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High-performance liquid chromatographic analysis of ondansetron enantiomers in human serum using a reversed-phase cellulose-based chiral stationary phase and solid-phase extraction

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Abstract

A stereospecific HPLC method was developed for the assay of *R*-(−)- and *S*-(+)-ondansetron enantiomers in human serum. The method involves the use of solid-phase extraction for sample clean-up and is also free of interference from 6-hydroxyondansetron, 7-hydroxyondansetron and 8-hydroxyondansetron, the three major metabolites of ondansetron. Chromatographic resolution of the enantiomers was performed on a reversed-phase cellulose-based chiral column (Chiralcel OD-R) under isocratic conditions using a mobile phase consisting of 0.7 *M* sodium perchlorate–acetonitrile (65:35, v/v) at a flow-rate of 0.5 ml/min. Recoveries at 200 ng/ml levels were more than 90% for both ondansetron enantiomers. Intra-day and inter-day precisions calculated as R.S.D.s were in the 0.3–5% and 2–8% ranges for both enantiomers, respectively. Intra-day and inter-day accuracies calculated as percent error were in the 0.3–11.5% and 0–3% ranges for both enantiomers, respectively. Linear calibration curves were obtained for each enantiomer in serum in the concentration range 15–750 ng/ml. The limit of quantitation of each enantiomer was 15 ng/ml. The detection limit for each enantiomer in serum using UV detection at 210 nm was 7 ng/ml ($S/N=2$).

Keywords: Enantiomer separation; Ondansetron

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1. Introduction

Ondansetron, 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one, is a selective inhibitor of type 3 serotonin (5-HT$_3$) receptors that exhibits antiemetic activity. Ondansetron is clinically used for the treatment of cancer chemotherapy- and radiation-induced and post-operative nausea and vomiting [1]. Ondansetron generally is well tolerated. The most frequent adverse effects of ondansetron in patients receiving the drug for the prevention of chemotherapy-induced nausea and vomiting involve the nervous system (e.g., headache) and GI tract (e.g., constipation) [2]. Studies on humans have shown that 6-hydroxyondansetron, 7-hydroxyondansetron and 8-hydroxyondansetron are the major metabolites of ondansetron [3].

Ondansetron possesses one asymmetric center and has been developed as the racemate (Fig. 1). Stereoselective 5-HT$_3$ receptor blocking has been observed with other 5-HT$_3$ antagonists such as

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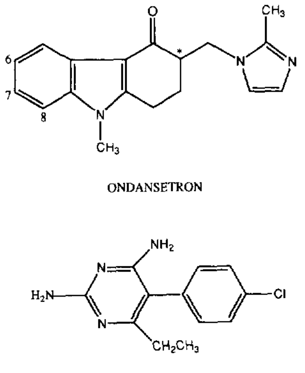

ONDANSETRON

PYRIMETHAMINE

Fig. 1. Chemical structures of ondansetron and pyrimethamine (I.S.).

zacopride and RS 56532 [4,5] although stereoselective differences between the *R*-(−)- and *S*-(+)-enantiomers have not yet been reported in the literature. Recently, a number of so-called racemic switches, which are chiral drugs that are already approved as racemates, have been redeveloped as single enantiomers due to demand on the worldwide market for single-enantiomer forms at the expense of racemic versions of chiral drugs. Ondansetron is one of those racemic switches which is in preclinical status and will be marketed as a single enantiomer in the future [6].

Racemic ondansetron in plasma has been assayed by high-performance thin-layer chromatography, HPLC and radioimmunoassay [7–9]. Resolution and determination of *R*-(−)- and *S*-(+)-ondansetron enantiomers have been reported [10]. It involved the use of a cellulose-derivatized stationary phase (Chiralcel OD) operated in the normal-phase mode.

In this paper, an isocratic HPLC method operated in the reversed-phase mode is presented that will separate and determine *R*-(−)- and *S*-(+)-ondansetron enantiomers in human serum using a Chiralcel OD-R column and solid-phase extraction. The method is linear over the range 15–750 ng/ml using UV detection at 210 nm. The detection limit of the procedure for each enantiomer was 7 ng/ml (*S*/*N*= 2). The procedure is free of interference from the major metabolites of ondansetron.

2. Experimental

2.1. Reagents and chemicals

Racemic ondansetron hydrochloride, 6-hydroxyondansetron hydrochloride, 7-hydroxyondansetron hydrochloride, 8-hydroxyondansetron hydrochloride and the *R*-(−)- and *S*-(+)-ondansetron enantiomers as maleate salts were supplied by Glaxo (Research Triangle Park, NC, USA). The internal standard pyrimethamine was obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade triethylamine and sodium perchlorate were obtained from Fisher Scientific (Pittsburgh, PA, USA). Drug-free human serum was obtained from Biological Specialty (Colmar, PA, USA). Cyanopropyl solid-phase extraction columns (100 mg/1 cc size) were obtained from J&W Scientific (Folsom, CA, USA). C_{18} and C_8 solid-phase extraction columns (100 mg/1 cc size) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). C_{18} and C_8 solid-phase extraction discs (15 mg/3 cc size) were obtained from 3M Industrial and Consumer Sector, New Products Department (St. Paul, MN, USA). The Vac-Elut vacuum manifold was obtained from Analytichem International (Sunnydale, CA, USA).

2.2. Chromatographic system

The HPLC system consisted of an Alcott Model 760 HPLC pump (Norcross, GA, USA), an Alcott Model 728 autosampler (Norcross, GA, USA) equipped with a 100-μl loop, a Kratos Model 757 variable wavelength UV–Vis detector (Ramsey, NJ, USA) set at 210 nm and a Hewlett-Packard Model 3395 integrator (Avondale, PA, USA). The Chiralcel OD-R column (10 μm, 250 mm×4.6 mm I.D.) equipped with an Opti-guard guard column RP C_8 (1.5 cm×1 mm I.D., Optimize Technologies, Portland, OR, USA) was obtained from Chiral Technologies (Exton, PA, USA) and operated at ambient temperature. The mobile phase consisted of 0.7 *M* aqueous sodium perchlorate–acetonitrile (65:35, v/v) and was delivered at a flow-rate of 0.5 ml/min. The solution was filtered through a 0.45-μm nylon membrane obtained from Alltech Associates (Deerfield, IL, USA) and sonicated prior to use.

2.3. Preparation of standard solutions

Stock solutions of *R*-(−)- and *S*-(+)-ondansetron enantiomers (maleate salts) calculated as ondansetron base were prepared in 10-ml volumetric flasks by dissolving 1.0 mg of each enantiomer in 2 ml of acetonitrile followed by the addition of deionized water to volume and stored protected from light at 4°C. A stock solution of internal standard pyrimethamine was prepared in 4 ml of ethanol and 0.4 ml of 0.3 *M* hydrochloric acid followed by the addition of deionized water to the volume and stored protected from light at 4°C. Stock solutions of ondansetron enantiomers and internal standard were stable for at least two weeks. Appropriate dilutions of the *R*-(−)- and *S*-(+)-ondansetron enantiomer stock solutions with deionized water gave 1, 10 and 15 μg/ml solutions which were used for spiking blank human serum.

2.4. Preparation of spiked human serum samples

Accurately measured aliquots (15 and 25 μl of the 1 μg/ml standard solutions, 10 and 40 μl of the 10 μg/ml standard solutions and 50 μl of the 15 μg/ml standard solutions) of *R*-(−)- and *S*-(+)-ondansetron enantiomers were each added into 1 ml volumetric tubes followed by the addition of 15 μl of 15 μg/ml of internal standard solution. Drug-free human serum was added to volume and mixed well to give final concentrations of 15, 25, 100, 400 and 750 ng/ml of each enantiomer.

2.5. Assay method

Cyanopropyl solid-phase extraction (SPE) cartridges were attached to a vacuum manifold and conditioned with 2 column volumes of absolute methanol followed by 2 column volumes of distilled water (Note: do not allow sorbent to dry). The blank and spiked human serum samples were transferred into the cartridges and the vacuum was applied. After the entire serum sample had been aspirated through the cartridge, the cartridge was washed with 500 μl of acetonitrile–water (20:80, v/v), and then dried under full vacuum for 20 min. The ondansetron enantiomers and internal standard were eluted with 1 ml of absolute methanol containing 0.1% triethylamine. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature. The residue was redissolved in 800 μl of mobile phase and triplicate 100-μl injections were made into the liquid chromatograph. Linear regression analysis of enantiomer concentration versus peak-height ratios of each ondansetron enantiomer to internal standard produced slope and intercept data which were used to calculate concentrations of *R*-(−)- and *S*-(+)-ondansetron enantiomers in each serum sample.

3. Results and discussion

Previous work in our laboratories showed that ondansetron enantiomers were resolved using a cellulose-derivatized stationary phase (Chiralcel OD) and a mobile phase of hexane–95% ethanol–2-propanol–acetonitrile (65:25:10:1, v/v) [9]. The Chiralcel OD-R column employs the same chiral selector [cellulose tris(3,5-dimethylphenyl carbamate)] as its normal-phase counterpart (Chiralcel OD), but is designed for analytical applications using reversed-phase chromatography. The chiral selectivity in both columns involves similar interactions such as hydrogen bonding, dipole–dipole interactions, π–π interactions and the formation of inclusion complexes. However, the Chiralcel OD-R column is more suitable than Chiralcel OD for the analysis of chiral drugs since many chiral drugs exist as salt forms and are more water-soluble. In addition, compared with the normal-phase mode, the reversed-phase mode is more environmentally friendly since less organic waste is produced and some harmful organic solvents such as hexane, chloroform and methylene chloride are not used in mobile phases.

Initial studies in our laboratories concerning the separation of *R*-(−)- and *S*-(+)-ondansetron enantiomers had shown that a mobile phase of 0.5 *M* sodium perchlorate–acetonitrile (65:35, v/v) produced a baseline separation ($R_s = 1.7$) of the enantiomers on a Chiralcel OD-R column with retention times of 17–20 min. The influences of sodium

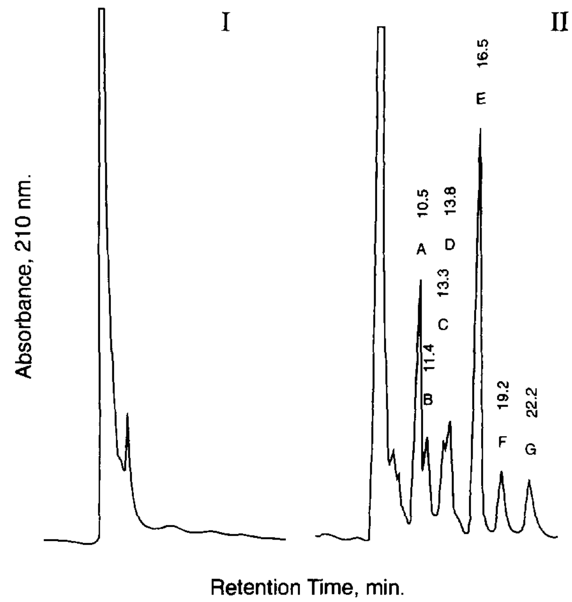

Fig. 2. Typical chromatograms of (I) blank serum and (II) serum spiked with 40 ng/ml of each enantiomer of ondansetron, 6-hydroxyondansetron, 7-hydroxyondansetron and 8hydroxy-ondansetron and 225 ng/ml of internal standard. Peaks: A=racemic 6-hydroxyondansetron and the first-eluting 7-hydroxyondansetron enantiomer; B=the second-eluting 7-hydroxyondansetron enantiomer; C and D=partially resolved 8-hydroxyondansetron enantiomers; E=internal standard; F=*R*-(−)-ondansetron; G=*S*-(+)-ondansetron.

perchlorate and acetonitrile concentrations in mobile phase on separation were investigated. A decrease in sodium perchlorate concentration and increase in acetonitrile concentration in mobile phase reduced the retention times at the expense of resolution. The final composition of the mobile phase was 0.7 *M* sodium perchlorate–acetonitrile (65:35, v/v). This mobile phase provided good resolution (R_s=2.1) of the ondansetron enantiomer peaks (F and G) and suitable retention times (16–23 min) and sensitivity in the desired ng/ml range. The method was also shown to be free of interference from human serum containing the three major metabolites of ondansetron. Typical HPLC chromatograms for blank human serum and serum spiked with 40 ng/ml of each enantiomer of ondansetron, 6-hydroxyondansetron, 7-hydroxyondansetron, 8-hydroxyondansetron and 225 ng/ml of internal standard are shown in Fig. 2. No interferences were observed in blank human serum at the retention times of *R*-(−)- and *S*-(+)-ondansetron peaks.

The selection of pyrimethamine as internal standard was based on its suitable retention time (16.6 min) and separation factors (α) of 1.25 between pyrimethamine (E) and the first eluting *R*-(−)-ondansetron (F) and of 1.34 between pyrimethamine (E) and the second eluting 8-hydroxyondansetron enantiomer (D). Quantitation was based on the plot of the concentration of each enantiomer versus peak-height ratios of each ondansetron enantiomer to internal standard.

The suitability of the system for the separation of the ondansetron enantiomers is shown in Table 1. The retention times of *R*-(−)- and *S*-(+)-ondansetron and internal standard pyrimethamine were 22.05±0.12, 19.13±0.08 and 16.46±0.06 min, respectively (n=5). Retention factors (k') for *R*-(−)- and *S*-(+)-ondansetron enantiomers and internal standard were 2.21±0.01, 2.70±0.02 and 1.76±0.01, respectively (n=5). The calculated theoretical plates for *R*-(−)- and *S*-(+)-ondansetron enantiomers were 2602±22 and 3457±38 per 25 cm column (n=5). Relative retention of the *R*-(−)- and *S*-(+)-ondansetron enantiomers expressed by the

Table 1
Chromatographic parameter data (mean±S.D., n=5) for ondansetron enantiomers and internal standard in spiked human serum

Analyte	R_s	α[a]	k'	t_R (min)	N[b]
Pyrimethamine (I.S.)			1.76±0.01	16.46±0.06	–[c]
	1.9	1.25			
R-(−)-Ondansetron			2.21±0.01	19.13±0.08	2602±22
	2.1	1.23			
S-(+)-Ondansetron			2.70±0.02	22.05±0.12	3457±38

[a] α, separation factor, calculated as k'_2/k'_1.
[b] N, theoretical plates, calculated as $N=16\ (t_R/w)^2$.
[c] Not calculated.

separation factor α, was calculated to be 1.23. Resolution (R_s) of the internal standard (E) and first eluting R-(−)-ondansetron (F) peaks and of the internal standard (E) and the second eluting 8-hydroxyondansetron enantiomer (C) peaks and of the R-(−)- and S-(+)-ondansetron enantiomer peaks (F and G) were 1.9, 1.8 and 2.1, respectively.

Three solid-phase extraction cartridges (C_{18}, C_8 and cyanopropyl) and two solid-phase extraction discs (C_{18} and C_8) were investigated for serum sample clean-up prior to the HPLC assay. The C_8 cartridge and C_8 disc showed interfering endogenous serum peaks at the retention times of ondansetron enantiomer peaks. The C_{18} cartridge and C_{18} disc were also found to be unacceptable due to co-elution of endogenous serum components with R-(−)-ondansetron. The cyanopropyl cartridges provided the best results in terms of clean-up and recoveries of R-(−)- and S-(+)-ondansetron enantiomers. The triethylamine was essential to the extraction as it interacts with the cyanopropyl phase so that the ondansetron enantiomers are more easily eluted. Although racemization may be expected to take place in alkaline medium since a ketone carbonyl group is linked to the α position of the asymmetric center, a partial racemization was not found under the conditions of solid-phase extraction (methanol–triethylamine).

The recoveries of R-(−)- and S-(+)-ondansetron enantiomers from human serum were assessed by using spiked samples at several different concentration levels. The absolute recoveries of R-(−)- and S-(+)-ondansetron were determined by a comparison of the extracted analyte peak height with the unextracted analyte peak height. The results are shown in Table 2.

Linear calibration curves were obtained in the 15–750 ng/ml range for each enantiomer. Standard curves were fitted to a first degree polynomial, $y=ax+b$, where y is the concentration of ondansetron enantiomer, x is the ratio of drug/internal standard peak heights, and a and b are constants. Typical values for the regression parameters a (slope), b (y-intercept) and correlation coefficient were calculated to be 329.9276, −2.0562 and 0.9996 for the R-(−)-enantiomer, and 380.6349, 2.407499 and 0.9995 for the S-(+)-enantiomer, respectively ($n=10$). The precision and accuracy (percent error) of the method were determined by using human serum samples spiked at 40, 200 and 600 ng/ml levels (Table 3). The data indicates that intra-day precision was in the 0.3–2% range ($n=3$) and intra-day accuracy in the 0.3–11.5% range ($n=3$) for both ondansetron enantiomers and that intra-day precision was in the 2–8% range ($n=3$) and inter-day accuracy in the 0–3% range ($n=3$) for both ondansetron enantiomers.

The minimum detectable concentration of each enantiomer was determined to be 7 ng/ml ($S/N=2$). The lowest quantifiable level was found to be 15 ng/ml for each enantiomer: R-(−), 6.9% R.S.D., 6.7% error; S-(+), 8.0% R.S.D., 8.1% error.

In conclusion, an HPLC method has been developed and validated for the assay of R-(−)- and S-(+)-ondansetron enantiomers in human serum using a Chiralcel OD-R column operated in the reversed mode. The procedure is free of interference from 6-hydroxyondansetron, 7-hydroxyondansetron

Table 2
Recovery data for ondansetron enantiomers and internal standard in human serum

Analyte	Concentration added (ng/ml)	Recovery[a] (mean±S.D., $n=5$) (%)	R.S.D. (%)
R-(−)-Ondansetron	40	99.74±7.61	7.6
	200	95.17±4.74	5.0
	600	95.0±2.87	3.0
S-(+)-Ondansetron	40	92.65±4.53	4.9
	200	94.41±4.88	5.2
	600	95.05±3.0	3.2
Pyrimethamine (I.S.)	225	75.59±2.82	3.7

[a] Recoveries were calculated by a comparison of the extracted analyte peak height to the unextracted analyte peak height.

Table 3
Accuracy and precision data for ondansetron enantiomers in human serum

Analyte	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Error (%)	R.S.D. (%)
Intra-day				
R-(−)-Ondansetron	40	41.30±0.78	3.3	1.9
	200	209.08±0.83	4.5	0.4
	600	601.62±2.74	0.3	0.5
S-(+)-Ondansetron	40	44.58±0.73	11.5	1.6
	200	210.63±0.76	5.3	0.4
	600	598.06±1.69	0.3	0.3
Inter-day				
R-(−)-Ondansetron	40	40.01±1.97	0.03	4.9
	200	202.10±6.37	1.1	3.2
	600	592.89±17.06	1.2	2.9
S-(+)-Ondansetron	40	41.02±3.14	2.6	7.7
	200	201.60±7.82	0.8	3.9
	600	593.14±15.84	1.1	2.7

[a] Based on $n=3$ for the intra-day study and $n=9$ for inter-day study.

and 8-hydroxyondansetron, the major metabolites of ondansetron, and is suitable for the separation and quantification of each enantiomer in 15–750 ng/ml range.

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