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Development and validation of a rapid and sensitive LC-ESI-MS/MS method for ondansetron quantification in human plasma and its application in comparative bioavailability study

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ABSTRACT: The validation of a high throughput and specific method using a high-performance liquid chromatography coupled to electrospray (ES+) ionization tandem triple quadrupole mass spectrometric (LC-ESI-MS/MS) method for ondansetron quantification in human plasma is described. Human plasma samples were extracted by liquid–liquid extraction (LLE) using methyl tert-butyl ether and analyzed by LC-ESI-MS/MS. The limit of quantification was 0.2 ng/mL and the method was linear in the range 0.2–60 ng/mL. The intra-assay precisions ranged from 1.6 to 7.7%, while inter-assay precisions ranged from 2.1 to 5.1%. The intra-assay accuracies ranged from 97.5 to 108.2%, and the inter-assay accuracies ranged from 97.3 to 107.0%. The analytical method was applied to evaluate the relative bioavailability of two pharmaceutical formulations containing 8 mg of ondansetron each in 25 healthy volunteers using a randomized, two-period crossover design. The geometric mean and respective 90% confidence interval (CI) of ondansetron test/reference percent ratios were 90.15% (81.74–99.44%) for C_{max} and 93.11% (83.01–104.43%) for AUC_{0-t}. Based on the 90% confidence interval of the individual ratios (test formulation/reference formulation) for C_{max} and AUC_{0-inf}, it was concluded that the test formulation is bioequivalent to the reference one with respect to the rate and extent of absorption of ondansetron. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: ondansetron; mass spectrometry; pharmacokinetics; HPLC; bioequivalence

Introduction

Ondansetron, {1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-4*H*-carbazol-4-one} is a selective 5-hydroxytryptamine type 3 (5-HT3) receptor antagonist used in the treatment of postoperative as well as chemotherapy- and radiotherapy-induced nausea and emesis (Butcher, 1993; Currow *et al*., 1997; Gralla *et al*., 1998; Gregory and Ettinger, 1998; Hesketh, 2000; Loewen *et al*., 2000; Markham and Sorkin, 1993; Oge *et al*., 2000; Roila *et al*., 2006). The control of emetogenic side effects is of major importance for the success of cancer chemotherapy.

Oral ondansetron is well absorbed, with a bioavailability of approximately 60–70%, and it is well tolerated in both healthy volunteers and patients. Absorbed ondansetron is not highly bound to plasma proteins (70–76%) and undergoes extensive hepatic oxidative metabolism (95%) to form hydroxylated metabolites, which is mediated through multiple cytochrome P450 forms (Dixon *et al*., 1995; Pritchard, 1992).

In addition to the radioimmunoassay (RIA) (Wring *et al*., 1994) and chiral capillary electrophoresis (CE) (Siluveru and Stewart, 1997) methods for ondansetron quantification in humam plasma, the specialized literature also contains chromatographic techniques including achiral high-performance liquid chromatography (HPLC) (Bauer *et al*., 2002; Colthup *et al*., 1991; Colthup and Palmer, 1989; Depot *et al*., 1997) and chiral HPLC (Kelly *et al*., 1993; Liu *et al*., 1997). Normally, the HPLC methods used solid-phase extraction (SPE) of ondansetron from biological samples along

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Abbreviations used: 5-HT3, 5-hydroxytryptamine type 3; ANVISA, Brazilian National Sanitary Surveillance Agency; FDA, US Food and Drug Administration; MTBE, methyl tert-butyl ether.

with traditional silica-based columns and UV detection. In spite of using expensive solid-phase procedures for analyte separation, the validated limits of quantification were in the range 10–15 ng/mL.

More recently, some methods were based on HPLC coupled to electrospray ionization tandem mass spectrometric (HPLC–ESI–MS/MS) to determine ondansetron in human plasma (Armando *et al*., 2009; Dotsikas *et al*., 2006; Liu *et al*., 2008; Xu *et al*., 2000). Although a very low concentration range was reached, the run time still lasted several minutes in some of these methods or the plasma volume used for drug extraction was large. The aim of this work was to develop and validate a novel, fast and sensitive HPLC–ESI-MS/MS method for the determination of ondansetron in a small volume of human plasma applying ondansetron-D3 as the internal standard. This method was applied as a bioanalytical tool to assess the relative bioavailability of two ondansetron tablet formulations in healthy volunteers.

Experimental

Chemicals and Reagents

Ondansetron hydrochloride reference standard was obtained from United States Pharmacopeia (Rockville, MD, USA). Ondansetron-D3 was obtained from Synfine Research (Richmond Hill, ON, Canada). Acetonitrile, methanol (HPLC grade) and methyl tert-butyl ether (MTBE) were purchased from J.T. Baker (São Paulo, Brazil). Ammonium formate was obtained from Fluka-Sigma-Aldrich (São Paulo Brazil). Ultrapure water was obtained from a Milli-Q system (Millipore, Sao Paulo, Brazil). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant EDTA (BD Vacutainer®, BD, Franklin Lakes, NJ USA). Blank pooled plasma was prepared and stored at –20°C until needed.

Calibration Standards and Quality Control

All sample analysis was carried out in a GLP-compliant manner and in accordance with the current Brazilian National Sanitary Surveillance Agency (ANVISA) requirements and the US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance.

Stock solutions of ondansetron and ondansetron-d3 were weighed and dissolved in pure methanol to reach the final concentration of 1.0 mg/mL. Ondansetron working solutions were prepared by serial dilutions of the stock solutions in methanol–water (50:50, v/v) to obtain the final concentrations of 2, 5, 20, 50, 100, 200, 400 and 600 ng/mL. Ondansetron-d3 working solution was prepared by dilution of the stock solution in pure water to obtain the final concentration of 30 ng/mL. Both stock and working solutions were stored at 4°C until use. Calibration curves for ondansetron were prepared by spiking blank plasma with working solutions to obtain a ten times dilution giving the final concentrations of 0.2, 0.5, 2.0, 5.0, 10.0, 20.0, 40.0 and 60.0 ng/mL. Analyses were carried out in duplicate for each concentration. Quality control samples were prepared in blank plasma at concentrations of 0.2 (lower limit of quantification, LLOQ), 0.6 (quality control at low level, QCL), 30.0 (quality control at medium level, QCM) and 50.0 ng/mL (quality control at high level, QCH). The spiked plasma samples (standards and quality controls) were extracted in each batch of sample analysis.

Sample Preparation

Plasma samples were thawed at room temperature. A 50 µL portion of human plasma was introduced into a glass tube followed by 25 µL of the ondansetron-d3 working solution and 1.0 mL of MTBE. After vortex mixing for 20 s, samples were centrifuged at 2000***g*** for 3 min at 4°C and the organic phase was transferred to another set of clean glass tubes and evaporated to dryness under N_2 at 50°C. The dry residues were dissolved in 0.50 mL of acetonitrile–water (50:50, v/v), vortex mixed for 10 s to reconstitute the residue and transferred to 96-well plates.

Chromatographic Conditions

After extraction, samples were injected (10 µL) into a Agilent Zorbax Eclipse® C_{18}, 5 µm (12.5 × 4.5 mm i.d.) guard-column (Agilent, Santa Clara, CA, USA) followed by Phenomenex Gemini® (Torrance, California, USA) C_{18}, 5 µm analytical column (75 × 4.6 mm i.d.) operating at room temperature. The mobile phase was acetonitrile–water (50:50, v/v) with 0.25 mM ammonium formate at a flow-rate of 0.80 mL/min. Under these conditions, typical standard retention times were 2.0 ± 0.4 min for both ondansetron and ondansetron-D3. The autosampler was maintained at 4°C and the total run time was 3.0 min.

Mass Spectrometry Conditions

An LC/MS/MS system consisted of a liquid chromatography system (model 1200, Agilent, Santa Clara, CA, USA) coupled to an electrospray tandem triple quadrupole mass spectrometer (model 6410, Agilent, Santa Clara, CA, USA) equipped with electrospray ionization (ESI) source operating at the positive ion mode (ES^+) was used. Multiple reaction monitoring (MRM) mode was used for detection and mass analysis. The tuning parameters were optimized for ondansetron and ondansetron-D3. The electrospray capillary potential was set to 4000 eV and Nitrogen was used as drying gas for solvent evaporation (5 L/min). The vaporizer and drying gas temperatures were kept at 200 and 325°C, respectively. The dwell time was 200 ms and the collision energy was set to 30 eV for both compounds. Based on the full-scan MS/MS spectrum of each drug, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions, as follows: *m/z* 294.2 → 170.0 and *m/z* 294.2 → 184.0 for ondansetron (Fig. 1a), *m/z* 297.2 → 173.2 and *m/z* 297.2 → 187.0 for ondansetron-D3 (Fig. 1b). The fragmentation route was proposed (Fig. 2). Data acquisition and analysis were performed using the MassHunter B01.03 software.

Linearity

The standard calibration curves were constructed using the peak area ratios of ondansetron and IS vs ondansetron-D3 nominal concentrations of the eight plasma standards (0.2, 0.5, 2.0, 5.0, 10.0, 20.0, 40.0 and 60.0 ng/mL) in duplicate. Linear regression analysis, with weighting factor of $1/x^2$, was performed to assess the linearity, as well as to generate the standard calibration equation: $y = ax + b$, where y is the peak–area ratio, x the concentration, a the slope and b is the intercept of the regression line. In addition, a blank (non-spiked sample) and a zero plasma sample (only spiked with IS) were run to demonstrate the absence of interferences.

Ion Suppression

A procedure to assess the effect of ion suppression on MS/MS was performed using a continuous infusion of compound into the MS detector protocol (Hsieh *et al*., 2001; Muller *et al*., 2002). The experimental set-up consisted of an infusion pump connected to the system by a 'zero volume tee' before the spliter and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of ondansetron. The infusion pump was set to transfer a mixture of analyte and IS diluted in mobile phase to the connecting tube between the HPLC column and the mass spectrometer ion source. The concentrations of the analyte and IS mixture were selected in order to achieve at least 5 times the baseline. The reconstituted extract from blank plasma was injected into the HPLC system while the standard mixture was being infused. In this system, any ion suppression would be observed as a depression of the MS signal.

Recovery

The extraction recovery of ondansetron and IS were assessed by comparing peak area ratios obtained from extracted plasma samples with post-extracted spiked samples (matrix extracted standard solution) at the

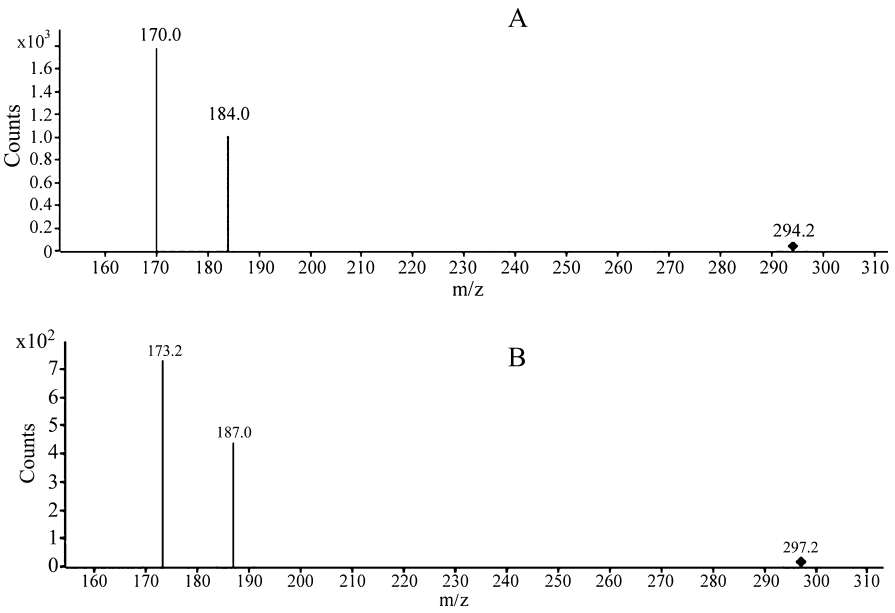

Figure 1. Precursor and product ion mass spectra of ondansetron (A) and ondansetron-D3 (B).

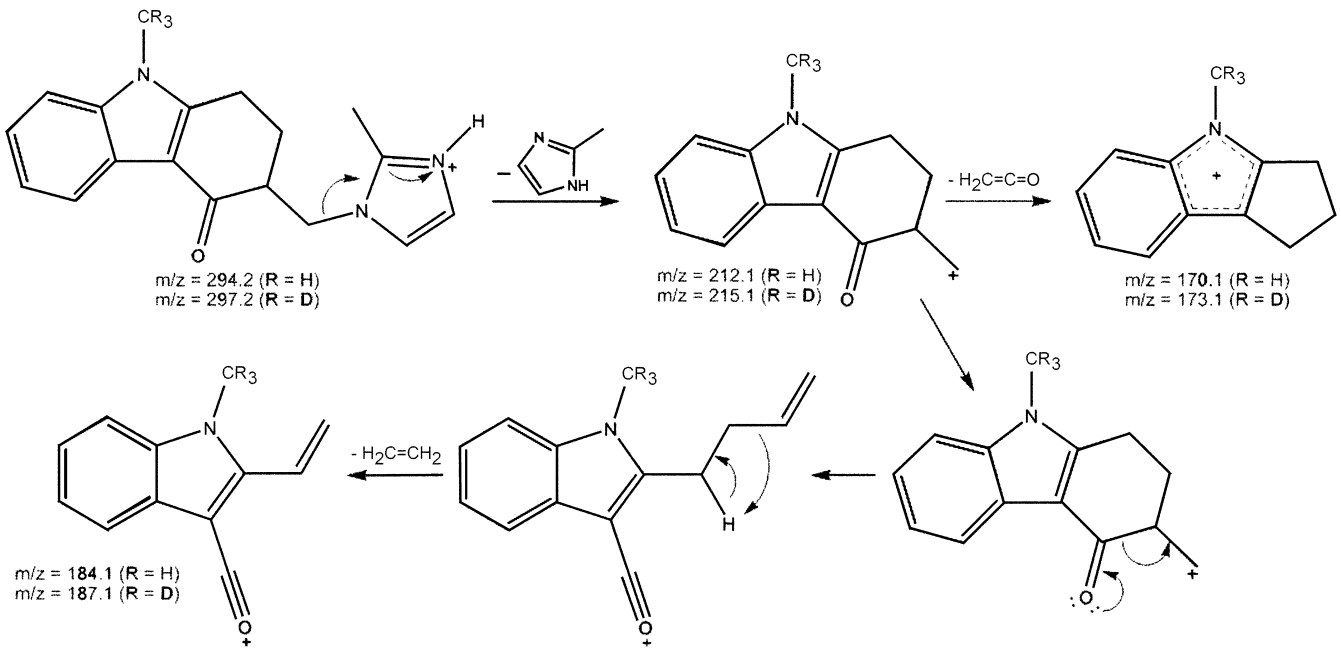

Figure 2. The fragmentation route for ondansetron and ondansetron-D3. The fragmentation route was proposed based on the transitions *m/z* 294.2 → 170.0 and *m/z* 294.2 → 184.0 monitored for ondansetron and transitions *m/z* 297.2 → 173.2 and 297.2 → 187.0 monitored for ondansetron-D3.

same concentration at each QC level (relative recovery). This procedure was performed using five aliquots from three different sources of human plasma at QCL (0.6 ng/mL), QCM (30 ng/mL) and QCH (50 ng/mL).

Precision and Accuracy

During the validation process, the precision and accuracy of the method were evaluated using three different batches of QCL, QCM and QCH samples of ondansetron. In addition, the intra-batch precision and accuracy were also calculated at the LLOQ concentration. For intra-batch assay precision and accuracy, six replicates of mentioned QC samples were assayed all at once within a day to obtain the CV(%) and accuracy values. The inter-batch assay precision and accuracy were determined by analyzing mean values of quality control samples from three plasma batches, yielding the corresponding inter-batches CV(%) and accuracy values.

Sensitivity

The LLOQ was determined for ondansetron, based on two criteria: (a) the analyte response at this concentration had to be at least five times baseline noise and (b) the analyte response at LLOQ could be determined with sufficient precision and accuracy, i.e. precision of 20% and accuracy of 80–120%. Calculations were based on eight replicates of three blank plasma batches.

Stability

Stability of ondansetron was assessed in five replicates of plasma spiked with ondansetron at the QCL (0.6 ng/mL) and the QCH (50 ng/mL) subjected to different conditions. Analysis of ondansetron concentrations were compared to fresh samples not subjected to the assay and expressed in percentage of degradation.

Initially, stability was evaluated after three freeze–thaw cycles of −20°C. In each cycle, frozen samples were allowed to thaw at controlled ambient temperature (22°C) and were subsequently refrozen for 24 h. Aliquots of all samples were quantified at the end of the third freeze–thaw cycle.

The post-processing stability was assessed for a 48 h period. Plasma samples spiked with QCs concentration were subjected to processing and stored after liquid–liquid extraction at room temperature prior to analyze by HPLC-MS/MS.

To evaluate the short term stability samples were initially thawed at room temperature (22°C) and remained on the bench top for a time exceeding the maximum period of time expected for routine sample preparation (7 h). Long-term stability was assessed over an 81 day period. In this assay, samples were subjected to frozen storage (−20°C) during the entire period covered by the bioequivalence study, i.e. from the first day of volunteer sample collection up to the last day of sample analysis. Storage stability was defined by comparing sample concentration to the mean values obtained during the first-day analysis.

Ondansetron stock and work solutions were prepared as described and stored at 25°C and five replicates were evaluated after 7 h. The stock and work solutions stability was also evaluated in samples stored at −20°C for 35 days.

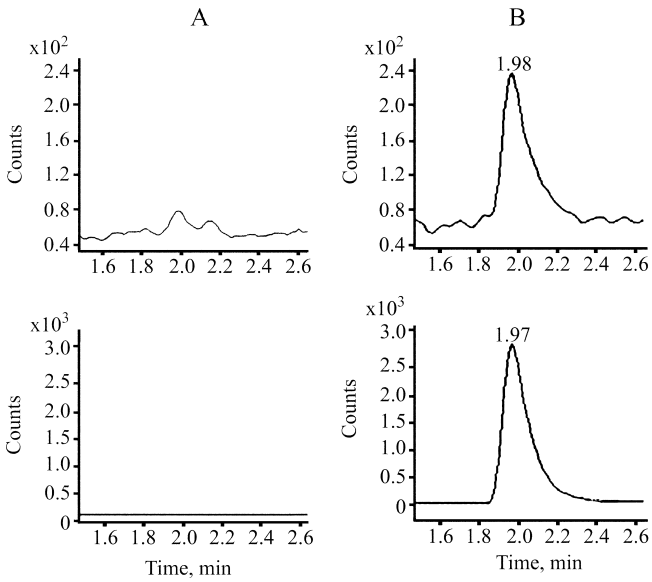

Figure 3. MRM chromatograms of (A) blank normal human plasma and (B) ondansetron at LOQ concentration (0.2 ng/mL) in normal plasma. The transitions *m/z* 294.2 → 170.0 and *m/z* 294.2 → 184.0 were monitored for ondansetron (upper panels) and transitions *m/z* 297.2 → 173.2 and 297.2 → 187.0 for ondansetron-D3 (lower panels).

Pharmacokinetic study. The analytical method developed here was applied to evaluate comparatively the ondansetron plasma concentration from two tablet formulations of ondansetron (8 mg) in healthy volunteers: Zofran® (lot no. R279435V, reference formulation from Glaxosmithkline, Rio de Janeiro, RJ, Brazil), and Ondansetrona (lot no. 07118501, test formulation from Cristália Produtos Químicos e Farmacêuticos Ltda, Itapira, SP, Brazil).

Twenty five healthy volunteers (12 men and 13 women) aged between 18 and 50 years and with body mass indices within 18.5–29.9 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, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, uric acid, hemoglobin, hematocrit, total and differential white cell counts, routine urinalysis and pregnancy test βHCG. All subjects were negative for HIV, HCV and HBV. All subjects gave written informed consent and the study was conducted in accordance with the revised Declaration of Helsinki, the rules of Good Clinical Practice (ICH-GCP) and resolution nos196/96 and 251/97 of National Health Council—Health Ministry, Brazil. The clinical protocol was approved by the Research Ethics Committee of University of Campinas, Unicamp, São Paulo, Brazil.

The volunteers had the following clinical characteristics expressed as mean ± SD (range): age 28.9 ± 7.0 years (18–44), height 169.0 ± 0.1 cm (153.0–189.0), body weight 63.9 ± 8.6 kg (48.0–82.0). The study was a single-dose, two-way randomized crossover design with a 7 day washout period between doses. The volunteers entered the Clinical Pharmacology Unit 10 h before drug administration and left the Unit 14 h after sampling. After time 0 sampling, each volunteer received a single dose of ondansetron (8 mg of either tablet formulation) with 200 mL of water. The volunteers were then fasted for 4 h, after which period a standard lunch was served. 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 soft drinks). The subjects were monitored throughout the study and the formulations were considered to be well tolerated. Blood samples were collected by in-dwelling catheter into EDTA containing tubes before dosing and 10, 20, 40, 50 min and also 1, 1.33, 1.67, 2, 2.5, 3, 4, 6, 8, 10, 12 and 24 h post-dosing. The blood samples were centrifuged at 2000***g*** for 10 min at room temperature and the plasma separated and stored in a polypropylene cryogenic screw-capped tubes at −20°C until analyzed for ondansetron content.

Statistical Analysis

Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under the curve (AUC) of plasma concentration until the last concentration observed (AUC_{last}) and the area under the curve between the first sample (pre-dosage) and the area under the curve extrapolated to infinity (AUC_{0-inf}). C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the ondansetron plasma concentration vs time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule.

The elimination rate constant (k_{el}) was obtained as the slope of the linear regression of the log-transformed concentration values vs. time data in the terminal phase. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$. The AUC0–inf was calculated as $AUC_{last} + C_t/k_{el}$, where C_t was the last measurable concentration.

Statistical calculations were defined at the level of $p \leq 0.10$ and bioequivalence was reached when the 90.0% confidence interval for C_{max}, AUC_{last} and AUC_{0-inf} felt within the range of 80.0–125.0% defined by both the FDA and the ANVISA. The software used included Equivtest® 2.0, MS Excel® 97, Tinn-R1.1, Win-Edit® 2.0 and Scientific Work Place® 5.0.

Results

Linearity and Specificity

The simplest regression method for the calibration curves of the ondansetron was $Y = a + bx$ from 0.2 to 60 ng/mL. Correlation coefficient ranged from 0.9918 to 0.9988. The chromatograms obtained from LLOQ (0.2 ng/mL) and extracted blank plasma are presented in Fig. 3. The ondansetron and IS retention times were both 2.0 ± 0.04 min and the signal-to-noise ratio was higher than 7. In the case of ondansetron and its IS, there was no significant

Table 1. Accuracy and precision data for ondansetron quantification in human plasma. Results were obtained during the validation of QC samples, including the LLOQ in human plasma

QC samples	Nominal concentration (ng/mL)	Intra-run accuracy[a]	Inter-run accuracy[b]	Intra-run precision[c] (%CV)	Inter-run precision[b] (%CV)
LLOQ	0.2	105.6	—	7.0	—
QCL	0.6	97.5	97.3	7.7	5.1
QCM	30	108.2	106.6	1.6	2.1
QCH	50	107.9	107.0	4.5	3.3

[a] Expressed as (found concentration/nominal concentration) × 100, $n = 6$.
[b] Values obtained from all three runs ($n = 18$).
[c] $n = 6$.

Table 2. Stability tests of ondansetron in human plasma

Initial mean concentration (ng/mL)		%CV	Final mean concentration (ng/mL)	% CV	Variation (%)
Freeze–thaw stability test (three cycles)					
QCL	0.58	5.0	0.57	6.1	–1.7
QCH	53.3	2.6	48.4	4.7	–9.2
Short-term stability test (7 h)					
QCL	0.58	5.0	0.60	13.5	2.4
QCH	53.3	2.6	48.5	2.0	–9.0
Post-processing stability test (48 h)					
QCL	0.58	5.0	0.58	7.7	0.0
QCH	53.3	2.6	54.7	2.0	2.6
Long-term stability test (81 days)					
QCL	0.59	4.2	0.61	2.9	3.3
QCH	49.6	4.6	53.6	4.6	8.3

$n = 5$ for each test. QCL = ng/mL; QCH = 50 ng/mL.

ion suppression in the region where the analyte and internal standard were eluted. In addition, there was no interfering peak when the analysis was performed using two other batches of hyperlipemic and hemolyzed plasma.

Recovery

Ondansetron and the IS showed recoveries [values ± CV (%), $n = 5$] for QCL, QCM and QCH as follows: 88.8 ± 5.7, 86.4 ± 6.8 and 91.8 ± 5.7%, respectively. The recovery of the IS was 83.0 ± 1.4%.

Accuracy and Precision

Intra-batch precision and accuracy of the assay was measured for ondansetron at each QC level and are presented in Table 1. The intra-assay precisions ranged from 1.6 to 7.7%, while inter-assay precisions ranged from 2.1 to 5.1%. The intra-assay accuracies ranged from 97.5 to 108.2%, while the inter-assay accuracies ranged from 97.3 to 107.0%. These results were within the acceptance criteria for precision and accuracy, i.e. deviation values were within ±15% of the nominal values, except for LLOQ, which could show a ±20% deviation.

Stability

As indicated by the stability test, there was no significant degradation of the stock solution after 7 h at room temperature. In this condition, the variation between fresh and stored samples was –9.0%. In addition, after 35 days at –20°C, the variation between fresh and stored samples was 6.9%.

The stability of ondansetron was assessed in human plasma and demonstrated no significant degradation after 7 h at room temperature, three freeze–thaw cycles, 48 h post-processing or 81 days at –20°C (Table 2).

Comparative Pharmacokinetic Study

Ondansetron was well tolerated at the administered doses and no significant adverse reactions were observed or reported. No clinically relevant change was observed in any measured biochemical parameter. A total of 25 volunteers finished the study (12 men and 13 women). The mean ondansetron plasma concentration vs time curves obtained after a single oral dose of each formulation is shown in Fig. 4. The plasma concentration of ondansetron did not differ significantly after administration of both formulations (test formulation and the reference one).

Table 3 shows the values of the pharmacokinetic parameters and Table 4 summarizes the bioequivalence analysis for ondansetron formulations. Briefly, the geometric mean and respective 90% CI of ondansetron test/reference percent ratios were 90.15% (81.74–99.44%) for C_{max} and 93.11% (83.01–104.43%) for AUC_{0-t}.

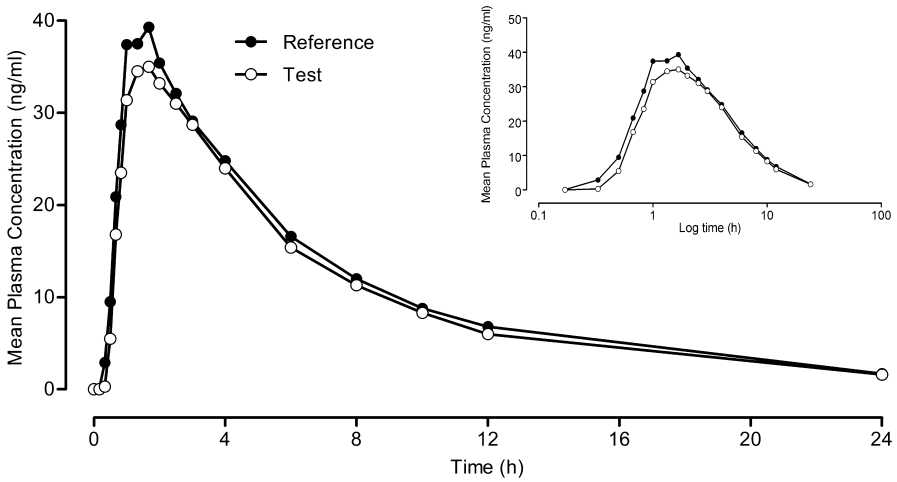

Figure 4. Ondansetron plasma mean concentrations versus time profiles obtained after the single oral administration of 8 mg of test and reference ondansetron formulations. The inner frame shows the semi-log representation of the data.

Table 3. Arithmetic mean pharmacokinetic parameters obtained from 25 volunteers after administration of each 8 mg ondansetron tablet formulation

	Test formulation		Reference formulation	
	Mean	SD	Mean	SD
C_{max} (ng/mL)	42.30	16.99	47.55	22.73
T_{max} (h)	1.51	0.62	1.30	0.46
$t_{1/2}$ (h)	5.66	0.96	5.79	1.68
AUC_{0-t} [(ng h)/mL]	247.14	113.82	266.88	133.23
AUC_{inf} [(ng h)/mL]	261.11	124.25	283.12	143.39

Table 4. Geometric mean of the individual AUC_{last}, AUC_{0-inf} and C_{max} ratios (test/reference formulation) and the respective 90% CIs

Parameters		Parametric (n = 25)		
	Geometric mean (%)	90% CI	Power (%)	CV (%)
AUC_{last} (% ratio)	93.11	83.02–104.43	99	23.99
AUC_{inf} (% ratio)	92.16	82.25–103.26	99	23.78
C_{max} (% ratio)	90.15	81.74–99.44	98	20.42

Discussion

The LC-MS/MS method described here for drug quantification is in accordance with both FDA and the ANVISA requirements for pharmacokinetic studies. This method includes a simple liquid–liquid extraction providing a clean extracted sample and a reproducible quantification allied to the high selectivity of the MRM mode on LC–ESI-MS/MS spectrometer. Blank plasma samples from all 25 volunteers showed a clear chromatogram with no significant peak at the analite retention time in all cases.

This method offers advantages over those previously reported using LC-MS/MS (Armando *et al*., 2009; Dotsikas *et al*., 2006; Liu *et al*., 2008; Xu *et al*., 2000), showing a low validated LLOQ (0.2 ng/mL) associated with a faster chromatographic run time (2.5 min) and very low plasma volume (50 µL) for ondansetron extraction. The sample preparation makes use of only a simple liquid–liquid extraction and this simple and reproducible protocol was enough to provide a reliable and effective cleanup.

In comparison to the previously LC-MS/MS published methods, our LLOQ (0.2 ng/mL) is significantly lower than the 1.0 ng/mL described by Xu *et al*. (2000) and the 2.0 ng/mL described by Armando *et al*. (2009). In addition, these two methods describe a much longer chromatographic run of 20 and 6 min, respectively. One additional advantage of our method is the significantly lower volume of human plasma used for the ondansetron extraction. While our method needed only 50 µL, others used from 200 µL to 1.0 mL (Armando *et al*., 2009; Dotsikas *et al*., 2006; Liu *et al*., 2008; Xu *et al*., 2000). Since a large number of blood samples are needed for a pharmacokinetics study in humans in each confinement period (17 in our study), the plasma volume used during analyte extraction became a critical parameter to be considered in order to reduce the amount of blood collected and the risks to volunteers during these confinement periods.

Some chromatographic techniques have been published for the determination of ondansetron in human plasma. Several

chiral methods have been developed for the determination of *R*(–) and *S*(+)ondansetron, using HPLC coupled to UV detection (Kelly *et al*., 1993; Liu and Stewart, 1997) or high-performance capillary electrophoresis with heptakis-(2,6-di-*O*-methyl)-*β*-cyclodextrin as a mobile phase modifier (Siluveru and Stewart, 1997). These methods showed poor sensitivity with a LLOQ that ranged from 10 to 15 ng/mL, and were not suitable for the measurement of ondansetron levels in pharmacokinetic studies. Colthup *et al*. (1989, 1991) described a laborious and expensive method for ondansetron quantification applying solid-phase extraction. However, the working range of 1–20 mg/mL was not appropriate for bioequivalence studies.

Concerning the extraction, an expensive solid-phase procedure has been used by some authors (Liu and Stewart, 1997; Xu *et al*., 2000), while others used liquid–liquid extraction with large volumes of solvent (Bauer *et al*., 2002; Chandrasekar *et al*., 2004; Depot *et al*., 1997). One exception is the method described by Dotsikas *et al*. (2006) that reported ondansetron extraction with a smaller volume (600 μL) of solvent, using the semi-automated extraction process.

Despite the lower LLOQ (0.1 ng/mL) described by Liu *et al*. (2008), the long chromatographic run of 12 min and the 200 μL of plasma needed for ondansetron extraction make the analytical method less attractive for bioequivalence studies. In addition, the method described by Dotsikas *et al*. (2006) describes a slightly higher LOQ (0.25 ng/mL) and a faster chromatographic run (2 min). However, these authors used a plasma volume 5 times higher. Probably, the performance of that method would be seriously compromised if only 50 μL plasma samples were used instead of the 250 μL originally described.

Since our method provides excellent analytical performance for ondansetron extraction and proved to be appropriate for analyzing human plasma samples, it has been successfully applied to human pharmacokinetic investigations of two ondansetron formulations. The tolerability of all preparations was excellent as indicated by the absence of any significant side effect. The respective 90% confidence intervals of the ratios of geometric means of C_{max}, AUC_{0-t} and AUC_{inf} values of dexchlorpheniramine absorbed from both test and reference formulations were included in the 80–125% interval as proposed by the FDA and the ANVISA.

Conclusion

This work describes a fast, sensitive and robust method to quantify ondansetron in human plasma using ondansetron-D3 as the internal standard. Extracted samples were analyzed by high-performance liquid chromatography coupled to ESI^+ tandem mass spectrometry. This method agrees with the requirements proposed by the FDA of high sensitivity, specificity and high sample throughput in comparative pharmacokinetic assays such as bioequivalence studies. The lowest concentration quantified was 0.2 ng/mL with appropriate accuracy and precision. The described method for ondansetron quantification in human plasma was successfully applied in a bioequivalence study of two ondansetron 8.0 mg tablet formulations using an open, randomized, two-period crossover design. Since the 90% CI for C_{max} and AUC ratios were all inside the 80–125% interval, it was concluded that the test formulation of ondansetron is bioequivalent to the reference formulation with respect to both the rate and the extent of absorption.

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