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Research Article

Development of LC Method for the Simultaneous Determination of Antidepressant Drug Combination Melitracen Hydrochloride and Flupentixol Dihydrochloride in their Combined Dosage Form

Usmangani K. Chhalotiya,[1] Kashyap K. Bhatt,[1] Dimal A. Shah,[1] Gautam R. Chauhan,[2] and Sunil L. Baldania[1]

[1] *Department of Pharmaceutical Chemistry and Analysis, Indukaka Ipcowala College of Pharmacy, Beyond GIDC, P.B. no. 53, New Vallabh Vidyanagar 388 121, India*
[2] *Sophisticated Instrumentation Centre for Applied Research and Testing, Vallabh Vidyanagar 388 120, India*

Correspondence should be addressed to Usmangani K. Chhalotiya, usmangani84@gmail.com

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A simple, specific and stability-indicating reversed-phase high-performance liquid chromatographic method was developed for the simultaneous determination of melitracen hydrochloride and flupentixol dihydrochloride in tablet dosage form. A Brownlee C-18, 5 μm column having 250 × 4.6 mm i.d. in isocratic mode, with mobile phase containing 0.025 M potassium dihydrogen phosphate: methanol (10 : 90, v/v; pH 7.3) was used. The flow rate was 1.0 mL/min, and effluents were monitored at 230 nm. The retention times of melitracen hydrochloride and flupentixol dihydrochloride were 7.75 min and 5.50 min, respectively. The linearity for melitracen hydrochloride and flupentixol dihydrochloride were in the range of 0.5–60 μg/mL. The recoveries obtained for melitracen hydrochloride and flupenthixol dihydrochloride was 99.81–100.77% and 99.42–100.12%, respectively. Both the drugs were subjected to acid and alkali hydrolysis, chemical oxidation, and dry heat degradation and photodegradation. The proposed method was validated and successfully applied to the estimation of melitracen hydrochloride and flupentixol dihydrochloride in combined tablet dosage form.

1. Introduction

Melitracen hydrochloride (MEL) is a white to off white powder and amorphous in nature. Chemically, it is 3-(10, 10-dimethyl anthracen-9-ylidene)-N, N-dimethylpropan-1-amine) [1, 2] (Figure 1(a)). It is a Tricyclic Antidepressant and work by inhibiting uptake of the neurotransmitters norepinephrine and serotonin by neurons. Flupentixol dihydrochloride (FLU) is white or almost white powder. Chemically, it is (Z)-4-[3-[2-(Trifluoromethyl)-9H thioxanthen-9-ylidene] propyl]-1-piperazin ethanol dihydrochloride [3] (Figure 1(b)). It is very soluble in water, soluble in alcohol and practically insoluble in methylene chloride [4]. Flupentixol acts by blocking the dopamine (a neurotransmitter) receptors in brain cells. Excess amount of dopamine receptors normally act to modify behavior and overstimulation resulting in psychotic illness. Flupentixol blocks these receptors to control psychotic illness. Thus it is neuroleptic with anxiolytic and antidepressant properties. The combination of FLU and MEL is indicated in the treatment of trigeminal neuralgia [5].

Literature survey revealed that melitracen hydrochloride can be estimated by spectrophotometry [6, 7] and by liquid chromatographic methods [8–11] individually or in combination with other drugs, and flupentixol dihydrochloride can be estimated by liquid chromatographic methods individually or in combination with other drugs [12–17]. LC/MS/MS method has been reported for simultaneous estimation of both melitracen and flupentixol human plasma [18]. A literature survey revealed no method reported for the determination of MEL and FLU in combined dosage form. Present study involves development of a stability liquid

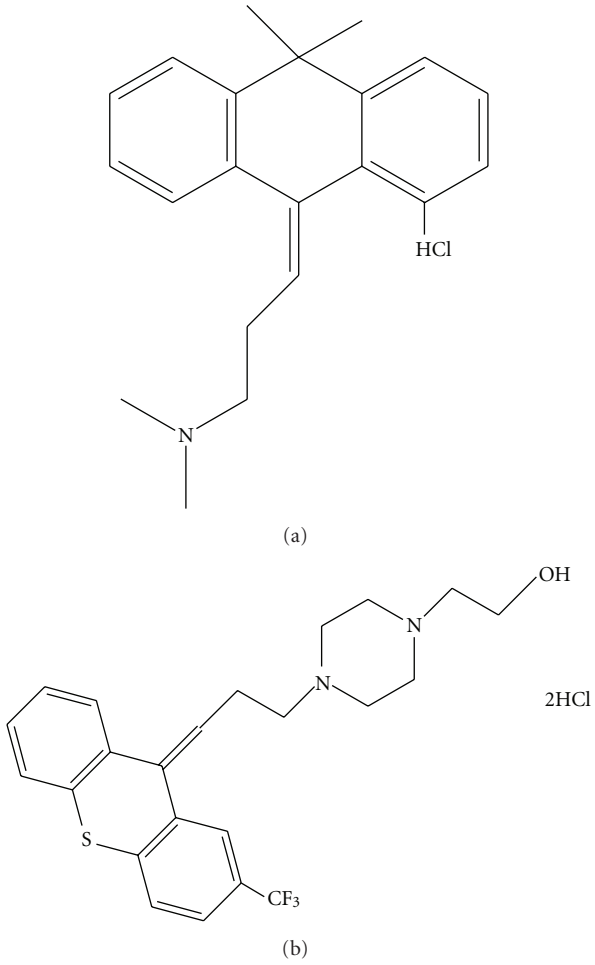

Figure 1: (a) Chemical structure of melitracen hydrochloride. (b) Chemical structure of flupentixol dihydrochloride.

chromatographic method for the determination of MEL and FLU in combination dosage form.

2. Experimental

2.1. Apparatus. The liquid chromatographic system of Perkin Elmer series 200 (Mumbai, India) containing quaternary gradient pump, variable wavelength programmable UV/Vis detector, and rheodyne injector with 20 μL fixed loop was used. A Brownlee C_{18} column with 250 × 4.6 mm i.d. and 5 μm particle size was used as stationary phase.

2.2. Reagents and Materials. Analytically pure MEL and FLU were procured as gift sample from Sun Pharmaceutical Pvt. Ltd., (Baroda, India). Methanol, water (E. Merck, Mumbai, India) was of LC grade, while potassium dihydrogen phosphate and triethylamine (S.D. fine chemicals, Mumbai, India) were of analytical grade and used for the preparation of mobile phase. Tablet formulation A (FRANXIT, Intas pharmaceuticals Ltd., Ahemadabad, India) containing labeled amount of 10 mg of melitracen hydrochloride and 0.5 mg of flupentixol dihydrochloride were purchased from local market.

2.3. Preparation of Mobile Phase and Stock Solution. Mobile phase was prepared by accurately weighing 0.340 g of potassium dihydrogen phosphate and dissolving in 100 mL of water. It was mixed with 900 mL of methanol, and pH was adjusted to pH 7.3 using triethylamine (TEA). The solution was filtered with Whatman filter paper no. 42 (0.45 μm). The solution was sonicated for 15 min for degassing prior to use.

Stock solutions were prepared by accurately weighing 10 mg each of MEL and FLU and transferring to two separate 10 mL volumetric flasks containing 3 mL of methanol. The flasks were sonicated for 10 minutes to dissolve the solids. Volumes were made up to the mark with methanol, which gave 1000 μg/mL of both the drugs. Aliquots from the stock solutions were appropriately diluted with mobile phase to obtain working standards of 100 μg/mL of each drug.

2.4. Chromatographic Conditions. A reversed phase C_{18} column (Brownlee) equilibrated with mobile phase comprising of methanol: 0.025 M potassium dihydrogen phosphate (90 : 10; pH 7.3) was used. Mobile phase flow rate was maintained at 1 mL/ min, and eluent was monitored at 230 nm. A 20 μL of sample was injected using a fixed loop, and the total run time was 10 min. All the chromatographic separations were carried out at controlled room temperature (25 ± 2°C).

2.5. Calibration Curves for MEL and FLU. Appropriate aliquots of MEL working standard solution were taken in different 10 mL volumetric flasks. Appropriate aliquots of FLU working standard solution were added to the same flasks. The volumes were made up to the mark with mobile phase to obtain final concentrations of 0.5, 1, 5, 10, 20, 40, and 60 μg/mL of MEL and FLU, respectively. The solutions were injected using a 20 μL fixed loop system, and chromatograms were recorded. Calibration curves were constructed by plotting peak area versus concentrations of the drug and regression equations were computed for MEL and FLU.

2.6. Analysis of Marketed Formulations. Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to 10 mg MEL and 0.5 mg of FLU was taken in 10 mL volumetric flask. A few mL of methanol was added to the above flask and the flask was sonicated for 15 minutes. The solution was filtered in another 10 mL volumetric flask using Whatman filter paper no. 42, and volume was made up to the mark with the same solvent.

Appropriate volume of the aliquot was transferred to a 10 mL volumetric flask and the volume was made up to the mark with the mobile phase to obtain a solution containing 20 μg/mL of MEL and 1 μg/mL of FLU. The solution was sonicated for 10 min. It was injected as per the above chromatographic conditions, and peak area was recorded. The quantifications were carried out by keeping these values to the linear equation of calibration curve.

2.7. Validation. The method was validated for accuracy, precision, specificity, detection limit, quantitation limit, and robustness.

2.7.1. Accuracy. The accuracy of the method was determined by calculating recoveries of MEL and FLU by method of standard additions. Known amounts of MEL (0, 10, 20, 30 μg/mL) and FLU (0, 0.5, 1, 1.5 μg/mL) were added to a prequantified sample solutions, and the amounts of MEL and FLU were estimated by measuring the peak area and by fitting these values to the straight-line equation of calibration curve.

2.7.2. Precision. The instrument precision was evaluated by injecting the solution containing MEL and FLU (20 μg/mL) six times repeatedly and peak area was measured. The results are reported in terms of relative standard deviation. The intraday and interday precision study of MEL and FLU was carried out by estimating the corresponding responses 3 times on the same day and on 3 different days (first, second and third day) for 3 different concentrations of MEL (0.5, 20, 60 μg/mL) and FLU (0.5, 20, 60 μg/mL), and the results are reported in terms of relative standard deviation (RSD). The specificity was estimated by spiking commonly used excipient (starch, talc, and magnesium stearate) into a preweighed quantity of drug. The chromatogram was taken by appropriate dilutions, and the quantities of drugs were determined.

2.7.3. Limit of Detection and Quantification. The detection limit is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using the following equation as per ICH guidelines. LOD $= 3.3 \times \sigma / S$ and LOQ $= 10 \times \sigma / S$, where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

2.7.4. Robustness. Robustness of the method was studied by deliberately changing the experimental conditions like flow rate, percentage of organic phase, pH of mobile phase, and also by observing the stability of the sample solution at 25 $\pm$ 2° for 24 h. The sample solution was assayed at every 6 h interval up to 24 h.

2.8. Forced Degradation Study. Stress degradation study using acid and alkali hydrolysis, chemical oxidation, and dry heat degradation was carried out, and interference of the degradation products was investigated. MEL and FLU were weighed (10 mg each) and transferred to two separate 10 mL volumetric flasks and diluted up to the mark with mobile phase. These stock solutions were used for forced degradation studies.

Forced degradation in basic media was performed by taking 1 mL stock solutions of MEL and FLU (1000 μg/mL) in two different 10 mL volumetric flasks and 1 mL of 2 N NaOH was added. Similarly, 1 mL aliquots of stock solutions of MEL and FLU were taken in same 10 mL volumetric flask, and 1 mL 2 N NaOH was added. All the flasks were stored at room temperature for 24 hrs. Solutions were neutralized with acid using pH meter and diluted up to the mark with mobile phase. The solutions were appropriately diluted with mobile phase to obtain final concentration of 10 μg/mL of MEL and FLU separately and in the mixture. Similarly, forced degradation in acidic medium was performed using 2 N HCl.

To perform oxidative stress degradation, appropriate aliquots of stock solutions of MEL and FLU (1000 μg/mL) were taken in two different 10 mL volumetric flasks and 1 mL of 3% hydrogen peroxide was added. Similarly, appropriate aliquots of stock solutions of MEL and FLU were taken in same 10 mL volumetric flaks, and 1 mL 3% hydrogen peroxide was added. All the mixtures were stored at room temperature for 24 hrs, and solutions were diluted up to the mark with mobile phase. The solutions were appropriately diluted with mobile phase to obtain final concentration of 10 μg/mL of MEL and FLU separately and in mixture.

To study dry heat degradation, solid drugs were exposed in oven at 80° for 2 h. After 2 h of heating, 10 mg each of MEL and FLU were weighed and transferred to two separate volumetric flasks (10 mL) and diluted up to the mark with the mobile phase. Solutions were further diluted by taking appropriate aliquots in different 10 mL volumetric flasks to obtain final concentration of 10 μg/mL of MEL and FLU.

To study photolytic (sunlight) degradation, solid drugs were exposed to sunlight for 3 days. After exposure, 10 mg each of MEL and FLU were weighed and transferred to two separate volumetric flasks (10 mL) and diluted up to the mark with the mobile phase. Solutions were further diluted by taking appropriate aliquots in different 10 mL volumetric flasks to obtain final concentration of 10 μg/mL of MEL and FLU.

All the reaction solutions were injected in the liquid chromatographic system and chromatograms were recorded.

3. Results and Discussion

3.1. Optimization of Mobile Phase. Optimization of mobile phase was performed based on resolution of the drugs and degradation products, asymmetric factor, and theoretical plates obtained for MEL and FLU. The mobile phase consisting of 0.025 M potassium dihydrogen phosphate: methanol (40 : 60 v/v, pH 7.3 adjusted with 1% TEA) was selected which gave sharp, well-resolved peaks for MEL and FLU (Figure 2(a)).

The retention times for MEL and FLU were 7.75 and 5.50 min, respectively. The asymmetric factors for MEL and FLU were 1.52 and 1.10, respectively. UV overlaid spectra of MEL and FLU showed (Figure 3) that all the drugs absorbed appreciably at 230 nm, so the same was selected as the detection wavelength during the studies.

3.2. Validation. The calibration curve was found to be linear over the range of 0.5–60 μg/mL for MEL and FLU. The data of regression analysis of the calibration curves are shown in Table 1.

The accuracy of the method was determined by calculating recoveries of MEL and FLU by method of standard additions. The recoveries obtained were 99.81–100.77% for MEL and 99.46–100.12% for FLU, respectively. The high

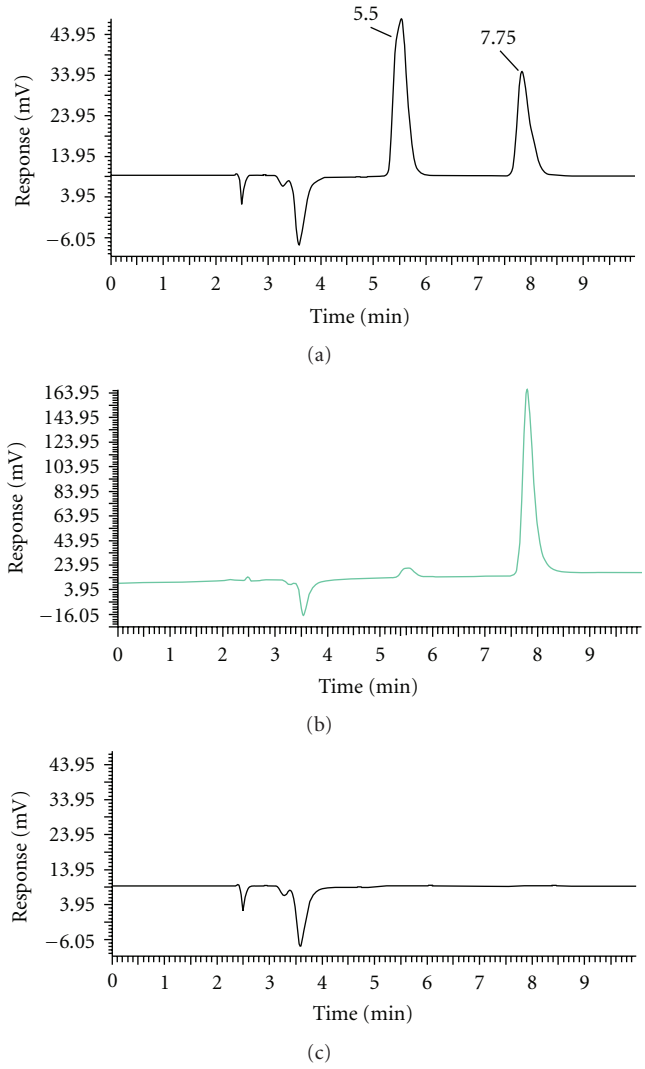

Figure 2: (a) Liquid chromatogram of MEL and FLU. (b) Liquid chromatogram of MEL and FLU in tablet dosage form. (c) Liquid chromatogram of placebo.

Table 1: Regression analysis of the calibration curve for the proposed method.

Parameters	MEL	FLU
Linearity range (μg/mL)	0.5–60	0.5–60
Slope	57663.83	96720.67
Standard deviation of slope	246.38	349.26
Intercept	96256.33	145690
Standard deviation of intercept	3342.37	7116.56
Correlation coefficient	0.9972	0.9971

values indicate that the method is accurate. Instrument precision was determined by performing injection repeatability test, and the RSD values for MEL and FLU were found to be 0.12% and 0.19%, respectively. The intraday and interday precision studies were carried out. For the intraday study, RSD values were found to be 0.14–0.58% for MEL and 0.11–0.57% for FLU, and for inter-day precision

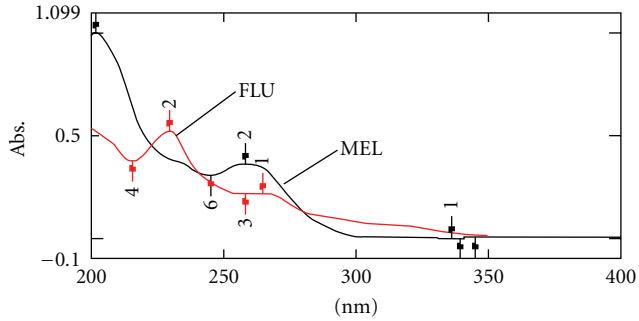

Figure 3: Overlain spectra of MEL (10 μg/mL) and FLU (10 μg/mL) in mobile phase.

Table 2: Summary of validation parameters.

Parameters	MEL	FLU
Detection limit (μg/mL)	0.07	0.02
Quantitation limit (μg/mL)	0.2	0.05
Accuracy(%)	99.81–100.77%	99.46–100.12%
Precision (RSD[a],%)		
Intraday precision ($n = 3$)	0.14–0.58%	0.11–0.57%
Interday precision ($n = 3$)	0.11–0.76%	0.13–0.42%
Instrument precision (RSD[a])	0.37 %	0.27 %

[a] RSD is relative standard deviation and "n" is number of determinations.

Table 3: System suitability test parameters for the proposed method.

System suitability parameters	MEL	FLU
Retention time (min)	7.75	5.50
Theoretical plates/ meter	5225	2539
Asymmetric factor	1.52	1.10
Resolution	2.08	—

study RSD values were found to be 0.11–0.76% for MEL and 0.13–0.42% for FLU, respectively. The low RSD values indicate that the method is precise. The detection limits for MEL and FLU were 0.07 and 0.02 μg/mL, while quantitation limits were 0.2 and 0.05 μg/mL, respectively. The above data shows that a nanogram quantity of both the drugs can be accurately and precisely determined. The validation parameters are summarized in Table 2, and the system suitability test parameters are shown in Table 3.

Robustness of the method was studied by changing the flow rate of the mobile phase from 1 mL/min to 0.9 mL/min and 1.1 mL/min. Using 1.1 mL/min flow rate, retention times for MEL and FLU were observed to be 7.69 and 5.48 min, respectively, and with 0.9 mL/min flow rate, retention times for MEL and FLU were observed to be 7.83 and 5.54 min, respectively, without affecting the resolution of the drugs. When mobile phase composition was changed to 0.025 M potassium dihydrogen phosphate: methanol (95 : 10, v/v) by increasing percentage of methanol, the retention times for MEL and FLU were observed to be 7.67 and 5.45 min, respectively. When pH of mobile phase was changed from 7.3 to 7.8 and 6.8 and at both this pH, there

Table 4: Data derived from robustness for proposed method.

Parameters	Normal condition	Change in condition	Change in % RSD	
			MEL	FLU
Flow Rate	1.0 mL/min	0.9 mL/min	0.33	0.21
		1.1 mL/min	0.48	0.11
pH	7.3	6.8	0.28	0.13
		7.8	0.31	0.18
Mobile phase ratio	90 : 10	95 : 5	0.17	0.13
		85 : 15	0.23	0.18

Table 5: Assay results of tablet dosage form using proposed method.

Formulations	Labelled amount (mg)		% Recovery [c]	
	MEL	FLU	MEL	FLU
A	10	0.5	100.77 ± 1.03	99.46 ± 0.57

[c] mean value ± standard deviation of three determinations; Tablet formulation A is FRANXIT (Intas pharmaceuticals Ltd., India) containing labeled amount of 10 mg of melitracen hydrochloride and 0.5 mg of flupentixol dihydrochloride.

was not seen any kind of change on the retention time. The robustness parameters are shown in Table 4. The solution stability study revealed that MEL and FLU solutions were stable for 24 h without detectable degradation, and the percentage recovery of both the drugs was found to be more than 98%.

3.3. Forced Degradation Study. Forced degradation study was carried out by subjecting both the drugs to acid and alkali hydrolysis, chemical oxidation, and dry heat degradation conditions. The MEL and FLU were found to be stable to oxidative stress degradation, dry heat degradation, and photolytic (sunlight) condition, acid and alkali hydrolysis. No degradation was observed in any of the stressed conditions, and MEL and FLU were found to be highly stable.

3.4. Analysis of Marketed Formulation. The proposed method was applied to the determination of MEL and FLU in their combined dosage form (Tablet A). The results for MEL and FLU were comparable with the corresponding labeled amounts (Table 5). The chromatogram of marketed formulation analysis is shown in Figure 2(b).

4. Conclusion

A validated stability-indicating RP-HPLC analytical method has been developed for the simultaneous estimation of MEL and FLU in bulk and in their combined tablet dosage form. The results of stress testing undertaken according to the ICH guidelines revealed that the method is selective and stability-indicating. Both the drugs were found to be stable to forced degradation study. The proposed method was validated and found to be simple, accurate, precise, and specific. The method was successfully applied for the estimation of combined tablet dosage form. The proposed liquid chromatographic method can be applied to the analysis of samples obtained during accelerated stability experiments to predict expiration dates of pharmaceuticals.

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