presence of the group effect is acceptable for this study. The observation of this effect has little influence in the analysis of data and occurs in about 10% of the bioequivalence studies (Jackson, 1994). The power of statistical test indicates that the sample size ($n = 23$) was adequate.

The 90% confidence intervals for AUC_{0-t} (89.3–107.2%), $AUC_{0-\infty}$ (89.7–106.0%) and C_{max} (87.5–103.8%) are within the 80–125% interval proposed by most regulatory agencies (FDA, EMEA, ANVISA).

It was concluded that the two formulations are bioequivalent in their rate and extent of absorption and, thus, may be used interchangeably, without any prejudice of therapeutic effect.

Acknowledgements

Authors would like to thank CAPES, and Biolab Farmacêutica.

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