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Development and validation of a rapid 96-well format based liquid–liquid extraction and liquid chromatography–tandem mass spectrometry analysis method for ondansetron in human plasma

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Abstract

A rapid and sensitive LC–MS/MS method for the quantification of ondansetron was developed and validated. The plasma samples were treated by a semi-automated liquid–liquid extraction (LLE) in 1.2 mL 96-well format micro-tubes. Ondansetron and the internal standard (IS) granisetron were analyzed by combined reversed phase LC–MS/MS, with positive ion electrospray ionization, using multiple reactions monitoring (MRM). The statistical evaluation for this method reveals excellent linearity, accuracy and precision values for the range of concentrations 0.25–40.0 ng/mL. The proposed method enabled the reliable determination of ondansetron in bioequivalence studies after per os administration of a 4 or 8 mg tablet.

Keywords: Ondansetron; Liquid chromatography–tandem mass spectrometry; 96-well plates; Pharmacokinetic

1. 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 5-hydroxytryptamine type 3 (5-HT_3) receptor antagonist used in the treatment of chemotherapy- and radiotherapy-induced nausea and emesis. So far, there are few published chromatographic techniques for the determination of ondansetron in human plasma [1–4]. All of these used solid phase extraction protocols with multiple steps for the extraction of ondansetron from biological samples along with conventional columns and UV detection. Subsequently, big run times, complicated chromatograms and inadequate, in terms of sensitivity, working concentration ranges characterize the previous methods. Other researchers developed two liquid chromatographic/electrospray ionization mass spectrometric LC–ESI–MS methods [5,6] to determine ondansetron and its hydroxyl metabolites. Although the low ng/mL range for ondansetron was reached, the run time of these methods still lasted several min.

In the present study, a novel, ultra-fast, semi-automated 96-well LLE, LC–MS/MS method for the determination of ondansetron in human plasma is presented, based on a protocol we had earlier developed [7].

2. Experimental

2.1. Chemicals and reagents

Ondansetron and granisetron were donated from Pharmathen S.A. (Athens, Greece). Ethyl acetate and methanol were obtained from Sigma–Aldrich (Athens, Greece) and were of HPLC grade. Formic acid, sodium carbonate and ammonium acetate were of analytical grade and were also bought from Sigma. All aqueous solutions and buffers were prepared using water de-ionized and doubly distilled (Resistivity > 18 MΩ cm) from a Millipore Milli-Q Plus System, Malva (Athens, Greece). All plasma samples for method validation were prepared with plasma purchased from Scandibodies (France).

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2.2. Instrumentation

Biological samples and other solutions were transferred using a pipette from Labsystems (Viodynamiki, Athens, Greece) into 1.2 mL 96-well format micro-tubes plates obtained from Abgene (Epsom, UK). The organic solvent for LLE was dispensed into the microtubes and removed from them via a Tomtec Quadra 96 robotic liquid handling system (Bidservice, NJ, USA). An Eppedorf 5810 R (Bacakos, Athens, Greece) centrifuge was also utilized during sample preparation. The supernatant organic solvent was transferred to 2.2 ml 96-well collection plates, which were purchased from Eppedorf (Bacakos, Athens, Greece). A Zymark TurboVap 96-well format plate evaporator (Malva, Athens, Greece) that applies nitrogen for solvent evaporation was also utilized. An Agilent Nitrogen Generator (Duratec, Hockenheim, Germany) that receives air from an Atlas Copco SF4 Air Compressor (Athens, Greece) supported the evaporator.

The HPLC system included a Waters Alliance HT 2795 pump (Malva, Athens, Greece) accompanied with an autosampler, a degasser and a column oven/cooler. A Micromass Quattro Micro tandem MS system (Hellamco, Athens, Greece) equipped with an electrospray ion source and operating under MassLynx 4.0 software, was used.

2.3. Chromatographic conditions

The isocratic HPLC mobile phase was composed of MeOH (A) and 20 mM ammonium acetate (B). The composition of the mobile phase was set (80% A, 20% B, v/v) via a gradient table of the MassLynx software. A flow rate of 0.7 mL/min was used for sample analysis on a YMC-Pack Cyano (Schermbeck, Germany) analytical column (50 mm × 4.0 mm i.d.). The column was maintained at room temperature (∼22 °C), whilst the autosampler temperature was 15 °C. The injection volume was 20 μL and the total run time was set for 2.0 min.

2.4. Mass spectrometric conditions

The ESI probe of the mass spectrometer operated in the positive ion mode. Tuning parameters were optimized for both analytes by infusing a solution containing 500 ng/mL of ondansetron and the IS at a flow rate of 10 μL/min via an external syringe pump (Harvard 11 plus) directly connected to the mass spectrometer. The source temperature was set at 100 °C, desolvation temperature was 300 °C, desolvation and cone gas flow were 700 and 70 L/h, respectively. Optimized cone voltage values for ondansetron was 35 V and for granisetron 45 V, respectively, while capillary voltage was set at 3.8 kV. The multiplier was set at 650 V and argon was used as the collision gas. Quantitation was performed using selected reaction monitoring (SRM) of the transitions *m/z* 294.3 → 169.9 for ondansetron and *m/z* 313.5 → 138.1 for granisetron, respectively, with a dwell time of 0.5 s per transition. The optimized collision energy of 25 eV was used for both the analyte and the IS. Data were acquired using the MassLynx software.

2.5. Preparation of standard and quality control/method validation samples

Stock solutions of ondansetron {100 μg/mL (SO_1) and 1000 ng/mL (SO_2)}and granisetron (700 ng/mL) were prepared by dissolving each of the accurately weighed reference compound in MeOH/H_2O 50/50 (v/v) for both analytes. Working solutions of 400, 200, 100, 40, 20, 10, 5 and 2.5 ng/mL for ondansetron were prepared by diluting SO_1 and SO_2. Two quality control-method validation (QC-MV) stock solutions (100 μg/mL and 1000 ng/mL, respectively) were prepared from a separate weighing of ondansetron. Dilutions were used to prepare four levels of QC working solutions, 300, 50, 7.5 and 2.5 ng/mL, which were stored at 4 °C.

Calibration standards, QC and MV samples were prepared in the same biological matrix (human plasma) as the samples to be analyzed. Calibration standards were prepared by diluting 10 times with human plasma the working solutions of ondansetron, obtaining final standard concentrations of 40, 20, 10, 4, 2, 1, 0.5 and 0.25 ng/mL. The following concentration levels of QC/MV samples were prepared: MV_L (0.25 ng/mL), MV_1/QC_1 (0.75 ng/mL), MV_2/QC_2 (5 ng/mL) and MV_3/QC_3 (30 ng/mL)

2.6. Sample extraction and preparation

Fifty microliters of the IS working solution and 125 μL of Carbonate buffer (Na_2CO_3, 1M) were added into the appropriate tubes of a 96-well plate rack. Next, 250 μL of each of the calibration, QC/MV sample were added and the samples were vortex-mixed for 10 min. Then, 600 μL ethyl acetate were added into all tubes. The samples were vortex-mixed for 20 min, centrifuged for 10 min at 3500 rpm and 4 °C and were frozen for 60 min at −30 °C. 400 μL of the supernatant organic solvent were transferred into the respective positions of a 96-deepwell plate. After evaporation by using a flow of nitrogen at 35 °C the residue was dissolved with 150 μL of reconstitution solution (50% 0.1% Formic acid in MeOH, 50% water). After vortex-mixing for 5 min the 96-well plate was transferred into the autosampler for injection.

3. Results and discussion

The liquid–liquid extraction in a 96-well format rack was a relatively fast and simple technique that allowed preparation of a big amount of samples daily The selected solvent provided a very satisfactory transfer from the plasma sample into the organic layer. Freezing, enabled an easier removal of the organic mixture, while reduced the possibility of transferring plasma sample elements.

Along with the extraction procedure, the LC–MS/MS system was used to separate and monitor ondansetron and the IS from the extracted samples. The MS spectra for both molecules are dominated by the $[M+H]^+$ ions [6,8]: *m/z* 294.3 for ondansetron and *m/z* 313.5 for granisetron, while the product ion spectra of the protonated molecules produced major product ions at *m/z* 169.9 and 138.1, respectively. Representative SRM LC–MS/MS chromatograms are shown in Fig. 1. As shown, the retention times

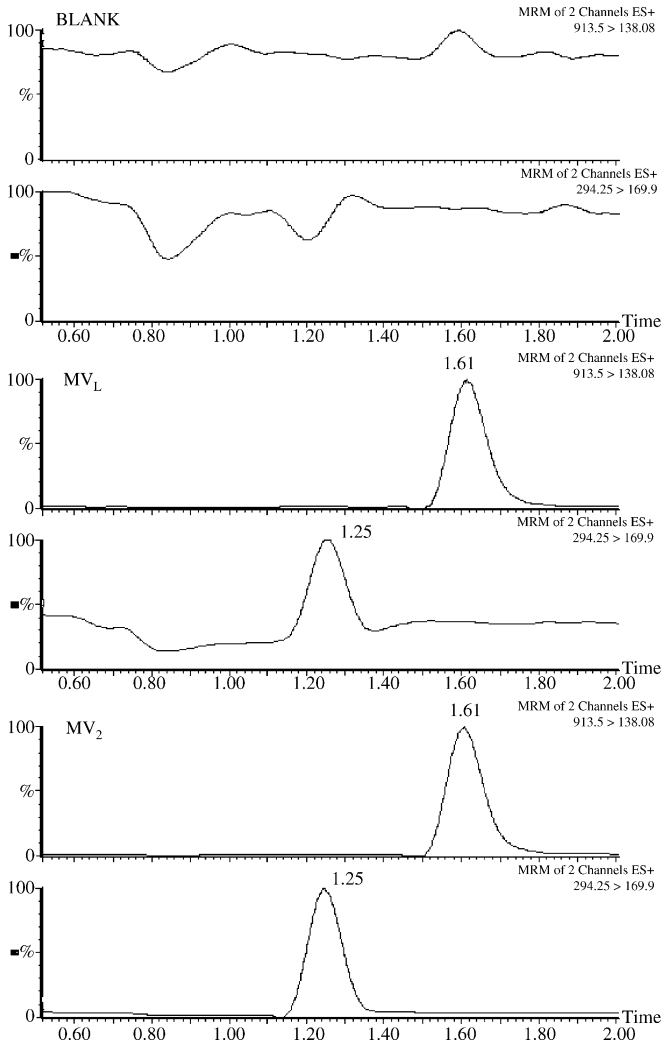

Fig. 1. Representative SRM chromatograms of onansetron and granisetron (top) obtained from a Blank, MV_L and MV_2 sample, respectively.

of ondansetron and the IS were 1.25 and 1.63 min, respectively. The first half min of the total run time the recorder was set off.

3.1. Standard curves

A full validation was performed by our GLP-compliant laboratory, according to currently presented US Food and Drug Administration (FDA) bioanalytical method validaton guidance [9].

To define the relationship between concentration and response, a calibration curve, containing 8 non-zero standards ranging from 0.25 to 40 ng/mL, for each analytical run, was prepared. This range was suitable for a pharmacokinetic study after per os administration of a 4 or even 8 mg tablet of ondansetron. The regression coefficients (*R*-squared) for the five runs were greater than 0.996, average linear slope was 0.966 ($S_a = 0.014$) and average intercept was 0.160 ($S_b = 0.225$). The experimental values of *F*-test (Mandel) were smaller than 2.985, when the (theoretical) threshold value of *F*-distribution (5%, one-sided) was 4.170. With all these results it can be concluded that the calibration curves were linear in the operating range. The result of the proportionality test was also positive; the *t*-test experimental value of 0.709 was greatly smaller than the theoretical value of 2.042 (5%, two-sided). Consequently, the 96-well liquid–liquid extraction procedure applied in this method was adequate of producing satisfactory concentration data for ondansetron standard samples.

3.2. Accuracy and precision

Precision and accuracy were assessed by analyzing MV samples in five runs on three separate days. In Table 1 the experimental values for accuracy and precision are presented. In all cases the values were within the acceptable range, certifying that the nominal concentration was, actually, observed and no systematic error was detected.

3.3. Extraction recovery

Data from five runs containing three QC samples of each type (QC_1, QC_2 and QC_3), obtained by the usual extraction process were compared with three samples for each concentration mentioned above, obtained by diluting working solutions directly in mobile phase (unextracted samples). Mean values of extraction recovery for ondansetron in QC_1, QC_2 and QC_3 were 49.4, 43.7 and 41.2, respectively, while mean granisetron recovery was estimated as 41.5%. These values were relatively small, but consistent and adequate for this concentration range. These values are absolutely justified considering the small quantity of organic solvent added. Tests for ion suppression effect were performed (data not shown) showing that

Table 1
Summary of % accuracy and precision of ondansetron from MV plasma samples

% Intra-run accuracy		% Inter-run accuracy		Intra-run precision	Inter-run precision
MV sample (ng/mL)	% Accuracy[a]	% Accuracy[b]	Wilcoxon-test[c]	% R.S.D.[d]	% R.S.D.[b]
MV_L (0.25)	97.68	94.61	2.00	11.55	6.20
MV_1 (0.75)	92.11	92.72	1.00	5.93	5.52
MV_2 (5)	104.49	97.50	3.00	7.22	7.77
MV_3 (30)	103.99	94.23	1.00	6.25	7.38

[a] ($n = 6$), expressed as 100 × (mean calculated concentration)/(nominal concentration).
[b] Values obtained from all 5 runs ($n = 30$).
[c] Experimental values. Lower and upper theoretical values were 0.00 and 14.00, respectively (5% two-sided).
[d] ($n = 6$).

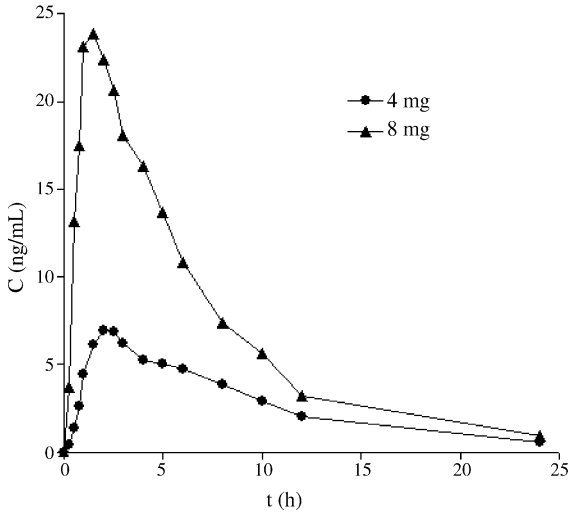

Fig. 2. Mean plasma concentrations–time curves from 24 subjects for ondansetron. Data obtained from two bioequivqlence studies (4 and 8 mg tablet, respectively).

this phenomenon had a minor effect (∼5%) on the recovery value.

3.4. Stability studies

Plasma samples containing two concentration levels of ondansetron were used for the stability experiments: Low-medium (S-l) 1 ng/mL and medium-high (S-h) 10 ng/mL. Freeze thaw stability, short term stability (6 h), autosampler stability (13 h) and long-term stability (23 days) were performed as described previously [7] and the results proved that ondansetron concentration in all samples remained intact (data not shown).

3.5. Application in pharmacokinetic studies

The present method was utilized for the analysis of plasma samples obtained from 24 healthy volunteers after the administration of a 4 mg and an 8 mg tablet of ondansetron, as part of two bioequivalence studies. The concentration–time profile of ondansetron in these volunteers in both studies is represented in Fig. 2 and it is indicative for the suitability of the current method for pharmacokinetic studies of ondansetron in human plasma.

4. Conclusions

We have presented a semi-automated method utilizing 96-well format plates including individual tubes and a high throughput LLE extraction method for the quantification of ondansetron in human plasma. The use of 96-well format tubes greatly decreased the time required for sample preparation, without any cross-contamination phenomena, and therefore hundreds of samples can be analyzed daily. Further advantages over previous methods was the quantity (only 250 μL) of human plasma used for analysis and the small run time (2.0 min). The developed method was validated over the concentration range of 0.25–40 ng/mL for ondansetron. This range is suitable for measuring ondansetron in plasma samples after per os administration of a 4 or 8 mg tablet in a pharmacokinetic or bioequivalence study.

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