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Short communication

Validation and application of a stability-indicating HPLC method for the *in vitro* determination of gastric and intestinal stability of venlafaxine☆

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Abstract

Gastrointestinal stability of venlafaxine was evaluated *in vitro* in simulated gastric (SGF) and intestinal (SIF) fluids using a stability indicating HPLC method. The method was validated using a 5 μm Ascentis® C_{18} column (150 mm × 4.6 mm) and mobile phase consisting of 30% acetonitrile in 20 mM potassium phosphate buffer (pH 6.5) delivered isocratically at a flow rate of 1 mL/min with UV detection at 228 nm. Venlafaxine in USP simulated gastric and intestinal fluids (0.4 mg/mL) was incubated at 37 °C in a shaking water bath. The gastric stability study samples were assayed at 0, 15, 30 and 60 min intervals while sampling for the intestinal stability study was at 0, 1, 2 and 3 h. System suitability determinations gave R.S.D.s of 0.68, 0.5 and 3.9% for retention factor (k'), peak area and tailing factor, respectively. The method was shown to be accurate, precise, specific, and linear over the analytical range. Intra- and inter-day precision was <5.3%. Forced degradation studies of drug substance in basic media at 70 °C as well as in H_2O_2 for 1 h and ultra-violet photostability studies at 255 and 365 nm for 24 h did not produce any detectable degradation products. Forced degradation studies of drug substance in acidic media at 70 °C for 1 h produced the dehydro-venlafaxine degradant. Venlafaxine was stable in SGF (pH ∼1.2) for the 1-h incubation period and in SIF (pH 6.8) up to 3 h with <1.5% relative difference (RD) between the amount of drug added and that found for all time points. This stability experiment in simulated gastric and intestinal fluids suggests that drug loss in the gastrointestinal tract takes place by membrane permeation rather than a degradation process.
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Keywords: Venlafaxine; *In vitro*; Stability; Gastric; Intestinal; Dehydro-venlafaxine

1. Introduction

The stability of a drug substance in gastric and intestinal fluids provides evidence whether drug loss from the gastrointestinal tract takes place by intestinal permeation or by a degradation process in the gastrointestinal fluids prior to membrane absorption. An FDA Guidance for Industry [1] concerning *in vivo* bioavailability and bioequivalence studies for immediate release solid oral dosage forms includes a discussion on gastrointestinal stability along with appropriate methodology to help in classifying a drug based on its intrinsic solubility, intestinal permeability and drug product dissolution. In determining drug substance permeability, the degree of drug degradation in the gastrointestinal fluid prior to intestinal membrane permeation should also be considered. Documenting that drug loss from the gastrointestinal tract is due to intestinal membrane permeation, rather than a degradation process, assists in establishing the drug's permeability classification [1]. Stability in the gastrointestinal tract may be confirmed by incubating the drug substance in gastric and intestinal fluids representative of *in vivo* drug exposure to these fluids, e.g., 1 h in simulated gastric fluids (SGF) and 3 h in simulated intestinal fluids (SIF). A validated stability-indicating assay is then utilized to measure drug concentrations. Significant degradation (>5%) of a drug assessed in this manner could suggest potential instability in the gastrointestinal tract [1].

Venlafaxine hydrochloride, (1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol) (Fig. 1), a third generation

☆ This scientific contribution is intended to support regulatory policy development. The views presented in this article have not been adopted as regulatory policies by the Food and Drug Administration at this time.

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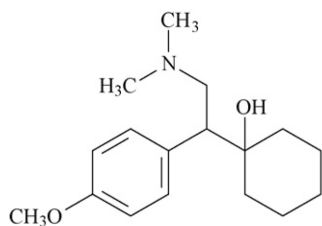

Fig. 1. Structure of venlafaxine.

antidepressant [2,3], inhibits the reuptake of serotonin, norepinephrine and to a lesser extent dopamine, is a serotonin noradrenalin reuptake inhibitor [4–6]. Venlafaxine is rapidly absorbed and metabolized in the liver to its active metabolite, *O*-des-methylvenlafaxine [7]. It is a highly soluble drug with a solubility of greater than 500 mg/mL in water and an octanol-water partition coefficient of 0.43 [8]. Approximately 92% of an oral dose is absorbed in the gastrointestinal tract. However, due to extensive first pass metabolism, only 12.6% is available in systemic circulation [9]. The bioavailability of venlafaxine does not differ significantly between a tablet and oral solution based upon maximum plasma concentration and area under the curve [6]. It is not known whether venlafaxine is metabolized or degraded before or after absorption as there is no information on gastrointestinal stability. A review of the scientific literature has identified only one stability-indicating high-performance liquid chromatography (HPLC) method for venlafaxine drug substance and application to a pharmaceutical formulation [10]. The majority of venlafaxine analytical methods documented in the scientific literature such as CE [11], HPLC [12–14] and LC–MS [15–17] are bioanalytical in nature and utilized for venlafaxine drug monitoring, metabolism and pharmacokinetics studies. Additionally two analytical methods, one electrochemical [18] the second, HPLC [19] have been identified for use in the analysis of venlafaxine in pharmaceutical formulations. Therefore a stability-indicating high-performance liquid chromatography method for venlafaxine based on that of Makhija and Vavia [10] was modified and validated for use in this study.

The purpose of this study was to examine the stability of venlafaxine in SGF and SIF to provide evidence whether the drug loss from the gastrointestinal tract takes place by intestinal membrane permeation or by a degradation process in the gastrointestinal fluids prior to intestinal membrane permeation.

2. Experimental

2.1. Reagents

Venlafaxine hydrochloride was obtained from Ceres Chemical Company, Inc. (White Plains, NY). Porcine pepsin and pancreatin, monobasic potassium phosphate, HCl and NaOH were all ACS reagent grade and purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile was HPLC grade and purchased from Burdick and Jackson (Muskegon, MI). Boron tribromide (BBr_3), anhydrous methylene chloride and heptane was purchased from Sigma–Aldrich (Milwaukee, WI). Deionized water was supplied in house from a Millipore Milli-Q System (Billerica, MA).

2.2. HPLC assay

A stability indicating high-performance liquid chromatography method for venlafaxine in SIF and SGF based on that of Makhija and Vavia [10] was modified and validated for use in this study. An HP 1090 Series II liquid chromatograph (Wilmington, DE) equipped with a diode array detector was controlled by computer via Agilent Technologies® (Wilmington, DE) ChemStation software. The column used was a 5 μm Ascentis® (Supelco, Inc., Bellefonte, PA) C_{18} column (150 mm × 4.6 mm) coupled to a C_{18} guard column (30 mm × 4.6 mm, 5 μm). UV detection was performed at 228 nm. The mobile phase consisted of 30% acetonitrile in 20 mM potassium phosphate buffer (pH 6.5) and was delivered isocratically at 1.0 mL/min for a runtime of 20 min. The injection volume was 50 μL.

2.3. System suitability

System suitability parameters (retention factor, k', peak area, tailing factor, T_f, and column efficiency, N), were evaluated to determine the daily system performance. The system suitability parameters were determined using a 6 μg/mL solution of venlafaxine–HCl in the mobile phase.

2.4. Method validation

The method was validated for specificity, precision, linearity, and accuracy using a set of calibration standards ranging in concentration from 1.05 to 10.5 μg/mL, and sets of three quality control (QC) standards at concentrations of 7 μg/mL (QC-high), 5 μg/mL (QC-intermediate) and 3 μg/mL (QC-low).

2.5. Forced degradation studies

Forced acid, base and oxidative degradation studies on 0.5 mL aliquots of 40 mg/mL venlafaxine solution in water were completed in triplicate by adding 0.5 mL of 2 M hydrochloric acid, 2 M sodium hydroxide or 0.5 mL 30% H_2O_2 to the drug solutions in glass vials for a final concentration of 20 mg/mL. The mixtures were incubated at 70 °C for 1 h. At the end of the incubation period 2.5 μL of the solutions were, respectively, neutralized with base or acid, and the solutions made up to 1 mL with mobile phase with 25 μL aliquots injected in to the chromatograph. For the H_2O_2 mixture, 2.5 μL was diluted with distilled water to 1 mL and 25 μL portions were injected. A set of control samples in which water was substituted for the degradation reagents, were subjected to same treatment as the test samples. Photostability studies of venlafaxine solution in water were placed in a Spectronics Corporation (Westbury, NY) ultra-violet–visible chamber at short UV (254 ± 10 nm) at 470 μW/cm^2 and at long UV (365 ± 10 nm) at 500 μW/cm^2 at a distance of 0.15 m for 0, 1, 4 and 24 h. Samples were prepared as above, diluted in mobile phase and injected onto the HPLC.

Table 1
Precision and accuracy of the HPLC method

QC sample (μg/mL)	Intra-day studies ($n = 6$)			Inter-day studies ($n = 18$)		
	Measured amount (μg/mL)	CV (%)	Relative error (%)	Measured amount (μg/mL)	CV (%)	Relative error (%)
3	2.9	5.3	3.3	2.9	2.8	3.3
5	4.8	1.1	4.1	4.9	2.5	2.0
7	7.1	2.5	1.4	7.1	1.9	1.4

2.6. Mass spectrometry

LC–MS analysis was performed on an Finnegan TSQ 7000 LC/MS–MS (San Jose, CA). Separation of the analytes was obtained on a Thermo Aquasil C_{18} (5 μM, 150 mm × 2.1 mm), RP-HPLC column with a Phenomenex C_{18} Security Guard cartridge (4 mm × 3.0 mm). The mobile phase was acetonitrile–aqueous 0.1% acetic acid (20:80, v/v) delivered isocratically for 3 min, then the percentage of acetonitrile was increased to 50% by 12 min The flow rate was 0.3 mL/min which was introduced into the MS without splitting. The analytes were ionized using atmospheric pressure chemical ionization (APCI) with the vaporizer set at 400 °C and the heated capillary set to 210 °C, and positive ions were analyzed. Product ion scans were obtained by charging Q2 with 1 mTorr argon, and increasing the voltage offset to −18 V.

2.7. Preparation of O-des-methylvenlafaxine

The *O*-des-methylvenlafaxine degradant was prepared by the demethylation of the methyl ester of venlafaxine by a modification (temperature and quenching, respectively) of two demethylation procedures [20,21]. A total of 12 mg (38 μmol) venlafaxine was dissolved in 4.5 ml of anhydrous methylene chloride and cooled to −78 °C in a dry ice bath. Approximately 650 μl of 1M BBr_3 (650 μmol) in heptane was slowly added to the venlafaxine solution and was allowed to react for 20 min. The reaction was stopped by addition of 2 ml of deionized H_2O, warmed to room temperature, and the two phases separated. Venlafaxine and *O*-des-methylvenlafaxine were identified in the aqueous phase, which was neutralized with 1 M K_2CO_3 to ∼pH 7.

2.8. Stability in simulated gastrointestinal fluids

The simulated gastrointestinal fluids were prepared according to USP specifications [22]. For SGF, 2.0 g sodium chloride and 3.2 g pepsin (from porcine stomach mucosa) were dissolved in 7.0 mL hydrochloric acid and a sufficient volume of water to make 1000 mL. The pH of SGF was approximately 1.2. For the SIF, 6.8 g monobasic potassium phosphate was dissolved in 250 mL water. To this, 77 mL 0.2 N sodium hydroxide and 500 mL water were added and mixed along with 10.0 g pancreatin (from porcine pancreas). The SIF solution was adjusted to pH 6.8 ± 0.1 with either 0.2 N sodium hydroxide or 0.2 N hydrochloric acid and then diluted with water to 1000 mL.

Venlafaxine solutions of 0.4 mg/mL were prepared in SIF and SGF. Aliquots of these solutions were pipetted into glass tubes, and placed in a 37 °C shaking water bath. At time points 15, 30 and 60 min for the gastric stability experiment and at 1, 2 and 3 h for the intestinal stability experiment, sample tubes, in triplicate, were removed and then diluted 1:50 in mobile phase to 8.0 μg/mL before HPLC analysis. The relative difference (RD) between the amount of venlafaxine added and that determined at the end of the incubation period was calculated as follows:

$$\mathrm{RD} = \frac{C_i - C_\mathrm{f}}{C_i} \times 100\%$$

where C_i is the amount of drug found at the zero time point and C_f is the amount found at the end of incubation. It gives a measure of the extent of any degradation of the drug in the presence of the gastrointestinal fluids.

3. Results and discussion

3.1. System suitability

The system suitability assessment for this venlafaxine HPLC method established instrument performance parameters such as the retention factor, k', peak area, column efficiency, N, and USP tailing factor, T_f, for venlafaxine. The mean ($n = 18$) k', peak area, T_f and N were 1.67, 662.6 mAU s, 1.2 and 37,800 plates/m with CVs of 0.68, 0.5, 3.9 and 11.7%, respectively, for venlafaxine.

3.2. Method validation

Table 1 summarizes the results of the method validation for venlafaxine. Calibration curves were linear over the range of concentrations used (1–10.5 μg/mL) with correlation coefficients greater than 0.998, a slope of 96.6 ± 1.6, and the y-intercept at 10 ± 2.5. Method reproducibility as indicated by the CVs for the quality control standards over the analytical range were <5.3% for intra- and inter-day precision as shown in Table 1. The accuracy, as measured by the relative errors, ranged from 95.9 to 101.4% for both intra- and inter-day samples. Peak purity determinations using the Agilent Technologies® spectral analysis software generated peak purity factors for venlafaxine peaks that were within threshold values indicating that no additional peaks were co-eluting with the venlafaxine peak.

3.3. Forced degradation studies

The forced degradation studies in acidic and basic media at 70 °C for 1 h as well as oxidation in H_2O_2 and photostability

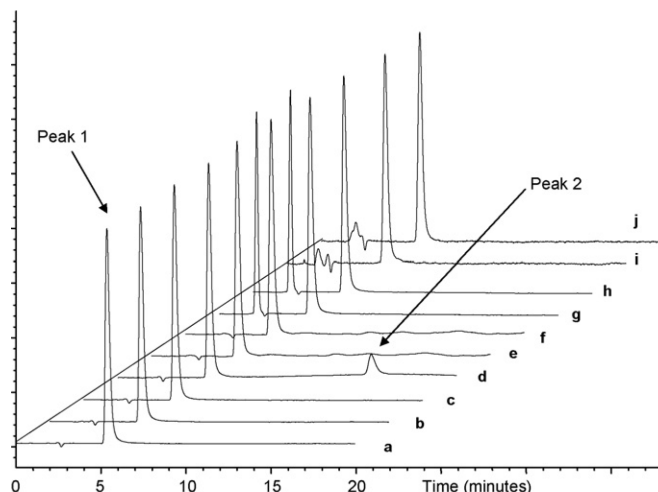

Fig. 2. Chromatograms of venlafaxine (peak 1) before exposure in (a) water, (c) acid, (e) base, (g) H_2O_2, and after exposure at 70 °C in (b) water, (d) acid,(f), base, (h) H_2O_2. Peak 2 in (d) acid degradant formed by the dehydration of venlafaxine. Chromatograms (i) and (j) are those of venlafaxine after 24 h exposure to UV (254 nm) and (365 nm) lights, respectively.

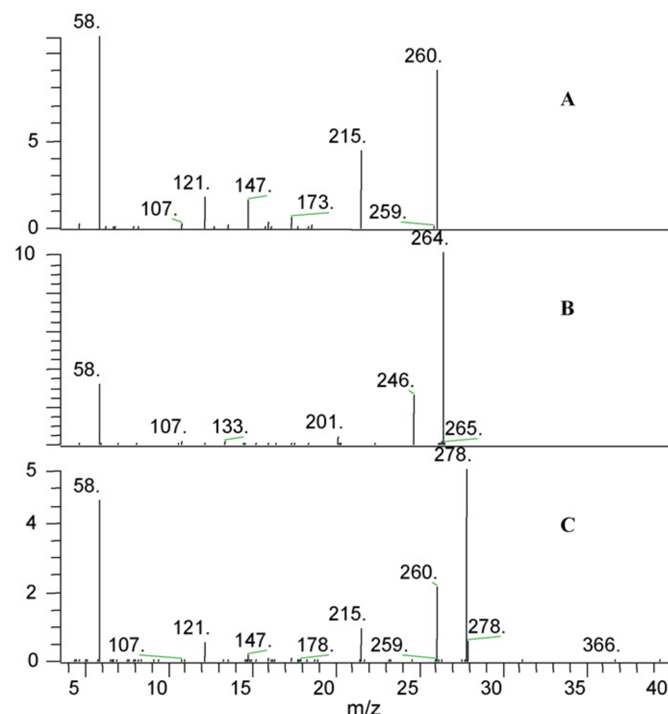

Fig. 3. Mass spectrum of (a) dehydro-venlafaxine, (b) *O*-des-methylvenlafaxine and (c) venlafaxine.

studies in the uv spectral region did not produce any detectable early eluting degradation products (Fig. 2). This is contrary to findings by Makhija and Vavia [10] who reported that acid degradation produced a peak, identified as the *O*-demethylation product of venlafaxine, with a shorter retention time than the venlafaxine peak. No such peak was detected in our study. However forced degradation studies in acidic media at 70 °C for 1 h did produce a late eluting peak (Fig. 2d). This late eluting peak (RT 15.2 min.) was identified by liquid chromatography–mass spectrometry as the dehydro-venlafaxine degradant (Fig. 3A). Comparison of peak parameters, such as retention times, peak heights and areas, of the control and test samples from the basic media, oxidation and photostability studies gave virtually identical characteristics; i.e., no reduction in peak sizes or retentions times to indicate changes from the degradation conditions. Peak purity determinations using the Agilent Technologies® spectral analysis software generated peak purity factors for venlafaxine peaks that were within threshold values indicating that no additional peaks were co-eluting with the venlafaxine peak. Additionally, LC–MS evaluation of the venlafaxine peak found only a mass determination of *m*/*z* of 278.1 (Fig. 3C) attributable to venlafaxine. No other mass determinations were observed.

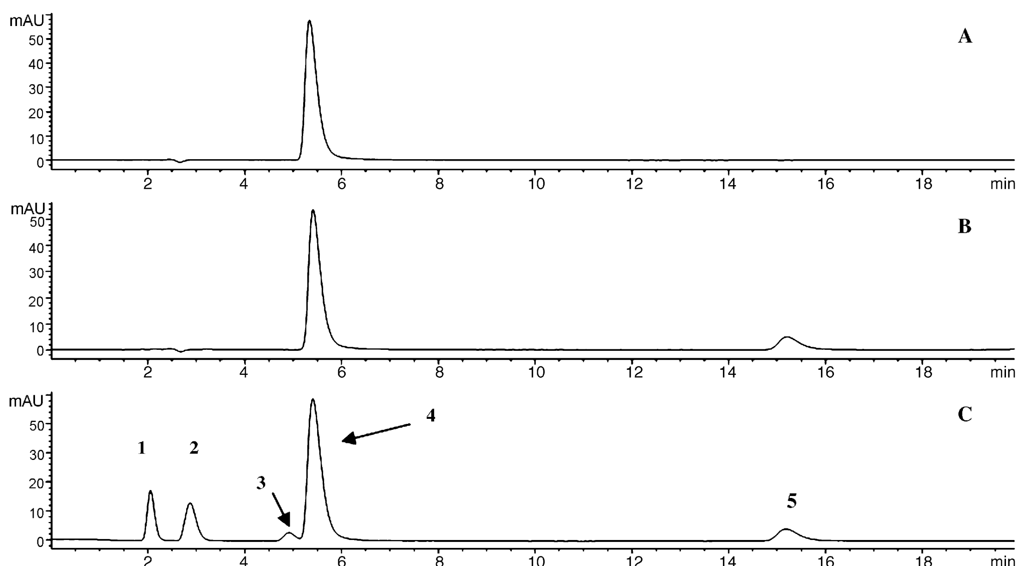

Fig. 4. Chromatograms of (a) venlafaxine in water, (b) venlafaxine and dehydro-venlafaxine, and (c) synthetic mixture: peak 1 synthetic reagents:peak 2 *O*-des-methylvenlafaxine (*m*/*z* 264.1):peak 3 *O*-des-methyl-dehydro-venlafaxine (*m*/*z* 246.1):peak 4 venlafaxine (*m*/*z* 278.1):peak 5 dehydro-venlafaxine (*m*/*z* 260.1).

Table 2
Venlafaxine stability in SGF and SIF

SGF			SIF		
Time point (min)	Concentration found[a] (μg/mL)	RD (%)	Time point (h)	Concentration found[a] (μg/mL)	RD (%)
0	8.09 ± 0.13	–	0	8.01 ± 0.01	–
15	8.04 ± 0.13	0.62	1	7.93 ± 0.06	0.99
30	8.09 ± 0.04	−0.04	2	8.04 ± 0.05	−0.29
60	8.02 ± 0.14	0.94	3	7.89 ± 0.06	1.59

[a] Mean ± S.D. ($n = 3$).

These results indicated that the method is stability-indicating and that the drug is stable under the conditions of this study.

3.4. Preparation and identification of the O-des-methylvenlafaxine

The *O*-des-methylvenlafaxine was successfully synthesized, separated by aqueous phase and identified by liquid chromatography–mass spectrometry. The *O*-des-methylvenlafaxine gave a mass determination of *m*/*z* 264.1 in the positive ion mode (Fig. 3B).

3.5. Co-elution study of venlafaxine and O-des-methylvenlafaxine

The synthetic mixture was diluted in mobile phase (50–500 μL) and chromatographically evaluated with our venlafaxine stability-indicating method to determine if the method is selective for venlafaxine. The *O*-des-methylvenlafaxine (Fig. 4C) eluted at 2.9 min (*m*/*z* 264.1) was well resolved from venlafaxine (*m*/*z* 278.1) at 5.4 min (Fig. 4C). Kirchherr and Kuhn-Velten previously identified a *m*/*z* 264.1 for *O*-desmethylvenlafaxine also using LC–MS in the positive ion mode [23]. Also identified in the synthetic mixture by mass spectrometry was the *O*-des-methyl-dehydro-venlafaxine (*m*/*z* 246.1 MS data not shown) at 4.9 min (Fig. 4C) and the dehydro-venlafaxine (*m*/*z* 260.1) at 15.2 min (Fig. 4B and C). These data indicate that the stability-indicating method is selective for venlafaxine in the presence of *O*-des-methylvenlafaxine and *O*-des-methyl-dehydro-venlafaxine.

3.6. Stability study in gastrointestinal fluids

Table 2 shows the results of the SGF and SIF experiments respectively. The extent of degradation or loss of the drug after incubation in SGF or SIF was assessed by the deviation, expressed as percentage, of the measured drug concentration at the end of incubation time period from that at the beginning. The relative deviation (%) of venlafaxine in SGF and SIF was no greater than 1.46 and 1.43%, respectively. Since the exposure of drug substances at 37 °C to SGF (1 h) or SIF (3 h) mimics the *in vivo* drug contact with these fluids, it was concluded that venlafaxine was stable in the gastrointestinal tract.

4. Conclusion

Significant degradation (>5%) of a drug evaluated in the manner presented in this study could indicate potential drug instability in the gastrointestinal tract [1]. Venlafaxine was stable in SGF (pH ∼1.2) for the 1-h incubation period and in SIF (pH 6.8) up to 3 h with <1.5% degradation. Based upon these results, venlafaxine drug substance is considered stable in SGF (1 h) and SIF (3 h) at 37 °C in accordance with FDA/CDER's BCS Guidance [1]. This study also suggests that venlafaxine would be stable in the gastrointestinal tract and that drug loss may take place by membrane permeation rather than a gastrointestinal degradation process.

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