ELSEVIER

Journal of Chromatography B, 693 (1997) 399–406

JOURNAL OF
CHROMATOGRAPHY B

High-resolution liquid chromatographic method using ultraviolet detection for determination of ondansetron in human plasma

Michelle Dépôt*, Suzanne Leroux, Gilles Caillé

University of Montreal, Faculty of Medicine, Department of Pharmacology, C.P. 6128, Succ. Centre-Ville, Main Building, R-401, Montreal, Quebec H3C 3J7, Canada

Received 30 September 1996; revised 30 December 1996; accepted 30 December 1996

Abstract

This paper describes a simple technique for extraction and a sensitive high-performance liquid chromatographic method for separation and quantitation of ondansetron in human plasma. The procedure involved liquid–liquid extraction of ondansetron from plasma, reversed-phase HPLC separation and ultraviolet detection at 305 nm. The internal standard method was applied for quantitation. The recovery of ondansetron was >85%. Linearity was good throughout the concentration range anticipated in human plasma from investigations in panic disorder (0.5–15 ng/ml, r^2 ranging from 0.9953 to 0.9988). This method was applied to the determination of plasma concentrations of ondansetron in humans.

Keywords: Ondansetron

1. Introduction

Developed by Glaxo Research Group in England, ondansetron is a serotonin antagonist structurally related to serotonin (Fig. 1). Since its introduction in our medical practice as an antiemetic, extensive pharmacological studies have indicated that ondansetron binds with high affinity to 5-HT_3 receptors and fails to interact with other neurotransmitter systems [1,2].

There is growing evidence from animal and clinical studies in support of a 5-HT hypothesis in panic disorder [3–9]. As an antiemetic, the usual dose is in the range of 8 to 32 mg per day whereas preliminary results from clinical investigations in panic disorder support the use of doses as low as 2 to

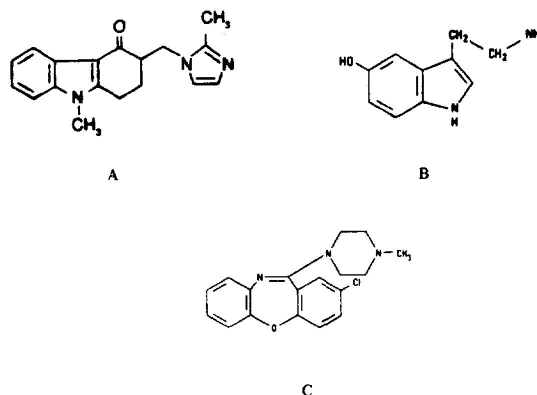

Fig. 1. Chemical structures of ondansetron (A), serotonin (B) and loxapine (C).

*Corresponding author.

0378-4347/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0378-4347(97)00068-6

4 mg per day. Consequently, the low plasma concentration range anticipated may require a very sensitive analytical method. HPLC analytical methods are available for analysis of ondansetron in human plasma [10–12]. Recently, a sensitive radioimmunoassay was developed to further enhance assay sensitivity [13]. These analytical methods all share a common extraction technique, solid-phase extraction.

The objective of this work was to develop a rapid and simple analytical method using a novel extraction technique suitable for assay of ondansetron in human plasma. This paper describes a sensitive reversed-phase HPLC assay combined with a liquid–liquid phase extraction technique for the determination of ondansetron in human plasma. Data pertaining to the specificity, stability, reliable limit of detection, recovery as well as precision and accuracy are presented herein.

2. Experimental

2.1. Materials

A pure powder sample of ondansetron free base (Lot No. GR38032X) was provided by Glaxo Research and Development (Middlesex, UK). Loxapine (Lot No. 2C0790A, Fig. 1), the acting internal standard, was supplied by Cyanamide Canada (Montreal, Canada). Acetonitrile, methanol and ethyl acetate were HPLC grade. The first two solvents were purchased from Fisher Scientific (Montreal, Canada). Ethyl acetate was purchased from Anachemia (Montreal, Canada). Sulfuric acid (H_2SO_4) was obtained from Baker Chemicals (Phillipsburg, NJ, USA). Certified buffer solution pH 9 (Lot No. SC5250211) was acquired from Fisher Scientific. All other chemicals were of analytical grade. Deionized water (Milli-Q water purification system, Millipore, Bedford, MA, USA) was used throughout the study. Control human plasma was obtained from the Canadian Red Cross.

2.2. Standard solutions

A 100 μg/ml ondansetron stock solution was prepared by dissolving 10 mg of ondansetron free base in 100 ml of methanol. Ondansetron powder was dissolved by 20-min sonication and Vortex-mixed. Dilutions to 1 μg/ml and 0.1 μg/ml of this stock solution were prepared in methanol. A 100 μg/ml internal standard (I.S.) stock solution was prepared by dissolving 10 mg of loxapine free base in 100 ml of methanol. The working I.S. solution was prepared by dissolving 600 μl of the stock solution in 100 ml of methanol. A final concentration of 0.6 μg/ml was obtained. All solutions (ondansetron and I.S.) were stored at 4±1°C.

The non-extracted ondansetron was prepared fresh by diluting the ondansetron stock solution with 0.025 *M* H_2SO_4 for final concentrations of 1 μg/ml and 0.1 μg/ml. The non-extracted I.S. was also prepared fresh by diluting the I.S. stock solution with 0.025 *M* H_2SO_4 for a final concentration of 0.6 μg/ml. Analytical aliquots were prepared with these solutions. The final volume was completed to 200 μl with 0.025 *M* H_2SO_4. All ondansetron solutions were protected from direct light as per storage instructions provided by Glaxo.

2.3. Calibrant and quality control samples

The selectivity of the assay method was established with independent sources of plasma. Plasma samples obtained from the Canadian Red Cross were screened and those tested that demonstrated no interfering peaks at the retention times of ondansetron and the I.S. were pooled to constitute the matrix for calibrant as well as quality control (QC) samples. Fig. 2 represents a chromatogram of an extracted human blank plasma.

Calibrant samples were prepared with drug free plasma that was spiked with increasing concentrations of ondansetron (0.5, 1.0, 2.5, 5.0, 10.0 and 15.0 ng/ml). Quality control samples were prepared with drug free plasma spiked with three different concentrations of ondansetron. Low, medium and high QC concentrations used were 1.5, 7.5 and 13.0 ng/ml, respectively.

2.4. Extraction procedures

Sample preparation comprised of a liquid–liquid extraction of ondansetron from control plasma samples spiked with standard samples (calibrant and

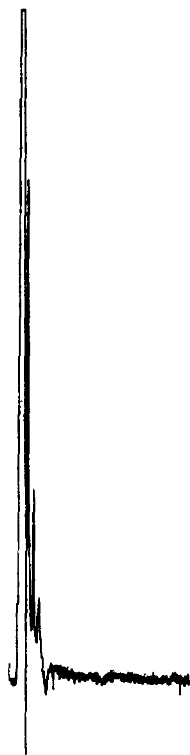

Fig. 2. Chromatogram of an extracted human blank plasma.

quality control samples). In a 17×120 mm polypropylene screw cap conical tube (15 ml) purchased from Sarstedt (Newton, NC, USA) was added 2.0 ml of plasma followed by 100 μl of a 0.6 μg/ml I.S. solution and 3.0 ml of certified buffer solution pH 9. After 15 s of vortex-mixing, 6.0 ml of ethyl acetate were added. The tubes were shaken for 15 min on an Eberbach shaker set at low speed and then centrifuged for 10 min at 1300 *g*. The organic layer was transferred to a clean 15 ml polypropylene conical tube. Thereafter, 200 μl of 0.025 *M* H_2SO_4 was added. The organic layer was discarded after vortexing the tubes for 60 s and centrifuging for 5 min at 1300 *g*. The excess of organic layer was then evaporated under a light stream of nitrogen for approximately 1 to 3 min at 45°C. A 100-μl aliquot of the aqueous phase was injected into the HPLC system. This process was carried out at room temperature, unless specified otherwise, and under reduced daylight.

2.5. Instrumentation and chromatography conditions

Plasma samples prepared according to the extraction procedures described in Section 2.4 were analysed by HPLC. The HPLC consisted of a Waters Model 501 solvent delivery system attached to a Shimadzu autosampler (Model SIL-9A), along with a Kratos Model 757 Spectroflow detector and a Shimadzu Model C-R6A Chromatopac integrator. The ultraviolet detector was operated at a wavelength of 305 nm. Ondansetron and I.S. were separated on a 10×0.46 cm Sperisorb reversed-phase column packed with 10 μm C_{18} material (ID No. 069638, Chromatographic Sciences Company, Montreal, Canada). The mobile phase consisted of acetonitrile–0.02 *M* sodium phosphate monobasic buffer ($NaH_2PO_4 \cdot H_2O$) adjusted to pH 3 with phosphoric acid 85% (60:40, v/v). The mobile phase was pumped at a flow-rate of 1.5 ml/min (pressure of 450 p.s.i. (1 p.s.i.=6894.76 Pa)). Chromatographic analysis was conducted at room temperature and protected from light.

3. Results and discussion

3.1. Optimisation of experimental conditions

Surface adhesion of ondansetron to glass represents a significant problem during the extraction process. Solid-phase extraction requires reconstitution of dry residue obtained from drying the organic phase under nitrogen. Ondansetron dry residue is also found on the sides of the extraction tubes and may hence troublesome the reconstitution. We have chosen a liquid–liquid extraction of ondansetron from plasma samples to circumvent this obstacle. Unlike ondansetron, the I.S. is a weaker base and required a more acidic solvent to ensure its solubility following extraction. Consequently, the optimum recovery of ondansetron and I.S. was achieved when the pH of the solvent was decreased from 3 to 1.8.

The mobile phase composition was optimised according to the ondansetron and I.S. retention times and the resolution of the chromatogram. We observed that the molarity of the buffer solution played a major role in the quality of the chromatogram. In

fact, lower molarity caused significant noise of the baseline. Increasing the molarity from 0.005 to 0.02 *M* dramatically improved the signal-to-noise ratio.

An ultraviolet spectrophotometer scan showed detection of ondansetron at four wavelengths: 216, 245, 268 and 305 nm. Wavelength of 216 nm produced further sensitivity improvements of a factor approaching 2.5. However, more interfering peaks emerged from the plasma. As a consequence, we selected a detection wavelength of 305 nm. The molarity and the wavelength together gave an excellent signal-to-noise ratio. Under these experimental conditions, retention times were reproducible. Ondansetron and the I.S. were eluted at 3.9 and 5.6 min, respectively.

Specificity for phase I metabolites was not assessed during this analytical work. However, ondansetron is first converted to the hydroxylated form followed by glucuronide or sulphate conjugation; *N*-demethylation is a minor metabolite route. All of these metabolites are more polar than the parent drug. Since we used a reversed-phase column for which the degree of separation depends on the polar properties of the solute molecules relative to the adsorbent, phase I metabolites will be eluted first and hence will not interfere with neither ondansetron nor loxapine.

3.2. Precision and accuracy

Between-run precision and accuracy were determined from QC samples spiked with three different concentrations of ondansetron (1.5, 7.5 and 13.0 ng/ml). A total of twelve replicates of each QC concentration were assayed on six different days. The QC concentrations were determined from six different calibration curves which were assayed with the QC samples. Precision was expressed as coefficient of variation (C.V.), while accuracy was measured as the nominal percentage of the theoretical value obtained according to the following equation:

$$\textit{Percentage of theoretical value} = (X/C_T) \cdot 100$$

where X = mean determined concentration of a quality control pool and C_T = theoretical concentration.

Results are shown in Table 1. The lower concentration (1.5 ng/ml) showed higher coefficient of variation and lower accuracy. Coefficients of variation and accuracy for all three concentrations were very good.

The within-run precision and accuracy were determined similarly to the between-run precision and accuracy. A total of six replicates of the low, medium and high QC concentrations were assayed. Their corresponding back-extrapolated concentrations were all calculated from one calibration curve covering the 0.5 to 15 ng/ml concentration range. The within-run coefficients of variation were small (less than 5%). The within-run accuracy was also good (Table 2).

This assay displayed a lower limit of quantification (LOQ) of 0.5 ng/ml (Fig. 3). Representative chromatograms of extracted ondansetron at low (1.5 ng/ml), medium (7.5 ng/ml) and high (13.0 ng/ml) QC concentrations are presented in Fig. 4.

3.3. Calibration curves

Linearity of the calibration curves was validated from 0.5–15 ng/ml and determined by weighted least squares regression analysis ($1/x^2$). Peak height ratios of ondansetron and I.S. were plotted versus their corresponding plasma concentrations. One calibration curve was assayed each day for six days ($n=6$). Calibration curves showed an average slope of 0.084 and an average *y*-intercept of 0.002. Precision and accuracy means for each concentration are

Table 1
Between-run ($n=12$) precision and accuracy for ondansetron

Theoretical concentration (ng/ml)	Observed concentration (mean (ng/ml)±S.D.)	C.V. (%)	Accuracy (%)
1.5	1.60±0.08	5.0	106.7
7.5	7.45±0.14	1.9	99.3
13.0	13.36±0.23	1.7	102.8

Table 2
Within-run ($n=6$) precision and accuracy for ondansetron

Theoretical concentration (ng/ml)	Observed concentration (mean (ng/ml)±S.D.)	C.V. (%)	Accuracy (%)
1.5	1.56±0.06	3.8	104.0
7.5	7.56±0.11	1.4	100.8
13.0	13.43±0.20	1.5	103.3

shown in Table 3. The square of the correlation coefficients (r^2) varied from 0.9953 to 0.9988.

3.4. Extraction yields of ondansetron and I.S. from human plasma

Peak heights of ondansetron and the I.S. extracted versus non-extracted equivalent concentrations of drug were compared under identical chromatographic conditions. The low (1.5 ng/ml), medium (7.5 ng/ml) and high (13.0 ng/ml) QC concentrations were used. The absolute recovery of both compounds was evaluated on two separate occasions. On the first occasion, all three QC concentrations were assayed in 6 replicates. On the second occasion, the low QC concentration was assayed in replicates of 4 while the medium and high concentrations were assayed in replicates of 3. The overall extraction yields were determined on pooled data for both compounds. The overall extraction yields of ondansetron in plasma were 94.0±14.7, 86.4±10.1 and 89.7±9.7% at 1.5, 7.5 and 13.0 ng/ml, respectively. The overall mean extraction yield of the I.S. was 81.8±7.6%.

3.5. Short term stability of ondansetron in human plasma

The short term stability of ondansetron was assessed with three replicates of stability samples

Fig. 3. Chromatogram of ondansetron at lower limit of quantification (0.5 ng/ml).

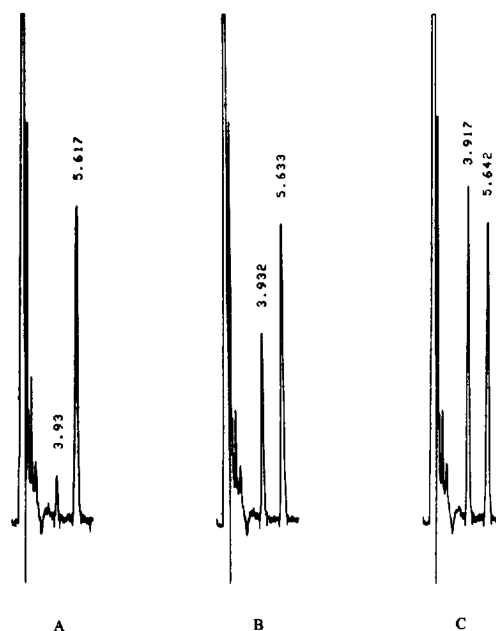

Fig. 4. Chromatograms of extracted ondansetron at low (A, 1.5 ng/ml), medium (B, 7.5 ng/ml) and high (C, 13.0 ng/ml) QC concentrations.

Table 3
Calibration curves ($n=6$) for ondansetron

Theoretical concentration (ng/ml)	Observed concentration (mean (ng/ml)±S.D.)	C.V. (%)	Accuracy (%)
0.50	0.50±0.01	2.5	100.0
1.00	0.99±0.07	7.2	99.0
2.50	2.54±0.10	3.9	102.0
5.00	5.00±0.13	2.7	100.0
10.00	9.69±0.15	1.5	96.9
15.00	15.34±0.19	1.3	102.3

which were kept at room temperature (22±4°C) for 5 h versus two replicates of freshly thawed comparison samples. All three QC concentrations, that is low (1.5 ng/ml), medium (7.5 ng/ml) and high (13.0 ng/ml), were used. The coefficients of variation observed either on the peak height ratios or on the back-calculated concentrations varied between 0.7 to 5.2%. The lower QC concentration showed the highest coefficient of variation. The results demonstrate that the short term stability of ondansetron was not compromised under this condition.

3.6. Stability of extracted ondansetron and I.S. at room temperature

Stability of extracted ondansetron and the I.S. was evaluated by leaving the extracted samples at room temperature (22±2°C) for 21–24 h. QC samples freshly extracted were immediately injected (time 0) and then re-injected 21–24 h after sitting in the autosampler at room temperature. Evaluation involved all three QC concentrations of ondansetron. The coefficients of variation determined on the mean ondansetron ratios at the three QC concentrations were all less than 5% (values ranged from 1.0 to 3.6%). The coefficients of variation observed with the mean peak height for both products at the three QC concentrations tested were less than 15% (ranging from 7.0 to 11.9%). The difference between the mean peak height of the I.S. injected at time 0 and after 21–24 h was 2.0%. Therefore, the stability of extracted ondansetron and I.S. from plasma on the autosampler was not compromised after 24 h.

3.7. Freeze–thaw stability of ondansetron in human plasma

Freeze–thaw stability involved analysis of stability samples: thawed once, twice and thrice versus replicates of comparison samples that have been freshly prepared. One low (1.5 ng/ml), one medium (7.5 ng/ml) and one high (13.0 ng/ml) QC concentrations were used. All plasma samples originated from the same batch. The inter-freeze thaw coefficients of variation determined on the peak height ratios and back-calculated concentrations of ondansetron/I.S. ranged from 8.2 to 10.9% for the low QC concentration and from 1.2 to 2.8% for the medium and high QC concentrations. The nominal percent for ondansetron varied from 100.7 to 106.7%. In conclusion, ondansetron freeze–thaw variability is observed to be small (less than 15%) up to thawing three times.

3.8. Stock solution stability of ondansetron and I.S.

Stock solution evaluation involved analysis of ondansetron stock solution freshly prepared compared to two ondansetron stock solutions stored at 4±1°C for nine weeks and eight months. We observed little variability between ondansetron stock solutions kept under the specified conditions. The coefficient of variation determined on pooled data was 1.6%. A coefficient of variation of 6.7% was measured for the I.S. stock solution kept for eight months at 4±1°C compared to the freshly prepared solution.

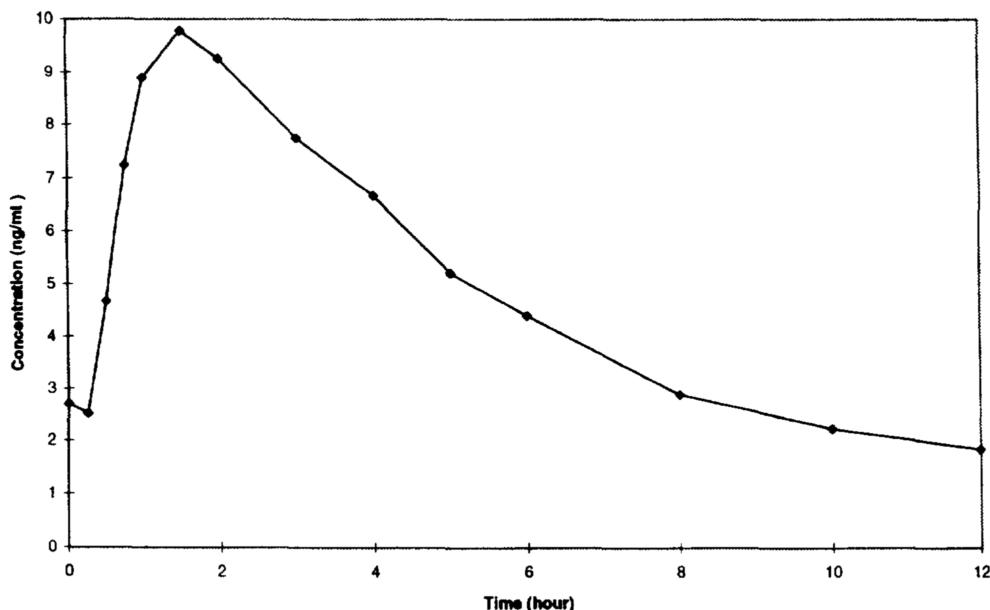

Fig. 5. Plasma concentration–time profile of ondansetron in a male healthy subject after administration of multiple oral doses of ondansetron.

3.9. Application of the method

The method was successfully applied to the determination of ondansetron plasma concentrations in five healthy male subjects. Each subject received ondansetron 2 mg twice daily during a 4-week period, for a total of 56 oral doses. The plasma concentration–time profile for one of the subjects is illustrated in Fig. 5. The pharmacokinetic characteristics derived from this profile show a mean plasma concentration of 5.4 ng/ml and a maximal concentration (C_{max}) of 9.8 ng/ml reached at 1.5 h (T_{max}). The elimination rate constant (z) was 0.1129 h^{-1} with a corresponding half-life of 6.1 h.

4. Conclusion

We described a reliable and reproducible analytical assay where the internal standard method is applied for quantitation of ondansetron. The chemical features of ondansetron complicate its extraction from plasma. The use of a liquid–liquid extraction technique resolves the problem of surface adhesion and provides a rapid way to extract many plasma samples. Results demonstrate that the analytical method described is precise and accurate. The rapid processing of numerous samples further supports the suitability of the method in pharmacokinetic studies.

Acknowledgments

We thank Glaxo Research and Development Limited, United Kingdom, for providing free base ondansetron powder which permitted completion of requirements for a doctoral degree.

References

[1] A. Butler, J.M. Hill, S.R. Ireland, C.C. Jordan and M.B. Tyers, Br. J. Pharmacol., 94 (1988) 397.
[2] R.J. Naylor and J.A. Rudd, Eur. J. Anaesthesiol., 9 (1992) 3.

[3] N.C. Tye, S.D. Iversen and A.R. Green, Neuropharmacology, 18 (1979) 689.

[4] J.T. Winslow and T.R. Insel, Neuropsychopharmacology, 3 (1990) 51.

[5] R.S. Kahn, H.G.M. Westenberg, W.M.A. Verhoeven, C.C. Gispen-de Wied and W.D.J. Kamerbeck, Int. Clin. Psychopharmacol., 2 (1987) 33.

[6] H.G.M. Westenberg and J.A. den Boer, Psychopharmacology, 98 (1989) 283.

[7] J.A. den Boer, H.G.M. Westenberg, W.D.J. Kamerbeek, W.M.A. Verhoeven and R.S. Kahn, Int. Clin. Psychopharmacol., 2 (1987) 21.

[8] S. Gloger, L. Grunhaus, D. Gladic, F. O'Ryan, L. Cohen and S. Codner, J. Clin. Psychopharmacol., 9 (1989) 28.

[9] J.A. den Boer and H.G.M. Westenberg, Int. Clin. Psychopharmacol., 3 (1988) 59.

[10] P.V. Colthup, C.C. Felgate, J.L. Palmer and N.L. Scully, J. Pharm. Sci., 80 (1991) 868.

[11] C.L. Graham, G.E. Dukes, C.-F. Kao, J.M. Bertch and L.J. Hak, Ann. Pharmacotherapy, 26 (1992) 768.

[12] I.A. Spahr-Schopfer, J. Lerman, N. Sikich, J. Palmer and U. Jorch, Clin. Pharmacol. Ther., 58 (1995) 316.

[13] S.A. Wring, R.M. Rooney, C.P. Goddard, I. Waterhouse and W.N. Jenner, J. Pharm. Biomed. Anal., 12 (1994) 361.