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# Quantitative Determination of Norepinephrine by HPLC in Rodent Urine Sample

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## Abstract

Norepinephrine, a key neurotransmitter that has been linked to a variety of neuropsychiatric diseases. However, there is limited work on employing HPLC-UV to estimate monoamines in biological samples using HPLC-UV method. The present study explores the detection of norepinephrine in rat urine sample, developing a easy, precise validated HPLC method. The o-phosphoric acid (80%):acetonitrile (70:30) combination was used as the mobile phase, and a flow rate of 0.5 ml/min was used to produce the chromatographic separation on a C8 column (250 x 4.6 mm, 5 m). The detection was observed at λmax 275 nm with better sensitivity. According to the parameters indicated in the ICH guidelines (Q2A; Q2B), the procedure was verified. The linearity range was selected from 10-35 µg/mL, $r^2$ =0.966, LOD (1.17 µg/mL), LOQ (3.55 µg/mL), ret. time (4 min). The technique has demonstrated that it is repeatable and recoverable within the given range. Thus can be used for routine analysis of norepinephrine in urine samples.

**Keywords:** Norepinephrine, HPLC-UV, method validation, rodent urine.

## 1. Introduction

Catecholamines (CA's), such as epinephrine, norepinephrine, and dopamine, are nerve-endogenous neurotransmitters having amine and catechol groups (DA). Because CAs and their metabolites are essential for the control of the central and peripheral nervous systems, they are used to diagnose and treat a broad spectrum of diseases[1]. Epinephrine was identified as a hormone produced by the adrenal medulla. Later, Norepinephrine was identified as a major neurotransmitter secreted by sympathetic neurons in the peripheral nervous system. Epinephrine and Norepinephrine, two neurotransmitters that are essential for the metabolism and regulation of sodium ions, are precursors to dopamine, a key neurotransmitter. Low-molecular-weight intercellular mediators called "biogenic amines" are involved in chemical communication [2,3].

Previously, catecholamines and small molecules were isolated from plasma proteins using an internal-surface reversed-phase column (octadecylsilane column), and they were analysed using liquid chromatography

(LC)/mass spectrometry (MS) employing electrospray ionisation time-of-flight MS [4], but Norepinephrine and Epinephrine methods cannot be analysed using HPLC and UV detector simultaneously. In comparison to other analytical procedures, HPLC methods are the most dependable and accurate.

The most frequent procedures for Norepinephrine and Epinephrine analysis are RIA [5, 6] and ELISA [7, 8] HPLC with diode array detector [7] and HPLC with fluorometric detector [8] were previously used, but both of these techniques involve numerous processes, take a long time, and yield variable results. As a result, this study's primary goal was to develop techniques for measuring catecholamines simultaneously using an RP-HPLC system with a C-8 column and a UV detector in urine sample [9].

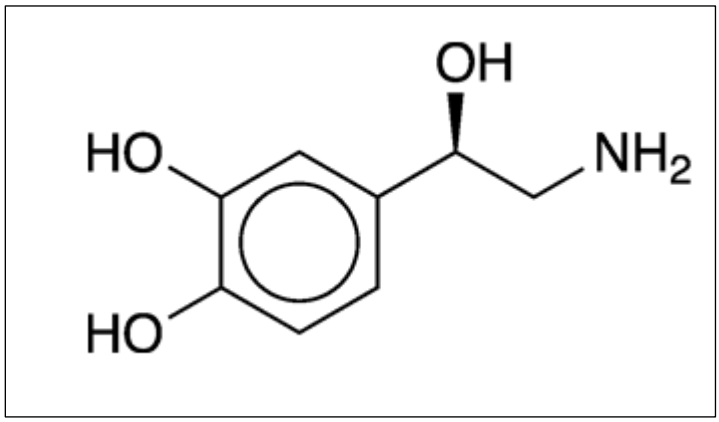


**Figure No. 1: Norepinephrine**

## 2. Materials And Methods

### 2.1 Chemicals and solvents

All of the compounds used in this investigation were of analytical grade. Ortho-phosphoric acid (85 percent, Sigma-Aldrich), glacial acetic acid (70 percent AR grade, SRL Chemical), formic acid, and norepinephrine standard (98.5 percent, Sigma-Aldrich) were utilised (98-99 percent AR grade, Research lab).

### 2.2 Liquid chromatography

The Kromasil C8, 5 m (250 X 4.6 mm) analytical column was used in low pressure gradient mode with an Agilent HPLC system to obtain the chromatographic separation. A mixture of acetonitrile and 0.1% orthophosphoric acid (pH 2) in a ratio of 30:70 v/v, pH 2.2, was degassed using sonication. Throughout the experiment, the flow rate and column temperature were maintained at 0.5 mL/min and 25ºC, respectively. The injection volume was maintained at 20 µL.

#### 2.2.1 Chromatographic conditions optimization

Based on available literature on HPLC and LC-MS, different mobile phases including acetonitrile, formic acid, methanol, and orthophosphoric acid were examined. Based on the findings of earlier research, a wavelength of 279 nm and a volume of 20 L were chosen for the injection. Testing flow rates ranging from 0.5 ml/min to 1 ml/min led to the identification of the flow rate that produced the greatest results. A particular retention time was used to separate the norepinephrine standard.

### 2.3 Standard stock solution preparation

Norepinephrine was weighed at approximately 1000 µg/ml using an analytical

balance (Shimadzu ATX224 precision balance), and then poured into a 1 mL eppendorf tube. It was dissolved in ultrapure water for labelling, and distilled water was used to make up the volume. The prepared stock standard solution contains 1000 µg/ml of active ingredient.

On the same day, successive dilutions of the standard stock solution using ultra pure water as a diluent resulted in the development of norepinephrine calibration standards with concentrations between 10-35 µg/ml.

**2.4 Detection of $\lambda_{max}$**

A UV spectrophotometer was used to scan a 10 µg/mL standard solution between 200 and 400 nm (Spectrophotometer: Shimadzu UV-1900). Max was calculated using the UV spectrum of a typical solution.

**2.5 Method validation**

Analytical Process Validation: The analytical method was validated using Q2 (R1) (ICH, 2005; Center for Drug Evaluation and Research (CDER), 1994). Linearity, precision, accuracy, specificity, the limit of detection (LOD), the limit of quantification (LOQ), and robustness were all addressed as validation parameters after the system validity and reliability were established.

**2.5.1 System suitability**

Following six replicate injections of the standard solution (10 µg/mL), the HPLC system's system suitability parameters (RSD percent for retention time, RSD percent for peak area, theoretical plates, and tailing factor) were evaluated.

**2.5.2 Linearity**

Five standard solutions with concentrations ranging from 10-35 g/mL were used to create the standard calibration. Each standard solution was chromatographed five times for a total of 10 minutes under optimum chromatographic conditions. The method's linearity was assessed using average peak area versus concentration data in a least squares linear regression analysis.

**2.5.3 Precision**

Precision was calculated by comparing the approach's intraday (repeatability assessed by checking the standard solution on the same day) and interday (repeatability assessed by studying the standard solution on three different days) variations. For these assays, a standard solution (10 µg/mL) that had been injected six times was used.

**2.5.4 Accuracy**

Recovery tests utilising the conventional addition approach were conducted to evaluate the method's accuracy. In this process, sample solutions that had already been analysed were added at three different levels of pure drug concentration—80, 100, and 120 percent—and norepinephrine recovery was assessed for each level.

**2.5.5 Selectivity/Specificity**

The analytical method's selectivity refers to its capacity to yield an analytical result in the presence of outside interference. The selectivity of the suggested approach was assessed by comparing the chromatograms of the mobile phase solution (blank) and the sample solution to the chromatogram of the standard solution. Retention time (Rt) and queuing factor values were chosen to illustrate the specificity and selectivity of the chosen method.

**2.5.6 LOD and LOQ**

As shown in the equations below, these values were computed using the standard error (s) and slope of the regression line (m).

LOD = 3.3 σ/S; LOQ = 10 σ/S

**2.5.7 Robustness**

A robustness study was conducted to examine the impact of subtle but regular changes to chromatographic settings. The robustness was checked with a few small changes in the parameters. Changes include different mobile phase flow rates (0.1 mL min-1) and concentrations (5%) as well as mobile phase pH values (0.010) and column temperatures (2.0 °C). After each adjustment, the sample solution was added to the chromatographic system to gauge the system's suitability for the parameters under consideration.

**2.5.8 Analysis in rat urine sample and sample pretretment**

The rat urine of 24-hour was collected, centrifuged at 8000 rpm for 3 minutes prior to analysis, filtered through a membrane filter (0.45 µm), and then 100 µL of urine was extracted with 0.4 M perchloric acid (100 µL) before being diluted to 1 ml with distilled water. 20 µL of urine were injected into the HPLC apparatus.

**3. Results and Observations**

**3.1 Determination of absorption maximum for Norepinephrine**

The spectra of norepinephrine were studied using a UV spectrophotometer, and was discovered at 275 nm, as shown in Figure 2.

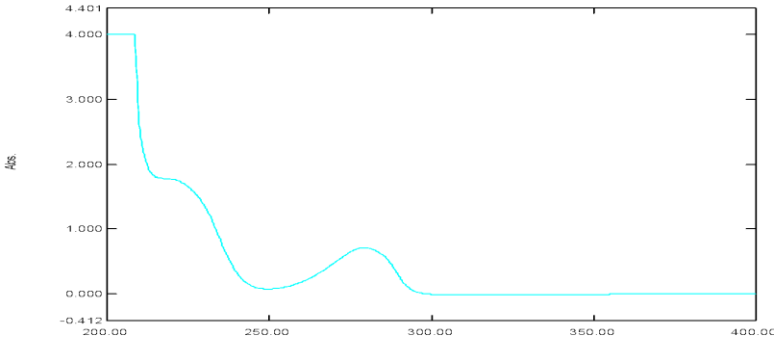


**Figure No. 2: λmax of Norepinephrine**

**3.2 Optimization of mobile phase and chromatographic conditions:**

Many reverse phase HPLC columns (C8 columns) and several mobile phases were used for the optimization experiment. It was unable to obtain proper peak symmetry because of the shorter columns. Proper peak symmetry was achieved on the kromasil C8 column (250 x 4.6 mm, 5 m). As a result, we employed the Kromasil C8 column (250 x 4.6 mm, 5 m) for the validation examination. Following the selection of the chromatographic column, the mobile phase was made up of a mixture of 0.1% o-phosphoric acid and acetonitrile (70:30). (pH 2.2). When selecting the mobile phase, environmentally harmful chemicals were avoided. The first low pressure gradient mode was gradually altered once the injection volume and column temperature were established.

**3.3 Method validation:**

**3.3.1 System suitability**

Using the same conditions as the chromatographic system, six injections of a 10 µg/mL standard solution were made into the HPLC system. As indicated in table 1, the number of theoretical plates, the symmetry

factor, the peak area, and the retention durations.

**Table No.1: Observational data for system suitability**

| System suitability parameters | Standard solution concentration (10µg/mL) |
|---|---|
| Symmetry factor | 1.52 |
| No. of theoretical plates | 3459 |
| Peak area (% RSD) | 6.22 |
| Retention time (% RSD) | 0.22 |

### 3.3.2 Linearity

The calibration curve for dopamine's peak-area vs concentration was then drawn. Least squares linear regression was used to analyse the peak area versus concentration. Data were used to test this strategy's linearity. Figure 3 and Table 2 both display the calibration curve and data for the linearity parameters, respectively.

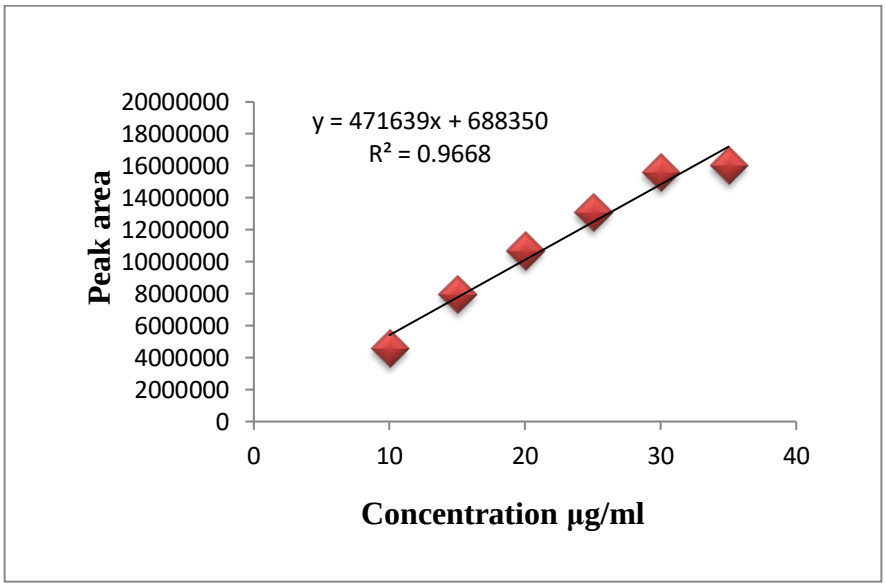


**Figure No.3: Calibration curve for Norepinephrine**

**Table No. 2: Linearity range (n=6)**

| Parameter | Observed values |
|---|---|
| Retention time (min) | 4.09 |
| Linearity range (µg/mL) | 10-35 |
| Regression equation | y=471639x+688350 |
| Correlation coefficient ($r^2$) | 0.966 |
| slope | 12635552.24 |
| Intercept | 688350 |
| LOD (µg/mL) | 1.17 |
| LOQ (µg/mL) | 3.55 |

### 3.3.3 Precision

The precision investigation employed six injections of standard solution at a concentration of 10 µg/mL. The precision Data are shown in Table 3. Yet, the percentage RSD readings of less than 2% demonstrate that the method is accurate and has a real-world application.

**Table No.3: Precision results (n=6)**

| Metabolite | Intra-day Precision (n=6) | | Inter-day Precision (n=6) | |
|---|---|---|---|---|
| | Mean ± SD | % RSD | Mean ± SD | % RSD |
| Norepinephrine | 4628999 ± 79435.51 | 1.72 | 5224038 ± 89442.72 | 1.71 |

### 3.3.4 Accuracy

The sample solutions that had previously been assessed at three levels 80%, 100%, and 120% were combined with a known quantity of standard solution. Three different concentrations have been used to calculate the amount of norepinephrine recovered. The accuracy information is in Table 4. percent The RSD values for every assay were under 2%, demonstrating the analytical method's accuracy and suitability for our applications.

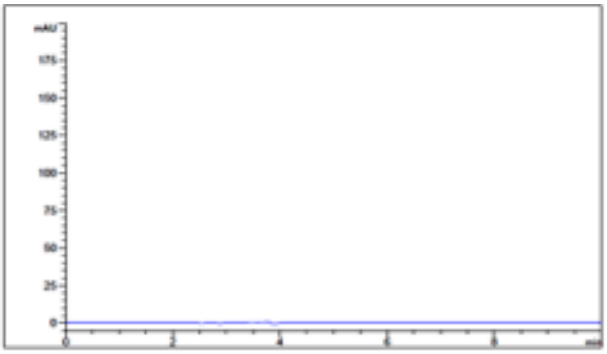

**Figure No.4. A) Chromatogram for blank solution**

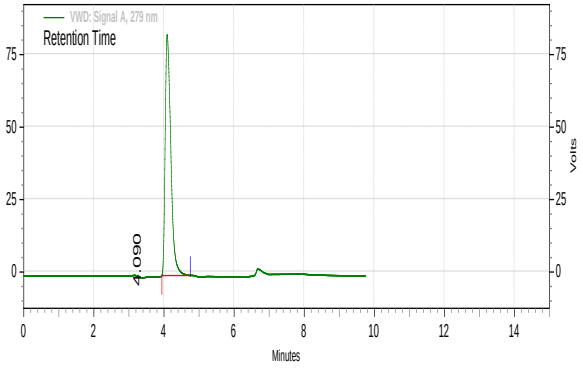


**Figure No.4. B) Norepinephrine standard chromatogram ( 8 µg/mL)**

**Table No.4: Accuracy (recovery data)**

| % Level spiked | Amount added | Amount recovered | % recovery | % Average | SD | % RSD |
|---|---|---|---|---|---|---|
| 30 | 30 | 25.97 | 86.57 | 86.87 | 0.16 | 0.632 |
| | 30 | 26.25 | 87.50 | | | |
| | 30 | 25.96 | 86.53 | | | |
| 50 | 50 | 41.99 | 83.98 | 85.29 | 0.57 | 1.327 |
| | 50 | 42.98 | 85.96 | | | |
| | 50 | 42.96 | 85.92 | | | |
| 60 | 60 | 55.92 | 93.20 | 91.94 | 0.66 | 1.190 |
| | 60 | 54.82 | 91.37 | | | |
| | 60 | 54.75 | 91.25 | | | |

### 3.3.5 Specificity/ selectivity

The chromatograms were shown in Figure 4. The chromatogram of the mobile phase blank showed no internally occurring peaks at the norepinephrine retention time (figure 4A). Dopamine hydrochloride had the same retention time in both the injectable sample solution (figure 4B) and standard solution (figure 4C) chromatograms. The selectivity of the suggested approach was demonstrated by the dopamine hydrochloride peak's lack of interference with mobile phase constituents. The tailing factor and retention duration parameters were computed to demonstrate the selectivity and specificity of the suggested technique. The parameters for the retention time and tailing factor are shown in Table 1.

### 3.3.6 LOD and LOQ calculations

The limit of detection was found to be 1.17 µg/mL and quantification limit 3.55 µg/mL for norepinephrine.

### 3.3.7 Robustness

Following each adjustment, the sample solution was fed into the chromatographic system, and the system suitability parameters were assessed. The results for % RSD are shown in Table 5.

| Conditions | Variations | % Assay | Standard Deviation | % RSD |
|---|---|---|---|---|
| Mobile phase flow rate (0.5 mL min-1) | 0.4 | 91.61 | 2.13 | 1.906 |
| | 0.6 | 92.02 | 1.18 | 1.261 |
| Column temperature (30 $^0$C) | 25 | 88.17 | 1.18 | 1.311 |
| | 35 | 86.99 | 0.98 | 1.200 |
| Mobile phase ratio (70:30) | 65:35 | 90.63 | 0.92 | 1.415 |
| | 75:25 | 85.28 | 0.66 | 1.549 |
| Mobile phase pH (2.2) | 2 | 91.24 | 1.85 | 1.608 |
| | 2.4 | 95.00 | 1.89 | 0.855 |

### 3.3.8 Analysis of norepinephrine in artificial urine

The amount of norepinephrine in each injected solution was estimated and found to be 94.78 ± 0.05 for norepinephrine by comparing the test and standard regions. The results are summarised in Table 6.

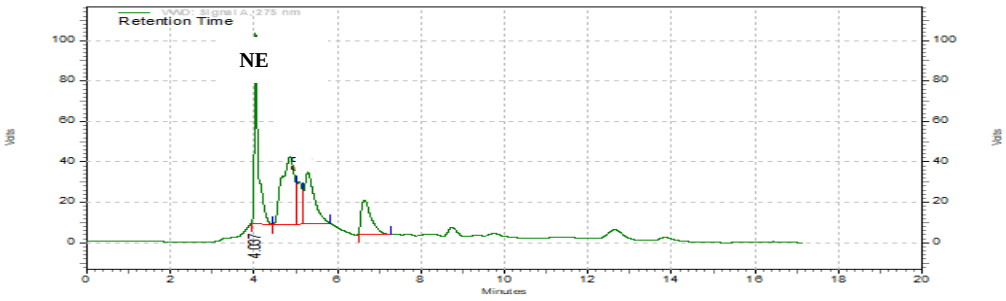


**Figure No 5: Chromatogram of norepinephrine in rat urine**

**Table No. 6: Analysis of norepinephrine in artificial urine (n=3)**

| Solution | Concentration added (µg/mL) | % Assay | mean ± SD |
|---|---|---|---|
| Norepinephrine | 20 µg/mL | 18.96 | 94.78 ± 0.05 |

## 4. DISCUSSION AND CONCLUSION

For evaluating norepinephrine, an effective HPLC method has been developed. The run duration for the analytical method was only 4 minutes, and it had a great peak shape and sufficient parameters for system suitability.

The analytical method was validated using the ICH Guidelines and was discovered to be linear, accurate, exact, and specific. The norepinephrine measuring technique has been successfully applied to rat urine. This method's high specificity and recovery for norepinephrine in the injectable solution has shown that it may be used to biological fluids. The technique can therefore be used to analyse norepinephrine levels in urine samples on a daily basis for quality control. The suggested method has the advantage of taking less time and using a phosphoric buffer that is compatible with chromatographic equipment for the mobile phase.

## 8. ACKNOWLEDGMENT

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