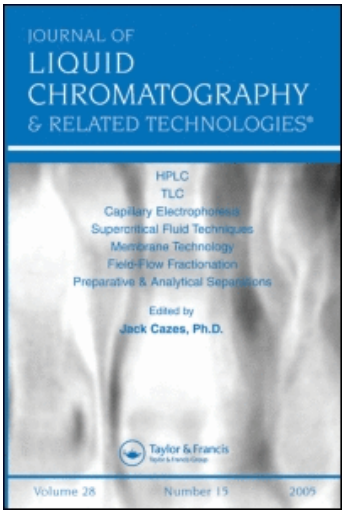

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Development and Validation of a Rapid HPLC Assay for the Simultaneous Determination of Three Psychoanaleptic Drugs in Pharmaceutical Formulations

V. Nagaraju[a]; A. M. Crouch[a]
[a] Department of Chemistry and Polymer Science, University of Stellenbosch, Stellenbosch, South Africa

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Development and Validation of a Rapid HPLC Assay for the Simultaneous Determination of Three Psychoanaleptic Drugs in Pharmaceutical Formulations

V. Nagaraju and A. M. Crouch

Department of Chemistry and Polymer Science, University of Stellenbosch, Stellenbosch, South Africa

Abstract: Atomoxetine, venlafaxine, and fluoxetine are classified as psychoanaleptics. While atomoxetine is used for the treatment of attention deficit/hyperactivity disorder (ADHD), the other two are for depressive disorders. A simple, rapid, isocratic, high performance liquid chromatographic (HPLC) method has been developed for the determination of atomoxetine, venlafaxine, and fluoxetine in pharmaceutical products. The HPLC method involves separation by reversed phase HPLC on a Nova-Pak C_{18} column (15 cm × 3.9 mm id) using acetonitrile-potassium dihydrogen phosphate buffer (0.05 M adjusted to pH 3.0 with phosphoric acid) (45:55 v/v) as the mobile phase, at a flow rate of 0.8 mL/min at room temperature (25°C), using UV detection at 226 nm. The method was validated for specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness. The excipients present in the formulations do not interfere with the assay procedure. The developed method was successfully applied to determine atomoxetine, venlafaxine, and fluoxetine in pharmaceutical formulations.

Keywords: Atomoxetine, Venlafaxine, Fluoxetine, Reversed phase HPLC, Pharmaceutical formulations

Correspondence: A. M. Crouch, Department of Chemistry and Polymer Science, University of Stellenbosch, Private Bag No. XI, Stellenbosch 7602, South Africa. E-mail: amc@sun.ac.za

INTRODUCTION

Psychoanaleptics, as their name implies, are drugs that stimulate the mood and correct depressive conditions. Atomoxetine (ATO), venlafaxine (VEN), and fluoxetine (FLU) are classified as psychoanaleptic drugs. ATO [(-)-N-methyl-3-phenyl-3-(o-tolyloxy)-propylamine] is a novel, non-stimulant, highly selective, noradrenaline reuptake inhibitor. It is a potent inhibitor of the presynaptic norepinephrine transporter with minimal affinity for other monoamine transporters or receptors.[1] ATO is used for the treatment of attention deficit/hyperactivity disorder (ADHD) in children, adolescents, and adults.[2,3] ADHD is a common behavioural disorder occurring in children and adolescents, and may persist into adulthood. The principal diagnostic features are inattentiveness, hyperactivity, and impulsive behaviour that are often disruptive and may become defiant and aggressive. ATO has been approved in the year 2002 by the US Food and Drug Administration for the treatment of ADHD in children, adolescents, and adults.

VEN [1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol] is a bicyclic antidepressant that inhibits the reuptake of serotonin, norepinphrine, and dopamine.[4] It is chemically unrelated to tricyclic, tetracyclic, or other available antidepressant agents. VEN is used in the treatment of depression, but is also used to treat obsessive compulsive disorder (OCD) or fibromyalgia (pain in the muscle, ligaments, and tendons).[5] OCD is an anxiety disorder and is characterized by recurrent, unwanted thoughts (obsessions) and/or repetitive behaviours (compulsions).

FLU [N-methyl-3-phenyl-3-(α,α,α-trifluoro-p-tolyloxy)-propylamine] is an antidepressant belonging to the class of selective serotonin reuptake inhibitors.[6] It is used in the treatment of depression, body dysmorphic disorder, OCD, bulimia nervosa (an eating disorder), premenstrual dysphoric disorder, hypochondriasis and panic disorder, and also been tried in obesity and alcohol abuse.[7]

A number of analytical methods have been published for the determination of VEN and FLU in pharmaceutical formulations based on HPLC,[8–14] GC,[15,16] and CE.[17,18] A couple of methods were reported for the determination of ATO in human plasma by HPLC.[19,20] Gavin et al.[21] evaluated a strategy for multi-sourced active pharmaceutical ingredient starting materials by considering phenyl methyl amino propanol as the starting material used for ATO and FLU. The determination of the enantiomer and positional isomer impurities in ATO with HPLC using polysaccharide chiral stationary phases was also reported.[22]

A thorough literature search has revealed that none has been published for the assay of ATO in pharmaceutical formulations using HPLC and simultaneous determination of ATO, VEN, and FLU in formulations, or in any matrix. In this paper, we describe a simple and rapid isocratic reversed phase HPLC method for the determination of ATO in pharmaceutical formulations. The method was also applied to the determination of VEN and FLU in pharmaceutical formulations.

EXPERIMENTAL

Materials and Reagents

All reagents were of analytical reagent grade unless stated otherwise. HPLC grade acetonitrile and potassium dihydrogen orthophosphate was obtained from Sigma-Aldrich (Steinheim, Germany). Working standards of ATO, VEN, and FLU were gifted by Metropolitan Overseas Limited, Hirehalli, Tumkur, India. Formulations of ATO, VEN, and FLU were purchased from a local pharmacy. Deionized water was prepared by passing distilled water through a Millipore (Bedford, MA, USA) Milli Q water purification system.

Instrumentation

Liquid Chromatograph

A HPLC (Waters, Milford, MA, USA), model 510 pump with a 20 μL loop injector was used. A Waters, Lambda Max model 481 LC spectrophotometer was connected after the column. All the operations were performed using a Waters System Controller model 600E. A reversed phase Nova-Pak C_{18} (Waters, Milford, MA, USA) column (15 cm × 3.9 mm id, particle size 5 μm) was used for the determination of the three drugs. pH measurements were carried out with a Combined Conductivity/pH meter (Jenway, UK), model 3880, equipped with a combined glass calomel electrode, which was calibrated using standard buffer solutions of pH 4.0, 7.0, and 9.2 before measuring the pH of the solutions.

Chromatographic Conditions

The mobile phase was an acetonitrile-0.05 M potassium dihydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) (45:55 v/v); before delivering into the HPLC system it was filtered through a 0.45 μm PTFE filter and degassed using a vacuum. The analysis was carried out under isocratic conditions using a flow rate of 0.8 mL min^{-1} at room temperature (25°C) and UV detection at 226 nm.

Analytical Procedure

Preparation of Standard Solutions

The stock solutions of ATO, VEN, and FLU (2 mg/mL) were prepared in a water:methanol (2:8 v/v) mixture. Working concentrations were prepared by diluting the stock solutions with 0.05 M potassium dihydrogen phosphate buffer. Synthetic mixtures containing ATO, VEN, and FLU were prepared

and a 20 μL volume was injected and chromatographed under the above conditions.

Preparation and Analysis of Real Samples

Ten commercial capsules of each of ATO, VEN, and FLU were emptied separately and weighed accurately. For ATO and FLU, the contents of the capsules were in powder form, whereas for VEN formulations, the contents of the capsules were granules. These were converted to a powdered form by using a mortar and pestle.

A portion of the powder equivalent to 40 mg ATO, 75 mg VEN, and 20 mg FLU were weighed accurately and transferred separately into 50 mL volumetric flasks, dissolved in 30 mL of 0.05 M potassium dihydrogen phosphate buffer, sonicated for 15 min, and made up to the volume with the same buffer. After filtration through a Whatman No. 42, appropriate solutions were prepared by taking suitable aliquots of filtrate and diluting it with the buffer to get final solutions. Before injecting into the chromatograph, the samples were filtered through a 0.45 μm filter.

RESULTS AND DISCUSSION

Method Development

The reversed phase chromatographic system was selected for the analysis of ATO, VEN, and FLU, as most of the reported methods used reversed phase HPLC for the analysis of basic drugs. Figure 1 gives the molecular structures of ATO, VEN, and FLU studied in the present investigation. Initially, method development started by using methanol and water at different concentration ratios and at different pH's with a Nova-Pak C_{18} column to separate the three drugs, and no separation was observed. Methanol was then replaced with acetonitrile as an organic modifier. The composition, pH, and the flow rate of the mobile phase were changed to optimize the separating conditions for ATO, VEN, and FLU. There was no improvement in the separation of these compounds. Later, acetonitrile and potassium dihydrogen phosphate buffer (adjusted pH with phosphoric acid) were found suitable for separation of ATO, VEN, and FLU after several preliminary investigatory chromatography runs. The chromatographic conditions were optimized by modifying the concentration of organic modifier, ionic strength of potassium dihydrogen phosphate, and pH of the mobile phase. The effect of the percentage of acetonitrile on retention of ATO, VEN, and FLU was studied by changing the concentration of acetonitrile from 10% (v/v) to 90% (v/v) and found to be optimum at 45% (v/v). The influence of potassium dihydrogen phosphate (KH_2PO_4) buffer in the concentration range 0.02–0.08 M on retention of these compounds was studied, and stable retention times were observed

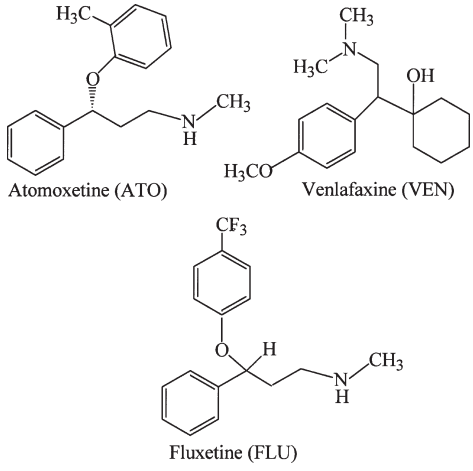

Figure 1. Chemical structures of atomoxetine, venlafaxine, and fluxetine.

from 0.05 M–0.08 M. Therefore, the concentration of 0.05 M KH_2PO_4 was chosen as an optimum value for further experimentation. The effect of pH on the separation of ATO, VEN, and FLU in the range 3.0–6.0 on the mobile phase was studied. pH values between 3.0–5.0 were found to be optimum and observed pH 3.0 is ideal with good peak shapes and base line separation. At pH above 5.0, the FLU peak is broad with severe peak tailing. The optimum chromatographic conditions to separate the compounds of interest was obtained using a Nova-Pak C_{18} column with acetonitrile: 0.05 M potassium dihydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) (45:55 v/v) at a flow rate of 0.8 mL min^{-1}, room temperature (25°C) and UV detection at 226 nm. A typical chromatogram of a synthetic mixture containing ATO, VEN, and FLU is shown in Figure 2. The peaks were identified by injecting and comparing the retention times with those of authentic standards. Reproducible peak shapes were obtained under the optimum conditions. The chromatographic data including retention time (t_R), relative retention time (RRT), retention factor (k'), tailing factor (T_f), and relative response factors (RRF) are given in Table 1. It could be seen from the Table 1, that the elution order of the drugs are VEN, ATO, and FLU and the response of the FLU is high when compared with the ATO and VEN.

Method Validation

Accuracy and Precision

Synthetic mixtures containing known amounts of ATO, VEN, and FLU were prepared and analyzed by HPLC. The accuracy of the method was checked for

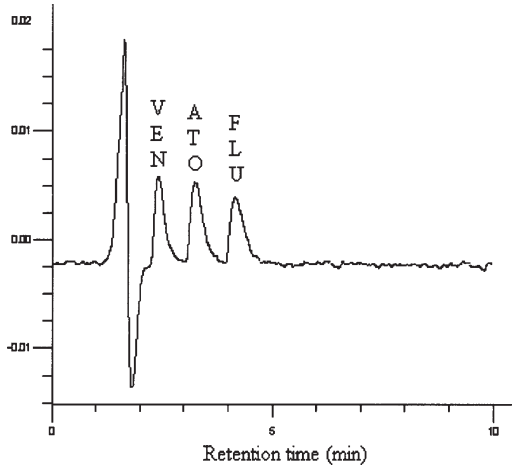

Figure 2. Typical chromatogram of a standard mixture containing Ven (0.75 μg), ATO (0.40 μg), and FLU (0.2 μg). Chromatographic conditions: mobile phase, acetonitrile-0.05 M potassium dihydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) (45:55 v/v); column, reversed phase Nova-Pak C_{18} (Waters, Milford, MA, USA) (15 cm × 3.9 mm i.d., particle size 5 μm); flow rate, 0.8 mL/min.; room temperature, 25°C; and UV detection at 226 nm.

three different concentration levels by the standard addition technique. Small quantities of ATO, VEN, and FLU were added to the placebo and chromatographed. It was found that these additions were accurately reflected in their peak areas. All estimations were repeated five times and standard deviations (S.D.) were calculated (Table 2). The repeatability (intra-day) was evaluated by replicate injections (n = 5) of standard solution containing ATO, VEN, and FLU during the same day and found with an RSD of 1.24%. The intermediate precision (inter-day) was studied similarly for 5 different days, using the standard solutions that were freshly prepared daily and the RSD ranged from 1.18%–1.35%.

Table 1. Retention and response data of venlafaxine, atomoxetine and fluoxetine

Drug	t_R (min)	RRT	k'	T_f	RRF
Venlafaxine	2.39	1.00	0.14	1.11	1.00
Atomoxetine	3.43	1.44	0.63	1.21	1.88
Fluoxetine	4.45	1.86	1.01	1.25	3.75

t_R: retention time; RRT: relative retention time; k': retention factor; T_f: tailing factor; RRF: relative response factors.

Table 2. Accuracy data for standard mixtures containing VEN, ATO and FLU

Drug	Taken (10^{-6} g)	Found[a] (10^{-6} g)	Recovery (%)
VEN	0.20	0.195 ± 0.003	97.5
	0.60	0.605 ± 0.002	100.8
	1.20	1.229 ± 0.003	102.4
ATO	0.15	0.148 ± 0.002	98.7
	0.60	0.615 ± 0.004	102.5
	1.00	1.019 ± 0.003	101.9
FLU	0.15	0.146 ± 0.002	97.3
	0.60	0.609 ± 0.002	101.5
	1.00	1.021 ± 0.004	102.1

[a]Mean $\pm$ SD (n = 5).

Specificity

To demonstrate the specificity of the method, known amounts of ATO, VEN, and FLU were spiked to the placebo. The placebo was prepared by mixing the excipients such as starch, lactose, magnesium stearate, and microcrystalline cellulose with proportions of drug to excipients ratios. All three drugs were clearly separated and it was found that no peak was observed relating to the excipients and recoveries in the range of 97.5% to 102.8% were obtained. This gives an indication that the method is specific for the separation and determination of these drugs in formulations.

Linearity

Calibration graphs (concentration versus peak area) were constructed at six different concentrations for ATO (0.15×10^{-6} g–1.00×10^{-6} g), VEN (0.20×10^{-6} g–1.20×10^{-6} g), and FLU (0.15×10^{-6} g–1.00×10^{-6} g). Three independent determinations were carried out at each concentration and good linearity was found between the integral responses for each of the compounds examined. Table 3 gives the linear equation, mass range, and correlation coefficients for the three compounds.

Limits of Detection and Quantitation (LOD and LOQ)

The LOD and LOQ values were calculated for ATO, VEN, and FLU based on the three and ten times of noise level, respectively, and the values are given in Table 3.

Robustness

In order to evaluate the robustness of the method, the influence of small and deliberate variation of analytical parameters on the recovery of ATO, VEN,

Table 3. Linear regression data of VEN, ATO and FLU

Drug	Range (10^{-6} g)	Linear regression	Correlation coefficient	LOD (10^{-6} g)	LOQ (10^{-6} g)
VEN	0.20–1.20	52108x + 5014.5	0.9938	0.04	0.12
ATO	0.15–1.00	99012x − 385.13	0.9966	0.03	0.09
FLU	0.15–1.00	119955x + 5166.1	0.9974	0.03	0.09

and FLU was studied. The parameters selected were mobile phase composition (acetonitrile: 0.05 M potassium dihydrogen phosphate buffer (45:55; 50:50; 40:60 v/v), pH (3.0, 3.3, 2.7), flow rate (0.6, 0.8, 1.0 mL/min), and ionic strength of buffer (0.050 M, 0.045 M 0.055 M). Only one parameter was changed, while the others kept constant. The recoveries were found to be 98.5–102.3%, 97.8–101.8%, and 98.1–103.2% for ATO, VEN, and FLU, respectively. Thus, it was considered that the method is robust and suitable for determination of ATO, VEN, and FLU in pharmaceutical formulations.

Sample Solution Stability

To determine the sample stability in buffer, ATO, VEN, and FLU were stored in 0.05 M potassium dihydrogen phosphate buffer for 24 h and chromatographed on the following day. The solutions were found to be stable for 24 h and it was observed, that there is no degradation/increase in the percentage of areas and, also, no significant changes were observed. Replicate injections (n = 5) of ATO, VEN, and FLU were performed and the relative standard deviations of peak areas were determined with 1.35–1.58%, 1.47–1.75%, and 1.14–1.68% for ATO, VEN, and FLU, respectively.

Analysis of Pharmaceutical Formulations

The optimized reversed phase HPLC method was applied to the determination of ATO, VEN, and FLU in Straterra® capsules (10 mg and 40 mg of ATO), Efexor® XR capsules (75 mg and 150 mg of VEN), and Prozac 20 capsules

Table 4. Results of amounts of VEN, ATO and FLU in pharmaceutical formulations

Name of formulation	Amount claimed (mg per capsule)	Amount found (mg)	RSD (%)[a]
Efexor® XR	75	74.8	1.35
Straterra®	40	40.5	0.98
Prozac	20	20.3	1.23

[a]n = 5.

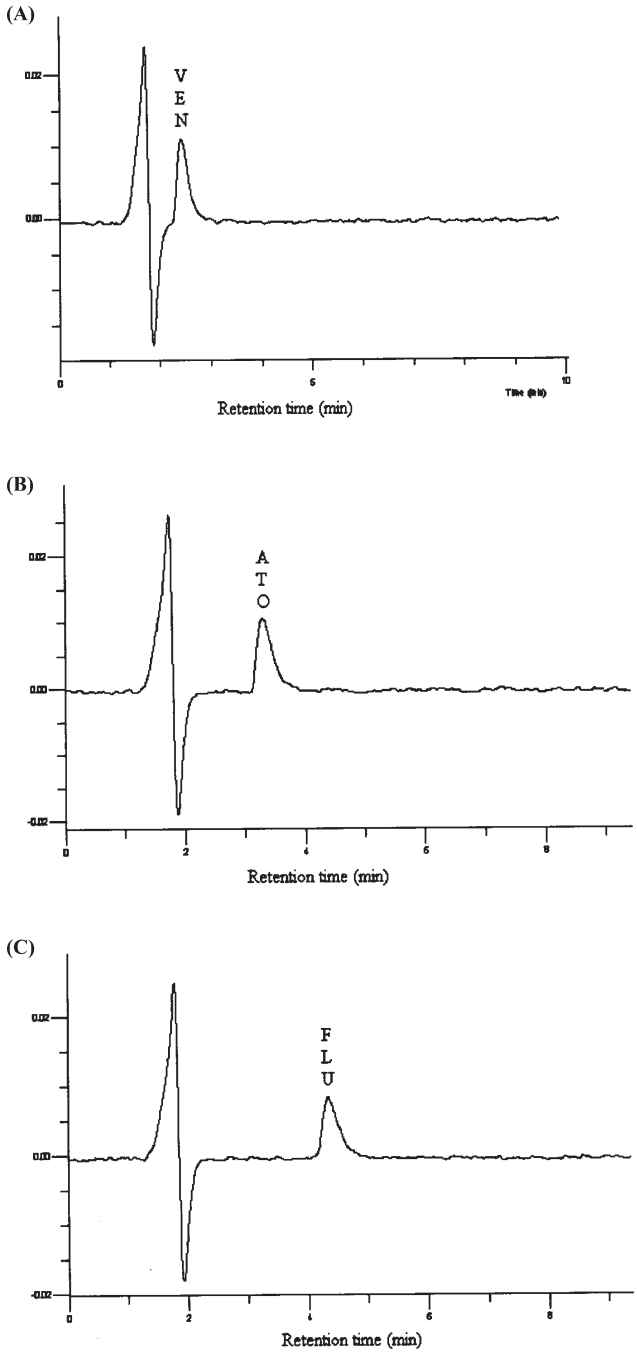

Figure 3. Typical chromatograms of formulations of A) venlafaxine (VEN), B) atomoxetine (ATO), and C) fluoxetine (FLU).

(20 mg of FLU). The samples were prepared as described in the experimental section. The content of each drug in the capsules was determined by five injections of each, on five independently prepared solutions. The results are recorded in Table 4. The chromatograms of VEN, ATO, and FLU are given in Figures 3A, 3B, and 3C, respectively. No interference was observed from the excipients present in the capsules. It could be seen from the results of Table 4, that the three drugs ATO, VEN, and FLU were successfully determined by using the present HPLC method.

CONCLUSION

An HPLC method was developed for the determination of ATO, VEN, and FLU in pharmaceutical formulations. The main advantage of the HPLC method is its rapidity and simplicity; using a single HPLC condition, one can determine three drugs in formulations without changing the experimental conditions. The three drugs elute within 5 min. Due to its simplicity, rapidity, and selectivity, the method could be used routinely in quality control laboratories of pharmaceutical industries.

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