ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ONDANSETRON HYDROCHLORIDE USING SODIUM HEPTANESULPHONATE AS A COUNTERION

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Abstract
A simple and selective isocratic HPLC method for the determination of ondansetron hydrochloride has been developed. The separation is achieved by ion-pair reversed-phase chromatography on a Ultrasphere C8 column. A mixture of 0.3% sodium heptanesulphonate in phosphate buffer solution pH 3.0 and methanol (40/60, V/V) is used as mobile phase. The influence of pH, mobile phase composition and concentration of organic modifier on capacity factor of ondansetron has been investigated. The HPLC system is applied to the quantitative estimation of the active substance in bulk and pharmaceutical dosage forms.

Keywords: ondansetron hydrochloride assay; ion-pair chromatography

Introduction
Ondansetron, (3RS)-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl] 1,2,3,9-tetrahydro-4H-carbazol-4-one hydrochloride dihydrate (Fig. 1) is a selective 5-HT3 receptor antagonist used as an antiemetic to treat nausea and vomiting following chemotherapy [1].

It is sparingly soluble in water and in alcohol, soluble in methanol, slightly soluble in methylene chloride and has a weak basic behavior (pK_a=7.4) [1, 2]. Due to its polarity, and also to its high molecular mass, it is
rather difficult to separate ondansetron using a classical HPLC method, as we presented elsewhere [3, 4].

![Structure of ondansetron hydrochloride dihydrate](image.png)

The classic reversed-phase liquid chromatographic separation (RP-HPLC) is based upon the non polar, hydrophobic interaction between non polar sample molecules and the non polar stationary phase. If the sample molecule has sufficient hydrophobic nature, it will be retained. Retention could be adjusted by altering the aqueous-to-organic content of the mobile phase. If the sample is not polar (for example, neutral molecules) it can be retained sufficiently only if a satisfactory change of the ionization form is developed [5-8].

When the sample contains ionic components, they can be too polar to be retained in a reverse phase (RP) mode. In the past, chromatographic separation of charged analytes had been achieved by ion suppression (the careful adjustment of the mobile phase pH to result in a non ionized analyte). Determining the optimum mobile phase pH in ion suppression, however, often requires extensive method development. Samples containing more than one ionizable component were often unusable. The limitations of ion suppression led to the development of a new, more generally applicable approach to separation of ionized components: ion pair chromatography [5-8, 15].

Developed by Dr. Gordon Schill in 1973, ion-pair chromatography (IPC) relies upon the addition of a counter ion to the mobile phase in order to promote the formation of ion-pairs with charged analytes [5-12]. These reagents are usually ionic compounds that contain an alkyl chain that imparts certain hydrophobicity so that the ion-pair can be retained on a reversed-phase column. When used with common hydrophobic HPLC phases in the RP mode, they can be used to selectively increase the retention of charged analytes. A negatively charged reagent having a charge opposite to the analyte of interest, such as one of the alkyl sulphonyc acids can be used to retain positively charged ionic bases. Similarly, a positively charged reagent, such as tetrabutyl ammonium chloride, can be used to retain negatively charged ionic acids (figure 2) [11].
Figure 2
Schematic representation of RP-IPC on a bonded phase (a) attached to silica particles (b). The ion-pair reagent (c), dissolved into the mobile phase, is adsorbed to the stationary phase. The sample ions could be free, unretained (e) or retained and separated on column (f), by IPC.

For example, at a low pH value, basic samples, as our analyte ondansetron hydrochloride, are sufficiently polar to be unretained in a reversed-phase mode. After addition into the mobile phase of an ion-pair reagent with an ionic end and a non polar tail, such as hexanesulphonic acid sodium salt, a new equilibrium state is obtained. An ion-pair reagent is similar to soaps; in fact, ion pairing was called "soap chromatography" by some in its early days. The non polar end of the reagent is strongly held by the typical non polar stationary phase (for example, C8 or C18), leaving the charged functional group sticking out into the mobile phase. Now ionic species of the opposite charge can be attracted to the immobilized ion-pair reagent, providing chromatographic retention. The ion-pair process takes place in the mobile phase, resulting in a neutral ion pairing between the sample and the reagent, which is then retained as a neutral species in the reverse phase mode [11].

Taking into account that IPC is reported in literature as an established and reliable technique which improves separation providing reduced retention times, highly reproducible results and sharper peak shapes in the separation of charged analytes [5-12], the objective of this work was to develop a new method for the optimal separation of our polar compound.

Materials and methods

Liquid chromatography system and chromatographic conditions

A Thermo Electron Finnigan Surveyor LC System with diode array detection and an Ultrasphere Octyl column (Serial No. OUE 0708, Part. No. 235332), 250 x 4.6 mm, 5 μm (Beckman) were used for the separation. The pH adjustment of buffer solutions was performed using a Mettler Toledo pH Meter type 1120.

The preliminary tests were performed using a mixture of phosphate buffer pH 3.0 / methanol, in different proportions. No peak was obtained on
a C18 column, proving that the substance is not retained. After changing the C18 column with a C8 column, the substance eluted (with a retention time of 2 to 8 minutes), depending upon the ratio of solvents into the mobile phase. Because all obtained peaks have big tailings (more than 3.5), no suitable separation was obtained.

Sodium heptanesulphonate 0.3%, as an ion-pair generator agent, was added into the phosphate buffer solution pH 3.0. The addition had a positive effect as far as the retention time was increased and the peak tailing decreased. Changing of pH value or reagent concentration has no remarkable effect neither on retention time, nor on peak shape (tailing). After optimization (ratio of the solvents, temperature, flow rate), the selected chromatographic conditions were as follows:

- mobile phase: solvent A / solvent B - 40 : 60 (V/V);
- solvent A: 0.3% sodium heptanesulphonate in a 10 mM sodium dihydrogen phosphate buffer (potentiometrically adjusted to pH 3.0 with phosphoric acid)
- solvent B: methanol
- UV detection: 309 nm (with full spectrum recording in the range 220-360 nm);
- flow rate: 1 mL/minute;
- sample solvent: mobile phase.

The samples were kept refrigerated (2-8°C) in the auto sampler during analysis.

The UV spectrum of the active substance solved into the mobile phase was recorded and it shows 4 absorption maxima. Because the solvent chromatogram has a more convenient baseline at 309 nm, this wavelength, corresponding to the forth peak in the ondansetron absorption spectrum, was selected for determination (Fig. 3).

Figure 3
The UV (a) and 3D-UV (b) spectra of ondansetron hydrochloride diluted into the mobile phase
Reagents

Working standards
Ondansetron hydrochloride dihydrate working standards were provided by Dr. Reddy’s (India). The substance had 9.8% water content.

Active substance and dosage forms
Ondansetron hydrochloride dihydrate active substance was also provided by Dr. Reddy’s (India). A value of 9.9% water content was determined by Karl Fischer titration.

As pharmaceutical product it was selected Osetron 4 mg, injectable solution (i.v.), manufacturer: Dr. Reddy’s, India (composition: 4 mg ondansetron as ondansetron hydrochloride dihydrate and excipients: citric acid monohydrate, sodium citrate, sodium chloride, water for injections to 2 mL).

Solvents and chemicals
All solvents were LiChrosolv® HPLC grade, obtained from Merck (Germany). Ultrapure water obtained with a Milli-Q UF Plus water purification system was used throughout the study.

Standard and sample solutions

Standard solution: an accurately weighed amount of 10 mg ondansetron hydrochloride dihydrate working standard, corresponding to 8 mg ondansetron, was dissolved in a 5 mL volumetric flask in mobile phase. After a brief sonication, it was brought to volume with the same solvent. A dilution of 1 mL in a 10 mL volumetric flask with mobile phase was prepared (0.2 mg/mL ondansetron hydrochloride dihydrate, corresponding to 0.16 mg/mL ondansetron).

Sample solution of active substance: was prepared in the same way as the Standard solution.

Sample solutions of dosage forms: 0.8 mL of dosage form (injectable solution) was diluted in a 10 mL volumetric flask with mobile phase.

Results and discussion

Ultrasphere Octyl column was equilibrated for at least 30 minutes with the mobile phase (the minimum required time for obtaining the equilibrium in IPC, according to literature data) [6,4,10]. Six replicates (10 μL) of standard solution and two replicates (10 μL) of 6 independent sample solutions were injected into the chromatographic column. The representative chromatogram obtained for the standard solution is presented in figure 4 (retention time of ondansetron ~ 5 minutes).
The results obtained both on active substance and pharmaceutical dosage forms are reported in table I.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Assay results (%)</th>
<th>Average %</th>
<th>RSD*</th>
<th>Confidence interval (n=6, P=95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active substance</td>
<td>99.67 100.56 98.98 100.70 100.31 100.29</td>
<td>100.08</td>
<td>0.64</td>
<td>99.41–100.76 % (as ondansetron hydrochloride)</td>
</tr>
<tr>
<td>Osetron</td>
<td>100.18 100.81 99.44 100.42 100.41 100.16</td>
<td>100.24</td>
<td>0.45</td>
<td>99.76–100.72 % (1.99–2.01mg/mL) (as ondansetron)</td>
</tr>
</tbody>
</table>

*Relative Standard Deviation

**Validation of chromatographic procedure**

The analytical procedure was validated according to International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) requirements for the following parameters: linearity, precision (repeatability), specificity, accuracy, robustness, limit of detection (LOD) and quantification (LOQ), solution stability [13].

**Linearity** study is carried out with the linearity solutions containing ondansetron in 5 different concentrations (i.e. 80, 90, 100, 110 and 120% of...
the target concentration). An accurately weighed amount of 20 mg of ondansetron hydrochloride dihydrate working standard, corresponding to 16 mg ondansetron, was dissolved in a 10 mL volumetric flask with mobile phase. Each concentration of the linearity solutions of 0.16–0.24 mg/mL ondansetron hydrochloride dihydrate was injected in triplicate. The obtained overlaid chromatograms for each concentration are presented in figure 5. A calibration curve is plotted in the figure 6 and the detector response factor is presented in figure 7. The results indicate a good correlation between the response and concentration.

Figure 5
Overlaid chromatograms for the calibration curve of ondansetron in the linearity range

Figure 6
Assay linearity – regression line

Figure 7
Assay linearity - detector response factor
Repeatability: RSD% (6 injections) for 0.2 mg/mL ondansetron hydrochloride dihydrate was calculated 0.10 and for diluted dosage form sample was calculated 0.57.

Specificity: no interference with solvent mixture (mobile phase) and placebo (figure 8) were detected.

Accuracy: the recovery of ondansetron hydrochloride working standard in 3 diluted samples with known concentration of 95, 100 and 105% from the target value of 0.20 mg/mL ondansetron hydrochloride dihydrate was determinated. The confidence interval (n=3, P=95%) was 97.30 – 102.67% (average 99.98%).

Synthetic mixtures with known quantity of ondansetron hydrochloride working standard (corresponding to 95, 100 and 105% of the target value of 2 mg/mL ondansetron) in placebo mixtures were prepared and diluted as prescribed. The confidence interval for the recovery in “Osetron synthetic mixtures” was 99.31 – 102.90% (average 101.11%).

LOD: 0.033 μg/mL ondansetron hydrochloride dihydrate (S/N=3), LOQ: 0.1 μg/mL ondansetron hydrochloride dihydrate (S/N=10).

Stability study of the test solutions (active substance and medicinal product) was performed. Because the recovery for the samples maintained at 25°C was 102.16% after 24 hours, it is recommended to preserve the solution in refrigerator (2-8°C), not more than 24 hours (recovery 99.29%).

A comparative study between the new established method for assay of ondansetron hydrochloride dehydrate (in bulk and dosage forms –
injectable solution) and the method presented in United States Pharmacopoeia (USP) monographs was realized [14].

The assay results are presented in table II. The results of ANOVA test (F value) proved that there is no significant difference between the two sets of measurements.

### Table II

Comparative results reported for the assay of ondansetron hydrochloride (n=6, P=95%)

<table>
<thead>
<tr>
<th>Sample type</th>
<th>C8 column; RP-IPC</th>
<th>Spherisorb CN column; RP-LC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average (%)</td>
<td>Confidence interval (%)</td>
</tr>
<tr>
<td>Active substance</td>
<td>100.08</td>
<td>99.41 – 100.76</td>
</tr>
<tr>
<td>Osetron 2mg/mL</td>
<td>100.24</td>
<td>99.76 – 100.72</td>
</tr>
</tbody>
</table>

### Conclusions

A simple and rapid new type of RP-HPLC method suitable for the routine assay of ondansetron hydrochloride in bulk and pharmaceutical dosage forms was developed using a mobile phase containing an ion-pairing reagent. The method was validated and the results were similar to those obtained using the current USP monographs.

### References

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