ORIGINAL ARTICLE

Simultaneous determination of nortriptyline hydrochloride and fluphenazine hydrochloride in microgram quantities from low dosage forms by liquid chromatography–UV detection

Safwan Ashour*, Nuha Kattan

Analytical Biochemistry Laboratory, Department of Chemistry, Faculty of Science, University of Aleppo, Aleppo, Syria

Abstract   A novel method for the simultaneous high-performance liquid chromatographic determination of nortriptyline hydrochloride and fluphenazine hydrochloride was developed and validated. Fluvastatin sodium was used as internal standard. The determination was performed on a Hypersil Gold C_8 column (250 mm × 4.6 mm i.d., 5 μm particle size) at 25 °C; the mobile phase, consisting of a mixture of formic acid (0.1 M, pH 2.16)-methanol (33:67, v/v), was delivered at a flow rate of 1.1 mL/min and detector wavelength at 251 nm. The retention time of nortriptyline, fluphenazine and fluvastatin was found to be 5.11, 8.05 and 11.38 min, respectively. Linearity ranges were 5.0–1350.0 and 10.0–1350.0 μg/mL with limit of detection values of 0.72 and 0.31 μg/mL, for nortriptyline and fluphenazine, respectively. Results of assay and recovery studies were statistically evaluated for its accuracy and precision. Correlation coefficients (r^2) of the regression equations were greater than 0.999 in all cases. According to the validation results, the proposed method was found to be specific, accurate, precise and could be applied to the simultaneous quantitative analysis of nortriptyline and fluphenazine.

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1.   Introduction

Nortriptyline, 3-(10, 11-dihydro-5H-dibenzo [a,d] cyclohepten-5-ylidene)-N-methyl-1-propanamine, is a tricyclic antidepressant drug widely used in the treatment of unipolar depression, since it is a non-selective serotonine uptake inhibitor [1]. Fluphenazine, 2-[4-[3-[2-(trifluoromethyl)-10H-phenothiazin-10-yl] propyl] piperazin-1-yl] ethanol dihydrochloride, is an antipsychotic drug in the phenothiazine class of compounds. The N-alkyl side chain is modified from a base phenothiazine structure [2].

Literature survey reveals that both nortriptyline hydrochloride and fluphenazine hydrochloride are official in British

*Corresponding author. Tel.: +963 933 604016.

E-mail address: profashour2010@myway.com (S. Ashour).

Pharmacopeia [2]. Few chromatographic methods for the determination of nortriptyline hydrochloride and fluphenazine hydrochloride in pharmaceutical preparations [3,4] or with other active ingredients [5] have been reported. Other techniques for the determination of the latter compounds in different pharmaceutical preparations have been developed including spectrophotometry [4,6–8], polymeric membrane electrodes [9], electrochemical [10] and HPTLC [11]. An increasing number of publications are appearing describing a chromatographic methods for the determination of nortriptyline hydrochloride and fluphenazine hydrochloride with other active ingredients in biological samples [12–20].

The purpose of the current study was to develop a sensitive, accurate and comparatively simple method for simultaneous quantification of nortriptyline hydrochloride and fluphenazine hydrochloride in raw materials and pharmaceutical formulations by HPLC.

2. Experimental

2.1. Materials

Working reference standards of nortriptyline hydrochloride (NOR), fluphenazine hydrochloride (FLU) and fluvastatin sodium (FVS) were supplied by Vasudha Pharma Chem Limited, Centaur Chemicals Private Limited (India) and Zhejiang Materials Industry Chemical Group Co., Ltd. (China), respectively. The structures of these compounds are shown in Fig. 1. Methanol and water (HPLC grade) were purchased from Labscan (Ireland). Formic acid (analytical grade) was purchased from Surechem Products Ltd. (England). Tablets were purchased from Syrian market, containing nortriptyline hydrochloride 10 mg and fluphenazine hydrochloride 0.5 mg per tablet.

2.2. HPLC system

The chromatographic system consisted of Hitachi (Japan) model L-2000 equipped with a binary pump (model L-2130, flow rate range 0.000–9.999 mL/min), degasser and a column oven (model L-2350, temperature range 1–85 °C). All samples were injected (10 µL) using a Hitachi L-2200 autosampler (injection volume range 0.1–100 µL). Elutions of all analytes were monitored at 251 nm using a Hitachi L-2455 absorbance detector (190–900 nm) containing a quartz flow cell (10 mm path and 13 µL volume). Each chromatogram was analyzed and integrated automatically using the Ezchrom Elite Hitachi Software.

2.3. Chromatographic conditions

Separation was achieved on a reversed phase Hypersil Gold C_8 column (250 mm × 4.6 mm, 5 µm particle size, Thermo

Germany) at 25 °C. The mobile phase was a mixture of methanol and 0.1 M formic acid (67:33, v/v) with a flow rate of 1.1 mL/min and was filtered and degassed by ultrasonic agitation before use. The injection volume was 10 µL.

2.4. Standard solutions

Standard stock solutions of nortriptyline hydrochloride (3.0 mg/mL) and fluphenazine hydrochloride (3.0 mg/mL) were prepared by direct weighing of standard substance with subsequent dissolution in methanol. Stock standard solution of fluvastatin sodium (1.0 mg/mL) was prepared by dissolving appropriate amount of the compound in methanol. These solutions were stored in the dark at 2–8 °C and found to be stable for three weeks at least.

2.5. Calibration graphs

A series of working standard drug solutions equivalent to 5.0–1350.0 µg/mL for NOR and 10.0–1350.0 µg/mL for FLU were prepared by diluting the stock standard solution with the methanol. In each sample 1 mL of FVS was added (100 µg/mL in the final volume). To construct the calibration curve five replicates (10 µL) of each standard solution were injected immediately after preparation into the column and the peak area of the chromatograms was measured. Then, the mean peak area ratio of NOR and FLU to that of the internal standard was plotted against the corresponding concentration to obtain the calibration graph.

2.6. Assay procedure for dosage forms

Twenty tablets containing NOR and FLU were weighed and finely powdered. Portions of the powder (each equivalent to the weight of five tablets) were accurately weighed into 50 mL volumetric flasks and 30 mL methanol was added. The volumetric flasks were sonicated for 15 min to effect complete dissolution of NOR and FLU, the solutions were then made up to volume with methanol. The sample solutions were filtered through 0.45 µm nylon filter. The aliquot portions of the filtrate were further diluted to get a final concentration of 900 µg/mL of NOR and 45 µg/mL of FLU in the presence of 100 µg/mL of internal standard. Finally, 10 µL of each diluted sample was injected into the column and chromatogram was recorded. Peak area ratios of NOR and FLU to that of FVS were then measured for the determination. NOR and FLU concentrations in the samples were then calculated using peak data and standard curves.
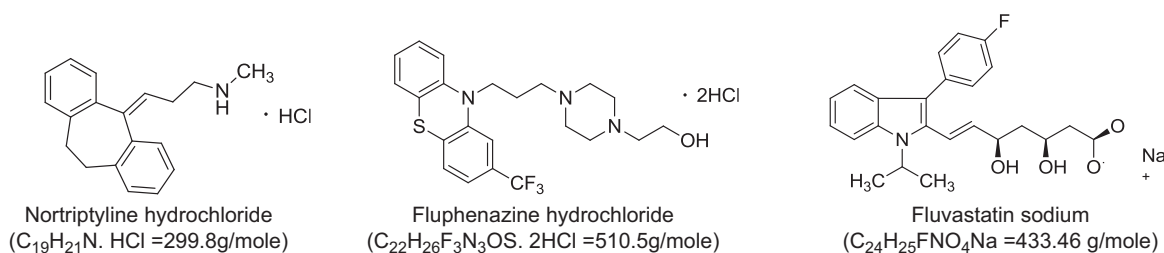

Nortriptyline hydrochloride
($C_{19}H_{21}N \cdot HCl = 299.8$ g/mole)

Fluphenazine hydrochloride
($C_{22}H_{26}F_3N_3OS \cdot 2HCl = 510.5$ g/mole)

Fluvastatin sodium
($C_{24}H_{25}FNO_4Na = 433.46$ g/mole)

Figure 1   Chemical structure of nortriptyline hydrochloride, fluphenazine hydrochloride and fluvastatin sodium (I.S.).

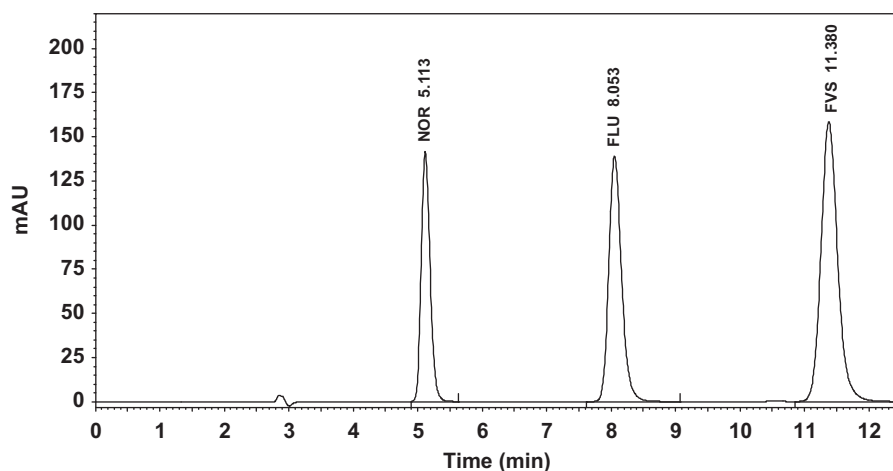**Figure 2** A typical chromatogram of a mixture of NOR (75 μg/mL), FLU (75 μg/mL) and FVS (100 μg/mL) at retention times 5.113, 8.053 and 11.380 min, respectively. Chromatographic conditions: RP-HPLC on C_8 column; mobile phase: formic acid (0.1 M, pH 2.16) and methanol (33:67, v/v); flow rate 1.1 mL/min and detection at 251 nm.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

During the analysis of drugs like NOR and FLU, one of the well known problem in pharmaceutical industry is peak tailing. Since these compounds strongly interact with polar ends of HPLC column packing materials, causing severe peak asymmetry and low separation efficiencies. High purity silica backbone and advances in bonding technology have alleviated the tailing problem of polar compounds in HPLC to a significant extent. During the optimization of the method, different columns (Nucleodur C_8, 250 mm × 4.6 mm, 5 μm; Nucleodur C_{18}, 250 mm × 4.6 mm, 5 μm; Hypersil Gold C_8, 250 mm × 4.6 mm, 5 μm and ODS Hypersil C_{18}, 250 mm × 4.6 mm, 5 μm) and two organic solvents (acetonitrile and methanol) were tested. The chromatographic conditions were also optimized using different buffers like phosphate, acetate and citrate for mobile phase preparation. After a series of screening experiments, it was concluded that formic acid gave better peak shapes than the other buffers. With acetonitrile as solvent both the peaks showed less theoretical plates and more retention time compared to methanol. The chromatographic separation was achieved on a Hypersil Gold C_8 (250 mm × 4.6 mm, 5 μm column) at 25 °C, using a mixture of methanol–formic acid (0.1 M, pH 2.16; 67:33, v/v) as mobile phase, and the peak shape of NOR, FLU and FVS was found symmetrical (Fig. 2). The retention time of NOR, FLU and FVS was 5.280, 8.320 and 11.793 min, respectively. The effect of composition of the mobile phase and flow rate on the retention time of NOR, FLU and FVS, was investigated. Results of the effect of methanol in the mobile phase are presented in Fig. 3. An increase in the percentage of methanol decreases the retention of compounds, namely, NOR, FLU and FVS. Increasing methanol percentage to more than 70% NOR and FLU peaks are eluted with the solvent front, while at methanol percentage lower than 60% the elution of FLU peak is seriously delayed. The optimum methanol percentage was found to be 67%. The effect of pH in the chromatographic elution of the compounds was also investigated by changing the concentration values of the aqueous component of the mobile phase from 0.03 to 0.15 M. For all experimental concentration values, the drugs are eluted in order of
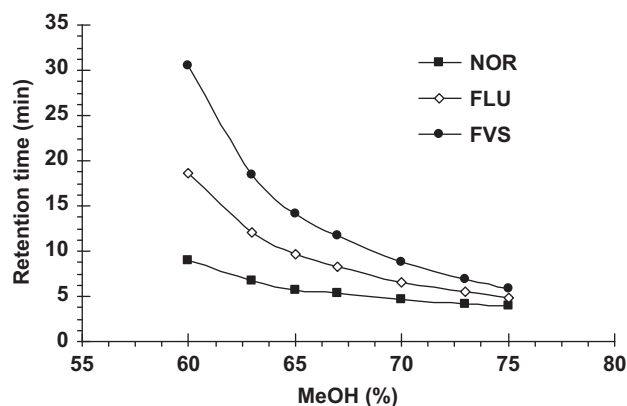

Figure 3 Plots of the retention time vs. methanol percentage in the mobile phase of NOR, FLU and FVS.

NOR, FLU and FVS. A concentration value of 0.1 M HCOOH was chosen for the optimum separation of the compounds, as at this concentration the analyte peaks were well defined and resolved. The optimum wavelength for detection was at 251 nm, at which the best detector responses for all substances were obtained.

3.2. System suitability

The system suitability was determined by making seven replicate injections and analyzing each solute for their peak area, resolution and peak tailing factor. The system suitability requirements for 75 μg/mL of NOR and 75 μg/mL of FLU in the presence of 100 μg/mL of internal standard were a %RSD for peak area less than 0.35, a peak tailing factor less than 1.2 and a resolution factor greater than 8.0 between adjacent peaks for all analytes. This method met these requirements.

3.3. Linearity and limits of quantification and detection

The calibration curves for NOR and FLU were linear over the concentration range 5.0–1350.0 μg/mL and 10.0–1350.0 μg/mL, respectively, as shown in Table 1. Correlation coefficients (r) of

Table 1 Calibration data for the estimation of nortriptyline hydrochloride and fluphenazine hydrochloride by HPLC.

Parameters	Nortriptyline hydrochloride	Fluphenazine hydrochloride
Optimum concentration range (µg/mL)	5.0–1350.0	10.0–1350.0
Regression equation[*]	$A_{NOR}=6.812C_{NOR}+39.867$	$A_{FLU}=10.217C_{FLU}+7.824$
Correlation coefficient ($n=5$)	0.9997	0.9998
Standard deviation of slope	0.015	0.034
Standard deviation of intercept	1.633	1.051
Regression equation[**]	$R_{NOR/FVS}=0.0063C_{NOR}+0.0376$	$R_{FLU/FVS}=0.0094C_{FLU}+0.0082$
Correlation coefficient ($n=5$)	0.9997	0.9998
Standard deviation of slope	0.0001	0.0003
Standard deviation of intercept	0.0028	0.0064
Limit of quantification, LOQ (µg/mL)	2.41	1.04
Limit of detection, LOD (µg/mL)	0.72	0.31

[*]Regression equation for the peak area of drug vs. concentration of drug in µg/mL.
[**]Regression equation for the ratio of peak area of drug to that of I.S. (FVS) vs. concentration of drug in µg/mL.

Table 2 Accuracy and precision of within and between run analysis for the determination of nortriptyline hydrochloride and fluphenazine hydrochloride by HPLC.

Compound	Nominal concentration (µg/mL)	Intra-day ($n=5$)			Intra-day ($n=5$)		
		Mean±SD (µg/mL)	RSD (%)	Recovery (%)	Mean±SD (µg/mL)	RSD (%)	Recovery (%)
Nortriptyline	5.00	5.09±0.07	1.29	101.76	5.08±0.06	1.27	101.56
	35.00	35.78±0.28	0.78	102.23	35.97±0.41	1.14	102.77
	75.00	77.00±0.39	0.50	102.67	75.68±0.79	1.04	100.91
	150.00	151.45±0.48	0.32	100.97	151.12±1.44	0.95	100.75
	300.00	311.75±0.61	0.20	103.92	300.90±2.56	0.85	100.30
	900.00	917.58±1.15	0.13	101.95	900.91±6.57	0.73	100.10
	1350.00	1350.55±0.97	0.07	100.04	1355.52±2.45	0.18	100.41
Fluphenazine	10.00	9.99±0.06	0.64	99.90	9.95±0.13	1.32	99.50
	35.00	36.34±0.21	0.59	103.82	35.37±0.41	1.16	101.05
	75.00	75.93±0.32	0.43	101.24	75.02±0.78	1.04	100.02
	150.00	150.81±0.49	0.32	100.54	151.25±1.35	0.89	100.83
	300.00	303.05±0.73	0.24	101.02	300.94±2.01	0.67	100.31
	900.00	903.80±1.84	0.20	100.42	899.74±4.79	0.53	99.97
	1350.00	1350.98±2.49	0.18	100.07	1348.54±1.90	0.14	99.89

the regression equations were greater than 0.999 in all cases. The minimum level at which the investigated compounds can be reliably detected (limit of detection, LOD) and quantified (limit of quantitation, LOQ) was determined experimentally. The LOD was expressed as the concentration of drug that generated a response to three times of the signal-to-noise (S/N) ratio, and the LOQ was 10 times of the S/N ratio. The LOD of NOR and FLU attained as defined by IUPAC [21], $LOD_{(k=3)}=k \times S_a/b$ (where b is the slope of the calibration curve and S_a is the standard deviation of the intercept), was found to be 0.72 and 0.31 µg/mL, respectively. The LOQ was also attained according to the IUPAC definition, $LOQ_{(k=10)}=k \times S_a/b$, and was found to be 2.41 and 1.04 µg/mL, correspondingly.

3.4. Accuracy and precision

The precision and accuracy of the method were determined by analysis of seven samples for drugs mixture. The specificity of the

chromatographic method was determined to ensure separation of NOR, FLU and the internal standard as shown in Fig. 2. Intraday assay variation was evaluated by injecting these samples in replicates of five in the same day. Interday assay variation was evaluated by injecting these samples in replicates of five on 5 different days from 1 to 25 after preparation. The standard deviation, relative standard deviation and recovery of different amounts tested were determined. The accuracy of the method is indicated by the excellent recovery and the precision is supported by the low standard deviation, as recorded in Table 2.

3.5. Application of the assay

The performance of the proposed method was assessed by comparison with the official method [4]. Mean values were obtained with a Student's t- and F-tests at 95% confidence limits for four degrees of freedom. The results showed
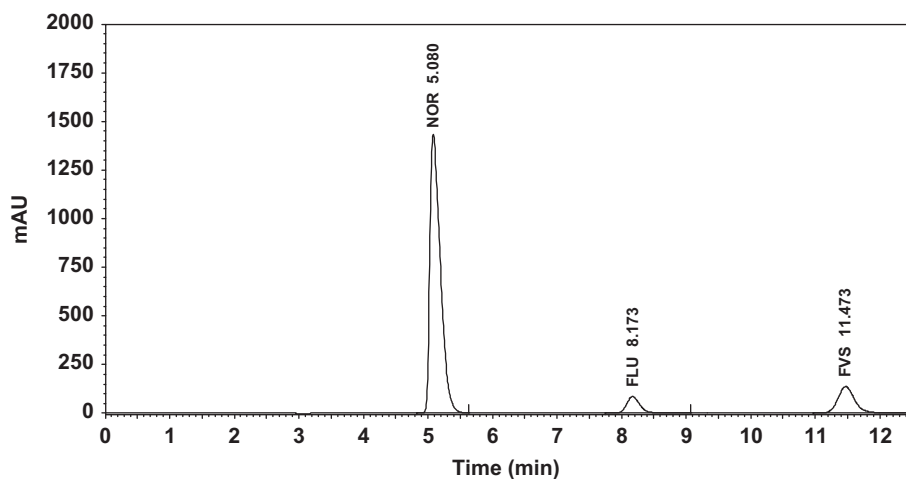
Figure 4 A typical chromatogram of a mixture of NOR (900 µg/mL), FLU (45 µg/mL) and the internal standard, FVS (100 µg/mL) in the mobile phase, prepared from Motival tablets. Chromatographic conditions: C_8 column; mobile phase: 0.1 M formic acid and methanol (33:67, v/v); flow rate 1.1 mL/min and detection at 251 nm.

Table 3 Determination of nortriptyline hydrochloride and fluphenazine hydrochloride in pharmaceutical formulations by the proposed method and official method.

Sample	Recovery (%)[a] ± SD			
	Nortriptyline hydrochloride		Fluphenazine hydrochloride	
	Proposed method	Official method	Proposed method	Official method
Pure	100.11 ± 0.12	101.50 ± 0.17	100.67 ± 0.32	101.29 ± 0.54
t-value	1.89		2.04	
F-value	2.00		2.85	
ADIVAL (10 mg NOR and 0.5 mg FLU/tablet)				
Mean ± SD[a]	103.07 ± 0.59	100.78 ± 0.60	101.40 ± 0.69	100.33 ± 0.94
t-value[b]	1.17	1.21	1.04	1.14
F-value[b]	1.03		1.86	
MOTIVAL (10 mg NOR and 0.5 mg FLU/tablet)				
Mean ± SD[a]	102.87 ± 0.39	100.65 ± 0.27	102.82 ± 0.82	101.08 ± 0.70
t-value[b]	1.37	1.09	1.24	1.33
F-value[b]	2.09		1.37	
NORTIVAL (10 mg NOR and 0.5 mg FLU/tablet)				
Mean ± SD[a]	104.84 ± 0.74	103.94 ± 0.51	103.50 ± 0.49	102.20 ± 0.36
t-value[b]	0.97	1.84	1.28	1.47
F-value[b]	2.11		1.85	

[a]Five independent analyses.
[b]Theoretical values for *t* and *F*-values at five degree of freedom and 95% confidence limit are $t = 2.776$ and $F = 6.26$.

comparable accuracy (*t*-test) and precision (*F*-test), since the calculated values of *t*- and *F*-tests were less than the theoretical data. The proposed procedures were applied to determine NOR and FLU in their pharmaceutical formulations (Fig. 4). The results in Table 3 indicate the high accuracy and precision. As can be seen from Table 3, the proposed method has the advantages of being virtually free from interferences by excipients such as glucose, lactose and starch or from common degradation products. The results obtained were compared statistically by the Student's *t*-test (for accuracy) and the variance ratio *F*-test (for precision) with those obtained by the official method for the samples of the same batch (Table 3). The values of *t*- and *F*-tests obtained at 95% confidence level did not exceed the theoretical tabulated value indicating no significant difference between the methods compared.

4. Conclusion

Conclusively, the HPLC method described in this paper is specific, sensitive, rapid and easy to perform. The proposed method enables simultaneous determination of nortriptyline hydrochloride and fluphenazine hydrochloride using fluvastatin sodium as internal standard with good separation and resolution of the chromatographic peaks. The sample recoveries from all formulations were in good agreement with their respective label claims, which suggested non-interference of formulations excipients in the estimation. Moreover, the present method is fast with respect to analysis time as compared to sophisticated spectrophotometric techniques. The method provided excellent specificity and linearity with a limit of quantification of 2.41 and 1.04 µg/mL and limit of detection of 0.72 and 0.31 µg/mL for NOR and FLU, respectively. The major advantage of this method is the wide range of linearity.

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