

# Quantitative Determination of Milnacipran by Simple Colorimetric Methods

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**Abstract** In the present study, two sensitive, precise and accurate spectrophotometric methods have been developed for the determination of milnacipran in bulk and capsule formulation. The first method is based on the reaction of milnacipran with ninhydrin in N,N'-dimethylformamide medium at 80 °C temperature to form a colored Ruhemann's purple, which exhibits absorption maximum at 575nm. The second method is based on extraction of milnacipran into chloroform as ion-pair with bromothymol blue. The yellow colored ion-pair complex exhibits absorption maximum at 410nm. Beer's law is obeyed in the concentration ranges 2.5-37.5µg/mL and 2-12µg/mL of milnacipran for methods I and II, respectively. The effect of experimental variables were investigated and optimized. The validation parameters like linearity, sensitivity, accuracy, precision and robustness were checked by following the ICH guidelines. The proposed methods were applied for the analysis of milnacipran in capsule formulation with good results.

**Keywords:** milnacipran, ninhydrin, bromothymol blue, Ruhemann's purple, ion-pair complex

## 1. Introduction

Milnacipran (MCN) or 1R 2S)-2 aminomethyl-N-N-dimethyl-1-phenyl cyclopropane carboxamine (Figure 1) is an antidepressant, effective in the treatment of fibromyalgia. MCN acts by inhibiting the reuptake of serotonin and nor epinephrine neurotransmitters in 1:3 ratio [1,2,3].

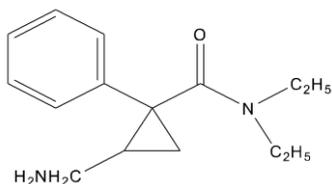


Figure 1. Structure of Milnacipran

The analytical methods reported for the determination of MCN are based on chromatographic techniques. They include RP-HPLC [4,5,6], LC-MS [7], U-HPLC [8] and HPTLC [9]. Most of the reported chromatographic methods have tedious extraction procedures, time-consuming, complex, multiple sample preparation steps, expensive and requires a skilled person to operate the instrument. Hence these methods are not suitable for routine analysis of MCN in quality control and quality assurance laboratories. Compared with the above mentioned chromatographic methods, spectrophotometry is considered as the most convenient analytical technique in many of the quality control and quality assurance laboratories because of its advantages, such as good

selectivity, less expensive, simple instrumentation and less time consuming.

To the best of our knowledge, there are only few reports on the determination of MCN using spectrophotometric method [10,11,12,13]. These methods have drawbacks such as insensitivity, lack of selectivity, use of expensive reagent and rigid control of pH. In the present paper, we report two spectrophotometric methods for the estimation of MCN in bulk and capsules by using ninhydrin and bromothymol blue as analytical reagents.

## 2. Materials and Methods

### 2.1. Apparatus

The absorption spectra and measurements were recorded using a systronics (Schimadzu Corporation, Tokyo, Japan), model visiscan-167, digital spectrophotometer with 1-cm matched quartz cells. Samples were weighed using a schimadzu electronic weighing balance (Schimadzu Corporation, Tokyo, Japan) BL 220H model.

### 2.2. Chemicals and Reagents

All reagents and chemicals were of analytical reagent grade

1. Milnacipran was kindly provided by Matrix laboratories (Hyderabad, India).

2. Milza capsules (Intas pharmaceuticals, Dehradun, India), labeled to contain 50 mg milnacipran/capsule, were purchased from hetero pharmacy (Guntur, India).

3. Ninhydrin (NHN) was prepared as 2% solution in N,N'-dimethylformamide (DMF) by dissolving 2gm of NHN (Merck specialties Pvt. Ltd. Mumbai, India) in 100mL of DMF (Merck specialties Pvt. Ltd. Mumbai, India).

4. Aqueous solution of 0.1% bromothymol blue (BTB) was prepared by dissolving 100mg of BTB (Thermo Fishers Scientific Pvt. Ltd. Mumbai, India) in 100mL of hot water.

5. 1N HCl was prepared by diluting 8.65mL of 11.6N HCl to 100mL with water.

6. Chloroform (Merck specialties Pvt. Ltd. Mumbai, India) was used for the extraction purpose.

7. Distilled water was used throughout the experiments.

## 2.3. Standard Solutions

An accurately weighed quantity (100mg) of MCN was transferred into a 100mL volumetric flask containing 50mL of water and mixed well. The volume was made up to 100mL with water. This stock solution (1mg/mL) was further diluted with water to produce working standard solutions of 250 $\mu$ g/mL (method I) and 100 $\mu$ g/mL (method II).

## 2.4. General Procedure

### 2.4.1. Method I

Aliquots (0.1-1.5mL) of standard solution (250 $\mu$ g/mL) covering the concentration range of 2.5-37.5 $\mu$ g/mL for MCN were transferred into a set of 10mL boiling test tubes. Then, 1.5mL of 2% NHN was added to each tube and they were heated in a boiling water bath for 35 minutes at 80 $^{\circ}$ C. The solutions were cooled, transferred to 10mL volumetric flasks and diluted to the mark with distilled water. The absorbance of the resulting solution was measured at 575nm against the reagent blank.

### 2.4.2. Method II

Aliquots (0.2-1.2) of standard solution (100 $\mu$ g/mL) covering the concentration range of 2-12 $\mu$ g/mL for MCN were transferred into a set of 125mL separating funnels. To each funnel, 1mL of 1N HCl and 1mL of 0.1% BTB were added and mixed well. Five mL of chloroform was added to each separating funnel and shaken vigorously. The funnels were kept aside for 5 mins for the separation of two layers. The colored organic layer was collected in a series of 10mL volumetric flasks and made up to the mark with chloroform. The absorbance of organic layer was measured at 410nm against the reagent blank.

In both the methods, the calibration curve was plotted with absorbance *versus* the final concentration of the drug ( $\mu$ g/mL). Alternatively, the corresponding regression equation was derived. The amount of the drug present in the sample solution was computed either from the corresponding calibration curve or from the corresponding regression equation.

### 2.4.3. Determination of MCN in Capsules

Twenty capsules were emptied, weighed and then mixed well. An accurately weighed quantity of the powder equivalent to 100mg of MCN was transferred into a small conical flask, extracted successively with 40mL of distilled water. The extract was filtered into a 100mL

volumetric flask and completed to mark with the same solvent. This solution was further diluted with the same solvent as appropriate to obtain the working standard solution of the concentrations 250 $\mu$ g/mL and 100 $\mu$ g/mL for methods I and II, respectively. The steps described under "General procedures for method I and method II" were followed. The nominal content of the capsule was determined either from the calibration curve or from the corresponding regression equation.

## 3. Results and Discussion

### 3.1. Method I

Ninhydrin is a widely used chromogenic reagent to quantify compounds with primary amine [14,15,16,17,18]. The NHN reacts with the primary amine to produce a colored Ruhemann's purple. The results obtained in the method I is based on the reaction of MCN with NHN in DMF medium via oxidative deamination of the primary amino group of MCN. In DMF medium, NHN is converted to o-carboxyphenylglyoxal which would reduce NHN to 2-hydroxyindan-1,3-dione. The 2-hydroxyindan-1,3-dione combines with primary amino group of MCN to form amino derivative. The amino derivative thus produced undergoes condensation with another molecule of NHN to give a colored Ruhemann's purple or diketohydrindylidene-diketohydrindamine, which shows absorption maximum at 575nm (Figure 2). The possible reaction mechanism, based on the reported methods [14,15,16,17,18], is given in Figure 3.

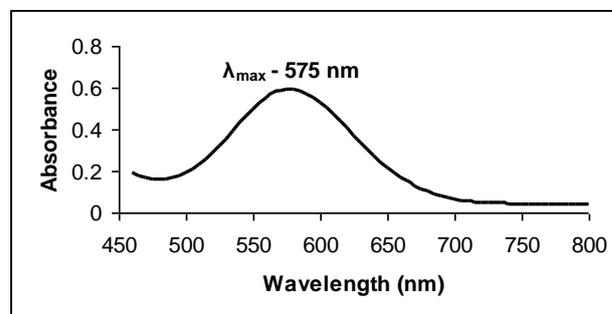


Figure 2. Absorption spectra of Ruhemann's Purple

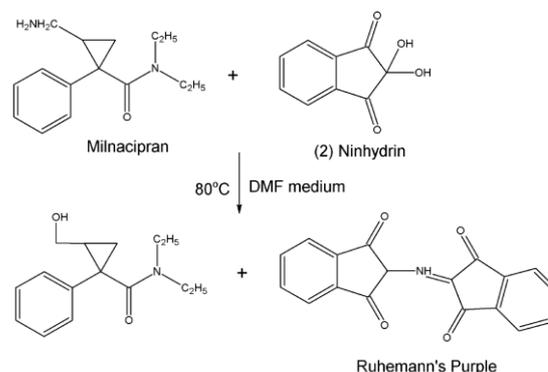


Figure 3. Possible Reaction between MCN and NHN

### 3.2. Method II

Ion-pair complex is formed through the electrostatic interaction between a cation and an anion. The property of

the compounds possessing basic moieties (primary, secondary or tertiary amino group) to form ion-pair with acidic dyes is suitable for their assay using extractive spectrophotometry. BTB, being an acidic dye is used in the determination of drugs with basic groups [19,20,21,22]. The method II is based on ion-pair complex formation between the MCN and BTB in acidic media. The ion-pair was most likely formed via electrostatic interaction between the most basic center in the MCN (amino group) and the carboxylate anion of the BTB. The MCN-BTB ion-pair complex shows the absorption maximum at 410nm against the reagent blank (Figure 4). The possible mechanism of the reaction pathway, based on the reported methods [19,20,21,22], is shown in Figure 5.

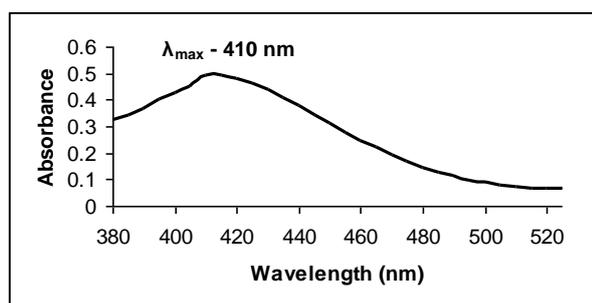


Figure 4. Absorption Spectra of MCN-BTB Ion-Pair Complex

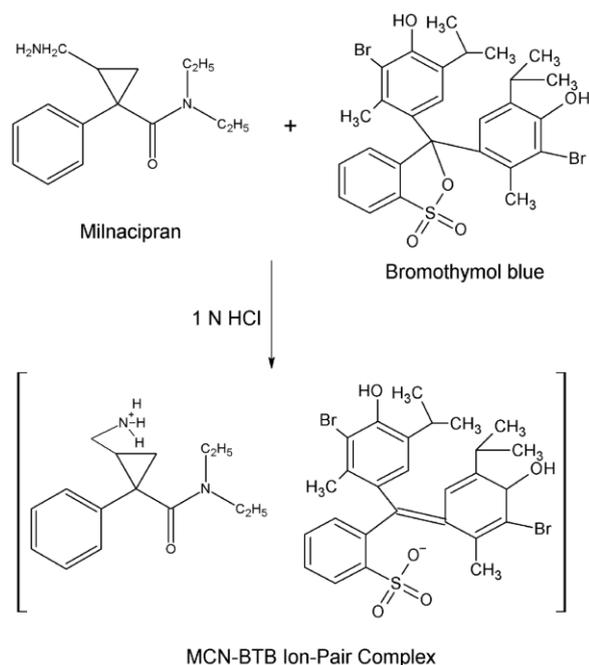


Figure 5. Possible Reaction between MCN and BTB

### 3.3. Optimization of the Experimental Conditions

#### 3.3.1. Method I

Different variables affecting the reaction between the MCN and NHN, including NHN concentration, temperature and heating time were studied to optimize the reaction conditions to give maximum absorbance. The optimum values of the variables were maintained throughout the experiment.

#### 3.3.1.1. Effect of Concentration of NHN

The influence of the NHN concentration was studied by treating 1mL of MCN (25  $\mu\text{g}/\text{mL}$ ) with varying volumes (0.5-2.5mL) of 2% NHN in DMF. It was found that 1.5mL of 2% NHN reagent solution gives the highest absorbance value as shown in Figure 6. Above this volume, the absorbance decreased. Thus, 1.5mL of 2% NHN in DMF was proved to be sufficient for the determination process.

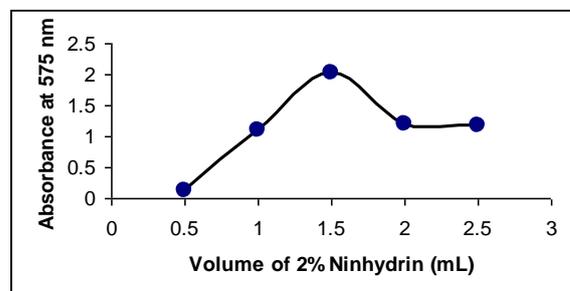


Figure 6. Effect of Concentration of Ninhydrin

#### 3.3.1.2. Effect of Temperature

The influence of the temperature on the rate of formation of the Ruhemann's purple was studied over the temperatures 20  $^{\circ}\text{C}$  -80  $^{\circ}\text{C}$ . It is evident from Figure 7 that the maximum absorbance was attained at 80  $^{\circ}\text{C}$ . At higher temperatures (>80  $^{\circ}\text{C}$ ) the solution was precipitated. Hence 80  $^{\circ}\text{C}$  was used as an optimum temperature.

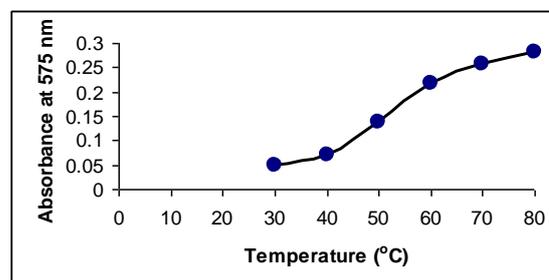


Figure 7. Effect of Temperature

#### 3.3.1.3. Effect of Time

To study the effect of heating time on the formation of the Ruhemann's purple, 1mL of MCN (25 $\mu\text{g}/\text{mL}$ ) was mixed with 1.5mL of 2% NHN in DMF. The contents of the mixture were heated upto 20min at 80  $^{\circ}\text{C}$ . The results revealed that the maximum intensity of color was obtained at 10min of heating and further increase in the heating time did not affect the absorbance intensity (Figure 8). Therefore, the optimum heating time was fixed at 10min.

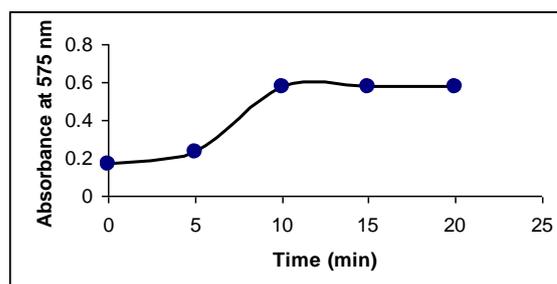


Figure 8. Effect of Time

### 3.3.2. Method II

#### 3.3.2.1. Effect of Concentration of BTB

The effect of the concentration of BTB in the formation of ion-pair complex was investigated by adding different volumes (0.2-1.6 mL) of 1.2% BTB and 1 mL of 1N HCl to 1 mL of MCN (10 µg/mL). It was found that the maximum absorbance of the yellow color was reached with 1 mL of 1.2% BTB, and remained stable with higher volumes (Figure 9). Thus, 1 mL of 1.2% BTB was used throughout the experimental investigations.

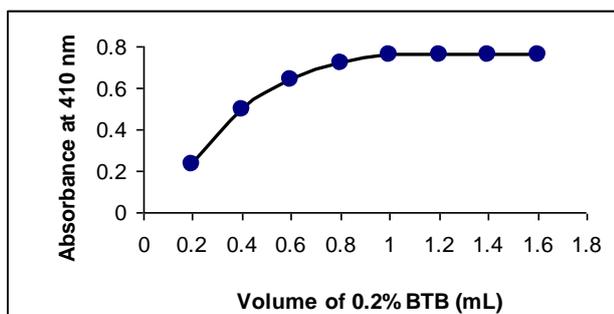


Figure 9. Effect of Concentration of Bromothymol blue

#### 3.3.2.2. Effect of Acidity

The influence of acidity on the development of ion-pair complex was studied by adding 1 mL of 1.2% BTB and different volumes (0.2-1.6 mL) of 1N HCl to 1 mL of MCN (10 µg/mL). The maximum color intensity was observed with 1 mL of 1N HCl, above this volume the absorbance remained constant (Figure 10). Therefore, 1 mL of 1N HCl was used throughout the experiment.

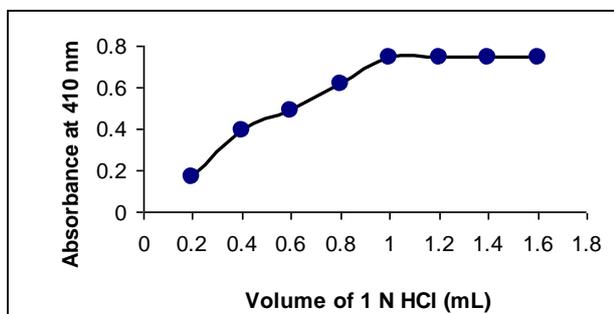


Figure 10. Effect of Acidity

#### 3.3.2.3. Effect of Extracting Solvent

To know the extraction efficiency, different organic solvents (benzene, toluene, carbon tetrachloride and chloroform) were used for the extraction of yellow colored of ion-pair complex. The results are shown in Table 1. Maximum absorbance was obtained while extracted with chloroform. So it is considered to be the good solvent for the extraction.

Table 1. Effect of Extracting Solvent

Solvent	Absorbance
Chloroform	0.157
Benzene	0.123
Carbon tetrachloride	No reaction
Toluene	No reaction

### 3.4. Validation of the Developed Methods

The developed methods were validated by following the ICH guidelines [23]. Different parameters like linearity, sensitivity, precision, accuracy and robustness were checked.

#### 3.4.1. Linearity

Linearity was studied in the concentration range from 2.5-37.5 µg/mL and 2-12 µg/mL MCN for method I and II, respectively. The drug showed good linearity in the tested range. The regression coefficient values for method I and II were found to be >0.9982. The results (Table 2) reveal a good and dynamic linearity ranges of the proposed methods.

#### 3.4.2. Sensitivity

The sensitivity of the proposed methods was estimated in terms of molar absorptivity, Sandell's sensitivity, limit of quantitation (LOQ) and limit of detection (LOD). The results (Table 2) showed the high sensitivity of the proposed methods.

Table 2. Optical and Regression Characteristics of the Proposed Methods

Parameters	Method I	Method II
$\lambda_{max}$ (nm)	575	410
Beer's Limit (µg/mL)	2.5-37.5	2-12
Molar Absorbitivity (L mole <sup>-1</sup> cm <sup>-1</sup> )	7.692 x 10 <sup>4</sup>	1.965 x 10 <sup>5</sup>
Sandell's sensitivity (µg cm <sup>-2</sup> /0.001 Absorbance unit)	0.367	0.00013
Stability of colored products (mins)	60	30
Regression equation (Y= mx + c) <sup>SS</sup>		
Slope (m)	0.027	0.075
Intercept (c)	0.01	0.0006
Regression coefficient (r <sup>2</sup> )	0.9982	0.9990
LOD (µg/mL)	0.244	0.087
LOQ (µg/mL)	0.740	0.260

<sup>SS</sup>Y = mx + c, where Y is the absorbance and x is the concentration of drug in µg/mL

#### 3.4.3. Precision and Accuracy

The precision and accuracy of the proposed methods were established by performing intra-day (repeatability) and inter-day (reproducibility) analyses of three different concentrations (2.5, 20 and 35 µg/mL - method I; 2, 7 and 12 µg/mL - method II), covering the linearity range of MCN, using the proposed methods. The results are reported as standard deviation, relative standard deviation, percentage of error and recoveries in Tables 3 and Table 4. The values are within the acceptable limits, indicating that both the methods have good repeatability and reproducibility.

Table 3. Intra-day Precision and Accuracy

Method	Amount of MCN (µg/mL)		RSD (%)	Recovery (%)	Error (%)
	Taken	Found <sup>S</sup> ± SD			
I	2.5	2.504 ± 0.013	0.519	100.16	0.16
	20	20.235 ± 0.163	0.805	101.17	1.17
	35	35.047 ± 0.210	0.599	100.13	0.13
II	2	2.005 ± 0.023	1.147	100.25	0.25
	7	7.007 ± 0.070	0.999	100.10	0.10
	12	12.001 ± 0.135	1.124	100.01	0.01

<sup>S</sup>Average of five determinations

**Table 4. Inter-day Precision And Accuracy**

Method	Amount of MCN ( $\mu\text{g/mL}$ )		RSD (%)	Recovery (%)	Error (%)
	Taken	Found <sup>s</sup> $\pm$ SD			
I	2.5	2.501 $\pm$ 0.017	0.679	100.04	0.04
	20	20.016 $\pm$ 0.177	0.884	100.08	0.08
	35	34.980 $\pm$ 0.219	0.626	99.94	0.06
II	2	2.008 $\pm$ 0.039	1.942	100.04	0.04
	7	6.99 $\pm$ 0.071	1.015	99.85	0.15
	12	11.99 $\pm$ 0.035	0.291	99.91	0.09

<sup>s</sup>Average of three determinations

### 3.4.4. Recovery Studies

The accuracy of the proposed methods was further determined by calculating the recovery of MCN by the method of standard addition. A known amount of MCN at three different levels (50%, 100% and 150%) was added to the preanalyzed sample solution and the amount of MCN was estimated by the proposed methods. The results are reported as relative standard deviation and percent recovery in Table 5. The recovery studies showed that there was no interference from excipients in the determination of the MCN by the proposed methods.

**Table 5. Results of Recovery Studies by Standard Addition Technique**

Method	Amount of MCN (mg)		Found <sup>s</sup>	RSD (%)	Recovery (%)
	Tablet	Spiked			
I	50	25	75.06	0.077	100.08
	50	50	100.06	0.112	100.06
	50	75	125.01	0.086	100.01
II	50	25	74.98	0.285	99.97
	50	50	99.95	0.103	99.95
	50	75	124.98	0.130	99.98

<sup>s</sup>Average of three determinations

### 3.4.5. Robustness

Robustness was performed to check whether the analytical performance of the proposed methods was affected by any small deliberate changes. Robustness of the proposed methods is demonstrated by the determination of the MCN at two different concentrations (within the linearity range) by the proposed methods with minor changes in the experimental variables, such as changing the volume of 2% NHN & temperature in method I and changing the volume of 1.2% BTB & volume of 1M HCl in method II. The relative standard deviation and recovery was calculated each time. The results (Table 6) indicated that the minor changes likely to take place, during the operation of the method did not adversely affect the analytical performance of the proposed methods.

## 3.5. Application of the Proposed Methods to Capsule Formulation

The proposed methods were applied for the determination of MCN in capsule formulation (Milza capsules, manufactured by Intas pharmaceuticals, Dehradun, India). The results obtained were compared with that of reported UV spectrophotometric method [10]. Paired t-test and F-test (at 95% confidence level) values were calculated. The results are reported in the Table 7. These results indicate that the proposed and reported methods did not show significant difference with respect to precision and accuracy.

**Table 6. Results of Robustness of the Proposed Methods**

Method	Parameter	Amount of MCN ( $\mu\text{g/mL}$ )		RSD (%)	Recovery (%)
		Taken	Found <sup>s</sup>		
I	Temperature (80°C $\pm$ 2°C)	2.5	2.52	1.23	100.80
		37.5	37.45	0.336	99.86
	2% NHN (1.5mL $\pm$ 0.2 mL)	2.5	2.49	0.481	99.60
		37.5	37.53	0.416	100.08
II	1.2% BTB (1 $\pm$ 0.1 mL)	2	2.02	1.089	101.00
		12	12.04	0.141	100.33
	1N HCl (1.0 $\pm$ 0.1 mL)	2	1.99	0.603	99.50
		12	11.98	0.191	99.83

<sup>s</sup>Average of three determinations

**Table 7. Assay of Milnacipran in Capsules**

Method	Amount of MCN (mg)		RSD (%)	Recovery (%)	F-value	t-value
	Tablet	Found <sup>s</sup>				
I	50	49.98	0.172	99.96	1.36	0.212
II	50	49.96	0.194	99.92	0.196	0.153
III*	50	49.97	0.126	99.92	---	----

<sup>s</sup>Average of five determinations

\* Reference method

## 4. Conclusion

The present study deals with the development and validation of two spectrophotometric methods for the determination of MCN using NHN (method I) and BTB (method II) as analytical reagents. The proposed methods offer the advantages of instrumental simplicity and high sensitivity. These methods showed satisfactory accuracy and precision. The results of recovery study proved that the methods are suitable for the determination of MCN in capsule formulations.

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## Statement of Competing Interests

The authors do not have any competing interest

## List of Abbreviations

MCN: Milnacipran  
 NHN: Ninhydrin  
 BTB: Bromothymol blue  
 DMF: N,N'-dimethylformamide

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