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LC determination and purity evaluation of nefazodone HCl in bulk drug and pharmaceutical formulations

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Abstract

A simple, selective and reproducible reversed-phase liquid chromatography (LC) method has been developed for the quantitative determination of nefazodone hydrochloride (I) in the presence of its related impurities, namely 5-ethyl-4-(2-phenoxyethyl)-2*H*-1,2,4-triazol-3-(4*H*)one (II), 1-(3-chlorophenyl)-4-(3-chloropropyl) piperazine hydrochloride (III) and $1,1^1$-trimethylene-bis[4-(3-chlorophenyl) piperazine] hydrochloride (IV). The separation was achieved using an Inertsil ODS-3V (250×4.6 mm^2) column and a mobile phase comprising 0.05 M KH_2PO_4 (pH 3.0), acetonitrile and methanol in the ratio 50:40:10 (v/v/v). The method has been completely validated and proven to be rugged. The limit of detection and limit of quantification for impurities II, III and IV were found to be 50, 79 and 91 ng/ml, and 152, 240 and 280 ng/ml, respectively. The intra- and inter-day assay precision of the method was within 1.2% relative standard deviations. The developed method was applied to the pharmaceutical dosage form (Tablet, Serzone-R) and the percentage recoveries ranged from 99.1 to 100.7. The percentage recovery of impurities ranged from 96.2 to 108.9. The stability studies were performed for nefazodone solution placed on laboratory bench and in the refrigerator for 60 days. The method was proved to be stability indicating in solution. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Nefazodone; Reversed phase; LOD; LOQ; Tablet; RSD

1. Introduction

Nefazodone hydrochloride is an antidepressant [1]. It acts both as a 5-hydroxytrytamine (5-HT) re-uptake inhibitor and a postsynaptic 5-HT2-receptor antagonist without cardiotoxicity or sedative side effects [2]. It increases prolactin levels and oral temperature by downregulation of 5-HT1C receptors or 5-HT1C receptor blockade [3]. The clinical antidepressant activity of nefazodone is attributed to the potentiation of the central serotonergic system. It is a strong inhibitor of neuronal uptake of serotonin and norephinephrine [4,5].

In the open literature, only two liquid chromatography (LC) methods were reported for the quantitative determination of nefazodone and its metabolites hydroxy nefazodone, *m*-chlorophenyl piperazine and triazole-dione in human plasma

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[6,7]. But no LC methods were reported for the purity evaluation and quantitative determination of nefazodone in bulk drugs and pharmaceutical dosage forms. This developed method is used for the purity evaluation and quantitative determination of nefazodone in bulk drugs and pharmaceutical dosage forms.

2. Experimental

2.1. Chemicals

Samples of nefazodone, 5-ethyl-4-(2-phenoxyethyl)-2*H*-1,2,4-triazol-3-(4*H*)one (II), 1-(3-chlorophenyl)-4-(3-chloropropyl) piperazinehydrochloride (III) and $1,1^1$-trimethylene-bis[4-(3-chlorophenyl) piperazine] hydrochloride (IV) were received from Process R&D, Dr Reddy's Research Foundation, Hyderabad, India. Five tablets of Serzone-R (Bristol-Mayer) were purchased from the market. Serzone-R is supplied as white hexagonal tablet weighing about 575 mg containing 250 mg active pharmaceutical ingredient (API) i.e. nefazodone hydrochloride, and the rest excepients namely microcrystalline cellulose, Providone, sodium starch glycolate, colloidal silicon dioxide and magnesium stearate. High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Merck, USA. Analytical reagent-grade potassium dihydrogen phosphate was purchased from s.d.-Fine Chemicals Ltd., India. Milli-Q water was prepared using the Milli Q plus purification system (Millipore, USA).

2.2. Equipment

The LC system, used in Laboratory A, consisted of a waters 600 solvent delivery system, a Rheodyne injector (7725i) fitted with a 10 μl loop, and a waters 486 tunable absorbance detector. The LC system used in Laboratory B consisted of a Perkin-Elmer series 200 LC solvent delivery system, a Rheodyne injector (7725i) fitted with a 10 μl loop and a waters 996 PDA detector. The output signal was monitored and processed using MILLENNIUM 2010 chromatography manager software (Waters) on a Pentium computer (Digital Equipment Co).

2.3. Chromatographic conditions

The chromatographic column used was a 250 × 4.6 mm^2 Inertsil ODS-3V with 5 μm particles. The mobile phase was aqueous potassium dihydrogen phosphate (0.05 M, pH 3.0), acetonitrile and methanol (50:40:10, v/v/v). The flow rate was 1.0 ml/min. The column was maintained at ambient temperature and the eluant was monitored at a wavelength of 220 nm. The injection volume was 10 μl.

2.4. Sample preparation

The stock solution of nefazodone (2.5 mg/ml) was prepared by dissolving the appropriate amount of the substance in mobile phase. Nefazodone solutions were prepared from the stock solution by taking 0.4, 0.6, 0.8, 1.0, and 1.2 ml in 5.0 ml volumetric flasks and making up to the volume with mobile phase.

3. Results and discussion

3.1. Method development

To develop a rugged and suitable LC method for the quantitative determination of nefazodone (I), different mobile phases and stationary phases were employed. Our preliminary trails using different compositions of mobile phases consisting of water, acetonitrile and methanol on different reversed-phase stationary phases did not give good peak shape and resolution between I, II, III and IV. Introduction of buffer (potassium dihydrogen phosphate) improved the peak shape of nefazodone and the related impurities. When the mobile phase consisting of 0.01 M KH_2PO_4 and acetonitrile in the ratio of 50:50 early elution was observed for nefazodone and related impurities, and the resolution between III and IV was poor. With the introduction of 10% methanol in the mobile phase, the separation was improved between III and IV. With the increase in buffer

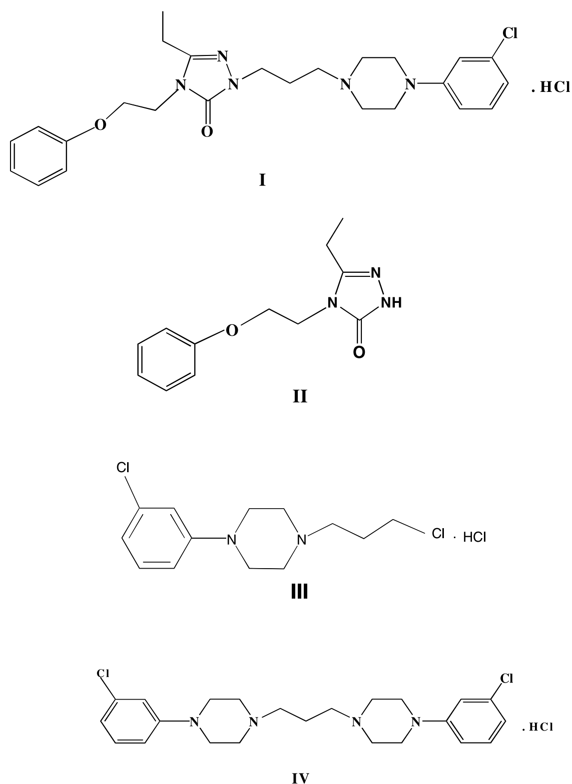

Fig. 1. Chemical structures of nefazodone (I) and its impurities II, III and IV.

concentration (0.05 M) of the mobile phase, the retention and the peak shape were improved for I, II, III, and IV, and the resolution between III and IV was further improved. Finally, by fixing buffer at pH 3.0, III and IV were resolved to the baseline. Above that pH value, there was a decrease in resolution between III and IV. The proposed conditions were found suitable for the purity evaluation and determination of nefazodone hydrochloride in bulk drugs and pharmaceutical formulations. Nefazodone hydrochloride and its related impurities, namely II, III and IV, were carried out using proposed chromatographic conditions. The chemical structures of I, II, III and IV are shown in Fig. 1.

Using the aforementioned column, the resolution between nefazodone and its impurities was found to be not less than 4, and all the known impurities were eluted in about 10 min. The system-suitability results are presented in Table 1. The retention times of I, II, III and IV were 8.6, 6.1, 4.6 and 3.6 min, respectively (Fig. 2).

3.2. Method validation

3.2.1. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of all the potential impurities.

For the specificity determination, 10 mg each of II, III, and IV (0.2 mg/ml) were weighed in a 50 ml volumetric flask containing 20 mg pure nefazodone (0.4 mg/ml) and the response of the analyte in the mixture was compared with the response of the pure nefazodone. It was found that the assay results were not changed in the presence of impurities. The assay results are presented in Table 2. Specificity was checked by stressing the pure nefazodone sample under UV light (254 nm) and temperature 70 ± 1 °C for 6 h. Specificity was also checked by applying some extreme conditions, such as refluxing the sample with 0.5 N HCl, 2.0 N NaOH, and 3% H_2O_2 solutions at 60 ± 1 °C for 6 h. The HPLC analysis was carried out at zero time and at an interval of 2 h. Nefazodone was not degraded when exposed to UV light (254 nm), temperature 70 ± 1 °C, and with 0.5 N HCl for 6 h. A new impurity, which is

Table 1
System-suitability report

Compound ($n = 3$)	Capacity factor	Resolution	Tailing factor	Number of theoretical plates
I	7.6	5.1	1.2	3962
II	5.1	4.9	1.1	4287
III	3.6	2.3	1.2	2914
IV	2.6	–	1.4	1708

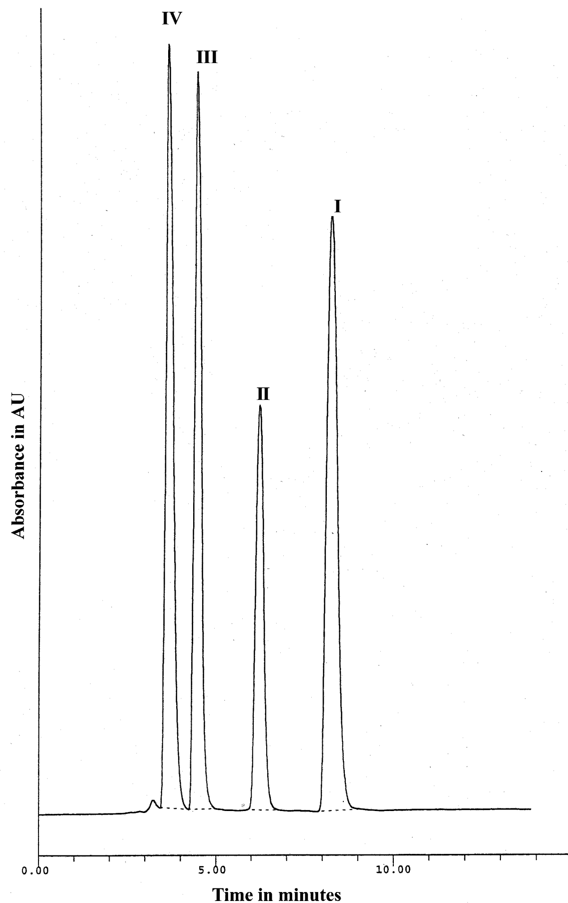

Fig. 2. HPLC chromatogram of nefazodone (0.4 mg/ml) spiked with its related substances.

Table 2
Specificity results of the method

	Pure sample	Sample spiking with all the impurities
	101.2	98.7
	100.4	99.3
	100.1	99.6
Mean	100.6	99.2
S.D.	0.6	0.4
% RSD	0.6	0.5

different from these three known impurities, was observed immediately after the addition of 3% H_2O_2. The extent of formation was 7, 17 and 30% for 2, 4, and 6 h, respectively. No degradation products were seen immediately after the addition of 2 N NaOH. When refluxed with 2 N NaOH, the sample was degraded with time and the extent of degradation was 3, 5 and 9% for the same intervals. The degradation products formed were well resolved from nefazodone. In the formulation samples of nefazodone, it was noticed that excipient peaks did not interfere with the peaks of interest (Fig. 3). This was confirmed by the peak purity results obtained by photodiode array detector. Hence, the method can be considered a stability indicating method.

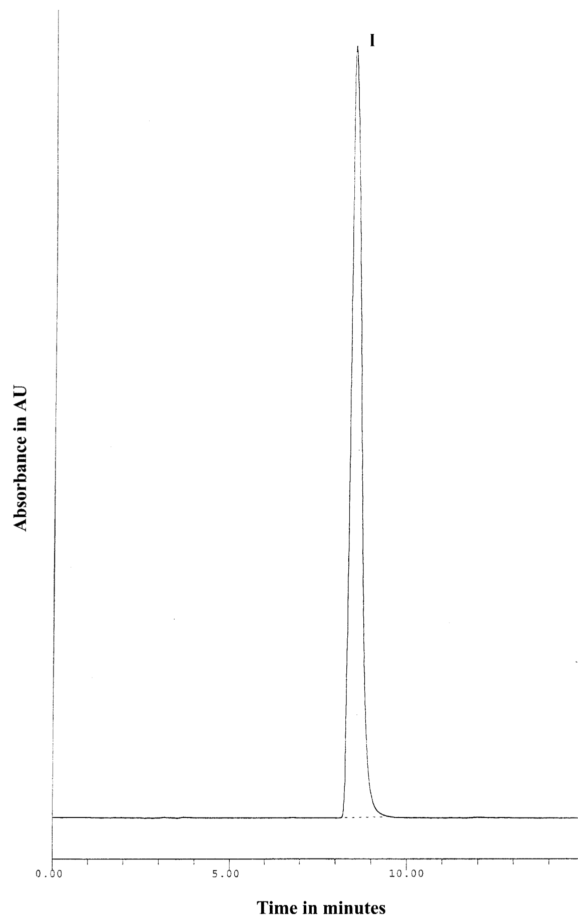

Fig. 3. HPLC chromatogram of formulated nefazodone (0.4 mg/ml).

Table 3
Intro-day and inter-day assay variations of nefazodone

	Mean of concentration (mg/ml) (n = 3)	S.D.	% RSD
Intra-day			
0 day	0.3341	0.0013	0.4
	0.4014	0.0012	0.3
	0.4795	0.0015	0.3
1 day	0.3302	0.0020	0.6
	0.4015	0.0017	0.4
	0.4791	0.0024	0.5
2 day	0.3289	0.0021	0.6
	0.4094	0.0020	0.5
Inter-day			
	0.4892	0.0036	0.7
	0.3310	0.0027	0.8
	0.4041	0.0045	1.1
	0.4826	0.0057	1.2

3.2.2. Linearity

The target analyte concentration of nefazodone was fixed at 0.4 mg/ml. The calibration curve for nefazodone was drawn by plotting the peak area ratio of nefazodone versus concentration of nefazodone, and yielded the coefficient of regression (r^2) of 0.9995 over the concentration range 0.2–0.6 mg/ml. The regression equation for nefazodone was $y = 19\,680\,269x - 36\,765$. Linearity was checked for three consecutive days for the same concentration range from the same stock solutions. The average slope value of nefazodone was $19\,680\,269 \pm 250$, and the relative standard deviation (RSD) value of intercept for the calibration curve was 4.5%.

3.2.3. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by assaying freshly prepared solutions in triplicate at concentrations of 0.32, 0.40, and 0.48 mg/ml nefazodone. The RSD ranged from 0.3 to 0.7% (Table 3). Inter-day precision and accuracy of the method calculated from the individual recovery data were evaluated by assaying freshly prepared solutions, in triplicate, for 3 days. The RSD ranged from 0.8 to 1.2% (Table 3). The accuracy results in terms of percentage recoveries are presented in Table 4.

3.2.4. Assay of nefazodone in the formulation sample

Five tablets of nefazodone (Serzone-R, 250 mg; Bristol-Mayer) were finely ground using an agate mortar and pestle. The ground material, containing the API, equivalent to 40 mg was weighed and transferred in a 25 ml volumetric flask and extracted into mobile phase by vortex mixing followed by ultrasonication. The resultant mixture was filtered through a 0.45 μm membrane filter. The filtrate was used as a stock solution for preparing test solutions. Test solutions were prepared in triplicate, taking 1 ml stock solution into a 5 ml volumetric flask and making up to the volume with the mobile phase. This solution would correspond to the API concentration of 0.32 mg/ml. Similar experiments were carried out to prepare two more test solutions that would contain API at concentrations of 0.40 and 0.48 mg/ml solution, respectively. The percentage re-

Table 4
Accuracy in the assay determination of nefazodone

Day of analysis	Taken (mg)	Recovered (mg) ($n = 3$)	% Recovery[a]	%RSD
0 day	0.3326	0.3341	100.4	0.4
	0.4026	0.4014	99.7	0.3
	0.482	0.4795	99.5	0.3
1 day	0.3282	0.3302	100.6	0.6
	0.4058	0.4015	98.9	0.4
	0.4854	0.4791	98.7	0.5
2 day	0.3315	0.3289	99.2	0.6
	0.4045	0.4094	101.2	0.5
	0.4824	0.4892	101.4	0.7

[a] % Recovery, Percent recovery of nefazodone from the sample against that taken.

Table 5
Assay results of formulations of nefazodone

S.NO	Concentration of nefazodone (mg/ml)		% Recovery	% RSD
	Recovered	Taken		
I				
1	0.3250	0.3265	99.5	0.8
2	0.3287	0.3265	100.7	
3	0.3235	0.3265	99.1	
II				
1	0.4113	0.4120	99.8	0.2
2	0.4124	0.4120	100.1	
3	0.4113	0.4120	99.8	
III				
1	0.4850	0.4820	100.6	0.5
2	0.4846	0.4820	100.5	
3	0.4810	0.4820	99.8	

coveries were ranged from 99.1 to 100.7% (Table 5).

3.2.5. Limit of detection and limit of quantitation

The limit of detection (LOD) represents the concentration of analyte that would yield a signal-to-noise ratio of 3 [8]. The LODs for II, III and IV were 50,79 and 91 ng/ml for 10 μl injection volume.

The limit of quantitation (LOQ) represents the concentration of analyte that would yield a signal-to-noise ratio of 10 [8]. The LOQs for II, III and IV were 152, 240 and 280 ng/ml for 10 μl injection volume.

3.2.6. Ruggedness

The ruggedness of an assay method is defined as the degree of reproducibility of assay results obtained by analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysts, different instruments, and different lots of reagents. The same samples of three concentration levels in triplicate of day 3 were analysed in Laboratory B with a different instrument (Perkin-Elmer series 200 LC solvent delivery system, a Rheodyne injector (7725i) fitted with a 10 μl loop, and a waters 996 PDA detector) by a different analyst. The data obtained from Laboratory B is well in agreement with the data of day-3 results obtained in Laboratory A.

3.2.7. Robustness

The robustness of a method is the ability to remain unaffected by small changes in parameters such as pH of the mobile phase, temperature, percentage organic solvent strength, and buffer concentration. To determine the robustness of the method, experimental conditions were altered and chromatographic characteristics were evaluated.

In the mobile phase buffer, the pH was 3.0. To study the pH effect on the resolution of nefazodone and its impurities II, III and IV, the buffer pH was changed by an increment of 0.1 U from 2.8 to 3.2. At pH 2.8, the resolution between III and IV was 2.9. By increasing the pH of the buffer, the resolution between III and IV was decreased and, at pH 3.2, it was 1.9. The effect of temperature on the retention characteristic (k) of nefazodone, II, III and IV was studied by changing the temperature in steps of 2 °C from 24 to 32 °C. A slight decrease in k was observed with an increase in temperature. Variation in temperature did not have a significant effect on resolution and peak shape. Effect of percent organic strength on retention was studied by varying the percentage of acetonitrile from -4 to $+4$%, while the other mobile phase components were held constant as stated in Section 2.3. The increase of percentage of acetonitrile in the mobile phase decreased the retention of nefazodone (from 12 to 7 min), while a slight decrease in retention of II, III, and IV was observed. Effect of buffer concentration was checked at five concentration levels, i.e. 0.01, 0.025, 0.04, 0.05, and 0.06 M. An increase in retention of nefazodone (from 5 to 9 min) was observed with the increase in buffer concentration of the mobile phase, whereas a slight increase in retention was observed for II, III, and IV.

3.2.8. Stability

The stability of 0.4 mg/ml solutions of nefazodone was evaluated. The solutions were stored in a tightly capped volumetric flask, on a laboratory bench and in the refrigerator. Recovery of these solutions was checked for 60 days at

an interval of 10 days against freshly prepared solutions. The samples kept on the laboratory bench and in the refrigerator were found to be stable (Table 6). Therefore, the method was stability-indicating in solution.

3.2.9. Standard addition and recovery of impurities

Standard addition and recovery experiments were conducted to determine the accuracy of the present method, for the quantification of impurities. The range of addition levels of impurities was 0.05–2.5 wt.% of the target analyte concentration [9]. The range of addition levels of impurities used in this study was 0.05–1.0 wt.%, and the recovery of each impurity was calculated from the slope and the intercept of the calibration curve drawn in the concentration range 200–4000 ng/ml. The response factors for impurities II, III and IV were found to be 0.95, 0.70 and 0.62, respectively. The equations for the calibration curves for impurities II, III and IV were $y = 20.00x - 221.72$, $y = 20.41x - 112.21$ and $y = 19.97x + 175.61$, respectively. The RSD values of the slopes for the calibration equations of II, III and IV were 1.2, 3.4 and 5.3, respectively. The RSD values of the intercepts for the calibration equations of II, III and IV were 2.3, 4.6 and 6.7, respectively. The percentage recoveries of impurities ranged from 96.2 to 108.9 (Table 7).

4. Conclusion

An isocratic reversed-phase LC method has been described for the determination of nefazodone hydrochloride in bulk drugs and pharmaceutical formulations. The method was also

Table 6
Assay results of stability studies of nefazodone solution

Day	Concentration of nefazodone (mg/ml) placed on bench top		% Recovery	% RSD[a]	Concentration of nefazodone (mg/ml) placed in refrigerator		% Recovery	% RSD[a]
	Recovered	Taken			Recovered	Taken		
0	0.4033	0.4058	99.4		0.4042	0.4058	99.6	
10	0.4052	0.4058	99.8		0.4050	0.4058	99.8	
20	0.4041	0.4058	99.6		0.4065	0.4058	100.2	
30	0.4068	0.4058	100.2		0.4072	0.4058	100.3	
40	0.4061	0.4058	100.1	0.5	0.4030	0.4058	99.3	0.5
50	0.4029	0.4058	99.3		0.4069	0.4058	100.3	
60	0.4070	0.4058	100.3		0.4023	0.4058	99.1	

[a] Calculated for seven determinations.

Table 7
Recovery of nefazodone impurities

Compound	Added (μg) (n = 3)	Recovered (μg)	% Recovery	% RSD
II	0.4530	0.4856	107.2	1.2
	0.8520	0.9281	108.9	0.8
	1.2725	1.3628	107.1	0.6
III	0.4896	0.5126	104.7	0.5
	0.8431	0.8534	101.2	1.3
	1.3143	1.3625	103.6	1.4
IV	0.4188	0.4373	104.4	1.3
	0.8881	0.8547	96.2	0.7
	1.3752	1.3861	100.8	0.4

applicable for the purity evaluation of nefazodone. The method was developed with a simple buffer when compared with other developed methods where ion-pairing reagents were used, which decreases the lifetime of the HPLC column. The stability of the method was tested for 60 days and found to be stable. The method was extensively validated and it was found to be rugged and robust.

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