Original Article

Assessment and quantification of Dopamine in rat urine by a validated HPLC- Photodiode Array method (HPLC-PDA)

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Abstract

Dopamine is a neurotransmitter that functions as a chemical messenger in the nervous system, allowing certain neurons to communicate with one another. For the determination of dopamine HCl in rat urine, an easy, precise, and precise HPLC approach was devised and validated. The separation was carried out on a C8 column (250 x 4.6 mm, 5 µm) with a combination of 0.1% ortho-phosphoric acid: acetonitrile (70:30) (pH 2.2) as mobile phase. The mobile phase flow rate was kept constant at 0.5 mL/min. UV detection at 279 nm was used to obtain high sensitivity. During the development of analytical methods, parameters according to ICH guidelines (Q2A; Q2B) were examined. Dopamine had a retention duration of 4.1 minutes. For dopamine 8-42 µg/mL range was selected , the approach has been validated (r2 =0.9871). This approach has proven to be highly reproducible and recoverable within the specified range. For routine analysis, this approach can be employed successfully for assessing and quantification in rat urine samples.

Keywords: Dopamine, HPLC-PDA, method validation, rat urine etc.

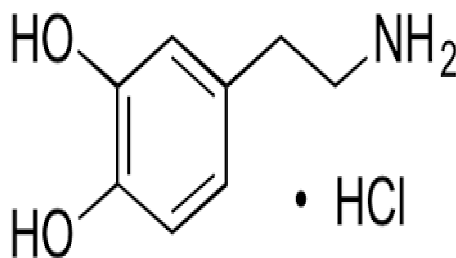
1. INTRODUCTION

Dopamine is a neurotransmitter that serves as a chemical transmitter in the nervous system, allowing certain neurons to communicate with one another. It belongs to the monoamine family of compounds, and more specifically, substances of catecholamine group. Dopamine functions as an stimulative or repressing mechanism within the central systema nervosum, looking on the situation of Intropin neurons and also the receptive characteristics of subsequent somatic cell cell within the chain (Berridge & Robinson, 2018). It is essential for endocrine control, respiration, movement, perception, and reward, among other brain activities (Moller et al., 2014). It's also important for the functioning of the heart, urinary, and hormonal changes. (Hussain and Lokhandwala, 2003; Ivan et al., 2005). Neurological diseases such as schizophrenia and PD have been linked to a decrease in dopamine-containing neurons (Guo et al., 2009). Dopamine determination must be sensitive and precise not only for diagnosis but also for pathological research.

Over the years, a variety of approaches have been employed to determine dopamine hydrochloride. To date, many methods for accurate dopamine analysis have been described, including fluorometry, chemiluminescence, HPLC with electrochemical processes, mass spectrometry, and capillary electrophoresis. LC-MS detectors (Carrera et al., 2007; Zhao & Suo, 2008), UV detectors (Liu et al., 2004), fibre optic detectors (Silva et al., 2009; Ferreira et al., 2009), electrochemical detectors (Sultan et al., 2014; Gupta et al., 2015) and fluorescent detector (Yoshitake et al., 2004) are mostly used in advanced HPLC analysis techniques.

These techniques are useful because of their sensitivity, accuracy, economical, and ease of use. Due to sophisticated and advanced instrumentation most of these are not easily available for use. Many of those approaches have additionally been developed for the detection of Dopamine in biological fluid solutions. For routine Dopamine determination in biological fluids is a need, thus a simple, rapid, precise, and accurate approach is required. The validation of dopamine in rat urine was assessed in this research work.

Figure 1: Dopamine hydrochloride



2. MATERIALS AND METHODS

2.1 Chemicals and solvents

All the chemicals used in this research were of analytical purity and were not further processed. The following substances were used: dopamine hydrochloride standard (98.5 percent, Sigma-Aldrich), o-phosphoric acid (85 percent, Sigma-Aldrich), and glacial acetic acid (70 percent AR grade, SRL Chemical), formic acid (98-99 percent AR grade, Research lab). Standards were dissolved in Millipore-purified distilled water (Merck, Direct O_2).

For animal study IAEC permission was granted with approval number (DYPIPSR/IAEC/Oct/2021-22/P-05)

2.2 Liquid chromatography

The chromatographic separation was achieved by using LC solutions Liquid Chromatography Shimadzu HPLC system in low pressure gradient mode using a Kromasil C8, 5 µm (250 X 4.6 mm) analytical column. Sonication was employed to degas a mixture of acetonitrile: 0.1 % ortho-phosphoric acid (pH 2) in the ratio (30:70 v/v) pH 2.2. The flow rate and column temperature were held constant throughout the experiment, at 0.5 mL/min and 25°C, respectively.

2.2.1 Optimization of chromatographic conditions

Various mobile phases such as acetonitrile, formic acid, methanol, and orthophosphoric acid were investigated based on published literature on HPLC and LC-MS. The injected volume was 20 µL, and wavelength of 279 nm was selected based on previous study results (Kremer T et al., 2021; Li H et al., 2022). The flow rate that generated the best results was discovered after testing flow rates ranging from 0.5 ml/min to 1 ml/min. Dopamine hydrochloride standard was separated at specific retention time.

2.3 Preparation of standard stock solution

An analytical balance (Shimadzu ATX224 precision balance) was used to weigh about 1 mg of Dopamine hydrochloride, which was then put into a 1 mL eppendorf tube. To label, it was dissolved in ultrapure water and the volume was made up using the same solvent. The prepared stock standard solution has a concentration of 1000 µg/mL

Serial dilution of the standard stock solution using ultra pure water as a diluent on the same day yielded a dopamine hydrochloride calibration curve in the concentration range of 8-42 µg/mL.

2.4 Determination of λmax

10 µg/mL standard solution scanning was performed on a UV spectrophotometer, between 200 and 400 nm (Spectrophotometer: Shimadzu UV-1900). λmax was derived from a conventional solution's UV spectrum.

2.5 Method validation

Validation of Analytical Procedure: Q2 (R1) (ICH, 2005; Center for Drug Evaluation and Research (CDER), 1994) were used to validate the analytical method. System validity and reliability was determined, linearity, precision, accuracy, specificity, the limit of detection (LOD), the limit of quantification (LOQ), and robustness were all addressed as validation parameters.

2.5.1 System suitability

The system suitability parameters (RSD percent for retention time, RSD percent for peak area, theoretical plates, and tailing factor) were tested in the HPLC system after 6 replicate injections of standard solution (8 µg/mL).

2.5.2 Linearity

The standard calibration was developed using six standard solutions with concentrations ranging from 8 to 42 µg/mL. In ideal chromatographic conditions, each standard solution was chromatographed five times for a total of 10 minutes. Least squares linear regression analysis of average peak area against concentration data was used to test the method's linearity.

2.5.3 Precision

Intraday (repeatability obtained by checking standard solution on the same day) and interday (repeatability determined by examining standard solution on three different days) variations of the technique were calculated to estimate precision. For these experiments, six times the standard solution (8 µg/mL) was injected.

2.5.4 Accuracy

Recovery tests were carried out using the standard addition approach to check the accuracy of the proposed method. In this procedure, 3 different levels of pure drug were added to previously examined sample solutions at 80 percent, 100 percent, and 120 percent, and Dopamine hydrochloride recovery was measured for each concentration.

2.5.5 Selectivity/Specificity

Selectivity refers to the analytical method's ability to obtain an analytic result when external interference is present. The chromatograms of the mobile phase solution (blank) and the sample solution were compared to the chromatogram of the standard solution to determine the selectivity of the proposed technique. The retention time (Rt) and queuing factor values were obtained to demonstrate that the selected strategy was particular and selective.

2.5.6 Detection limit and Quantification limit

The formula stated below was used for calculation:

LOD = 3.3 σ/S; LOQ = 10 σ/S

2.5.7 Robustness

To investigate the influence of mild but systematic alterations in chromatographic settings, a robustness study was done. Minor variations in parameters were used to verify the robustness. Different concentrations of the mobile phase (5%) and mobile phase flow rates (0.5 mL min-1) are among the changes, the mobile phase pH, and column temperatures (20 °C). By introducing the sample solution into the chromatographic system after each change, the system suitability parameters were tested.

2.5.8 Analysis in rat urine sample

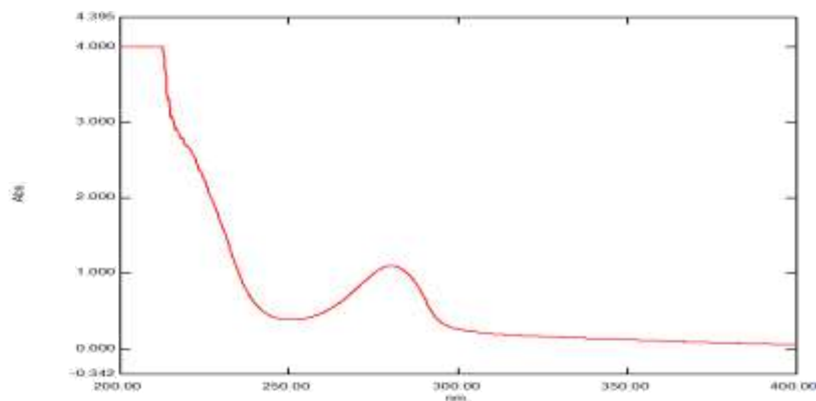
The 24 hr rat urine sample was collected and before analysis the urine extracted with 0.4M perchloric acid then centrifuged at 8000 rpm for 3 min and then it was filtered through a 0.45 µm membrane filter. Approximately 20 µL urine was injected through the HPLC system.

3. RESULTS AND OBSERVATIONS:

3.1 Determination of absorption maximum for metabolite

A UV spectrophotometer was used to study the spectra of dopamine, and the λmax of dopamine was found at 279nm shown in fig 2.

Figure 2: Dopamine hydrochloride λmax at 279 nm



3.2 Optimization of mobile phase and chromatographic conditions:

Several reverse phase HPLC columns (C8 columns) and different mobile phases were used in the optimization study. Because of the narrower columns, proper peak symmetry was hard to accomplish. Peak symmetry was achieved on the kromasil C8 column (250 x 4.6 mm, 5 m). As a result, we selected the kromasil C8 column (250 x 4.6 mm, 5 m) for the validation study. Following the selection of the chromatographic column, the mobile phase was chosen as a mixture of 0.1 percent o-phosphoric acid and 70:30 acetonitrile (pH 2.2). When selecting on the mobile phase, no environmentally harmful compounds were used.

The injection volume and column temperature were calculated, and the initial low-pressure gradient mode was gradually adjusted.

3.3 Method validation:

3.3.1 System suitability

Using the same conditions as the chromatographic system, an 8µg/mL standard solution was injected six times into the HPLC system. Table 1 shows the number of theoretical plates, the symmetry factor, the peak area, and retention durations.

Table 1: Results for system suitability

System suitability criteria	**Standard solution concentration (8 µg/mL)**
Symmetry factor	1.20
No of theoretical plates	10602
Peak area (% RSD)	1.34
Retention time (% RSD)	0.068

3.3.2 Linearity

The calibration curve for dopamine peak-area against concentration was then plotted. Least squares linear regression was used to examine the relationship between peak area and concentration. Data was used to test the strategy's linearity. Figure 3 depicts the calibration curve, while table 2 contains data on linearity parameters.

Figure 3: Calibration curve for dopamine hydrochloride

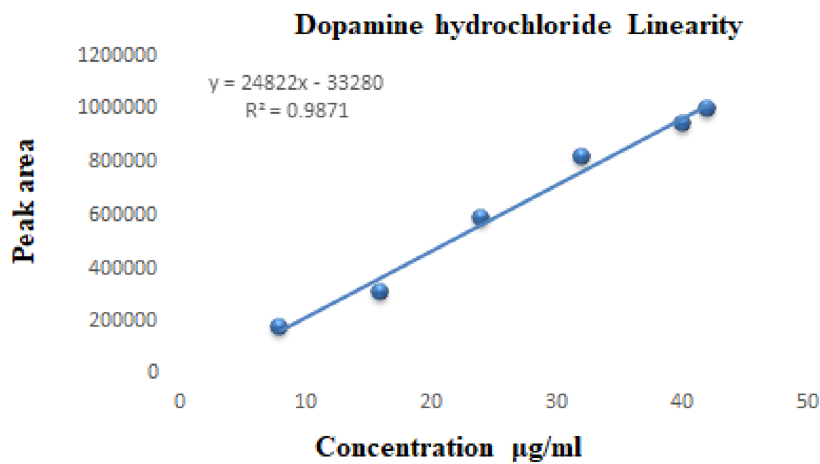


Table 2: Linearity range (n=6)

Parameter	Observed values
Retention time (min)	**4.1**
Linearity range (µg/mL)	**8-42**
Regression equation	**y = 248222x - 33280**
Correlation coefficient (r^2)	**0.9871**
slope	**7972569.37**
Intercept	**- 33280**
LOD (µg/mL)	**1.40**
LOQ (µg/mL)	**4.23**

3.3.3 Precision

6 injections of standard solution of 8 µg/mL concentration were used in the precision study. Table 3 shows the precision data. Regardless, the percentage RSD readings of less than 2% show that the approach is accurate and can be put into practical use.

Table 3: Precision results (n=6)

Metabolite	Precision (Intra-day) (n=6)		Precision (Inter-day) (n=6)	
	Mean ± SD	**% RSD**	**Mean ± SD**	**% RSD**
Dopamine hydrochloride	**178058.17 ± 2217.94**	**1.25**	**185353.17 ± 2588.33**	**1.40**

3.3.4 Accuracy

A known quantity of standard solution was added to the sample solutions that had previously been evaluated at three levels: 80 percent, 100 percent, and 120 percent. The amount of Dopamine hydrochloride recovered has been calculated at three different concentrations. Table 4 contains the accuracy data. percent The RSD values for all assays were less than 2%, showing that the analytical procedure is precise and suitable for our applications.

Table 4: Accuracy (recovery data)

% Level spiked	Amount added	Amount recovered	% recovery	Average %	SD	RSD
80	80	72.97	91.21	91.20	0.01	0.014
	80	72.95	91.19			
	80	72.96	91.20			
100	100	91.99	91.99	92.64	0.57	0.611
	100	92.98	92.98			
	100	92.96	92.96			
120	120	115.92	96.60	95.97	0.66	0.570
	120	114.82	95.68			
	120	114.75	95.63			

3.3.5 Specificity/ selectivity

The chromatograms were shown in Figure 4. There were no internally occurring peaks in the chromatogram of the mobile phase blank at the retention time of dopamine hydrochloride (figure 4A). Dopamine hydrochloride has the same retention time in injected sample solution chromatograms (figure 4B). The dopamine hydrochloride peak did not interfere with mobile phase components, indicating the proposed method's selectivity. The tailing factor and retention time parameters were calculated to demonstrate how selective and specific the suggested method was. Table 1 shows the calculated retention time and tailing factor parameters.

Figure 4. A) Blank solution chromatogram

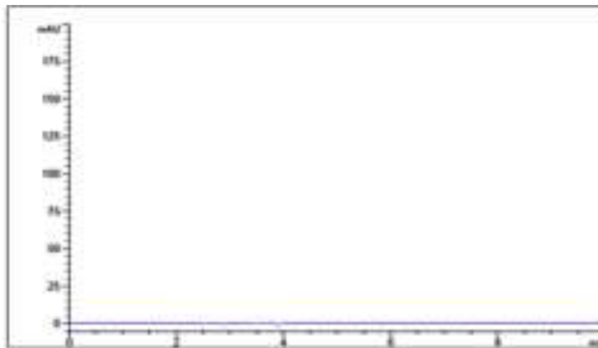
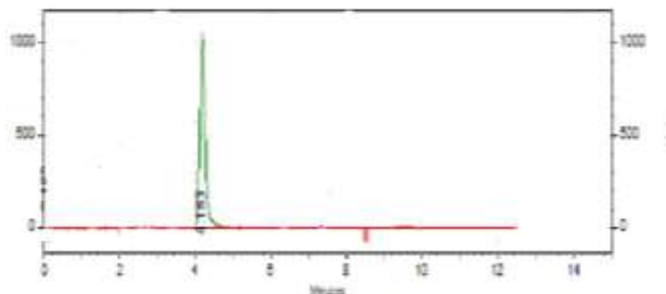


Figure 4. B) Dopamine hydrochloride standard chromatogram (8 µg/mL)



3.3.6 LOD and LOQ calculations

The values were calculated from the formula given below and shown in table 2:

3.3.7 Robustness

After each modification, the sample solution was injected into the chromatographic system, and the system suitability parameters were assessed. The percent RSD values are shown in Table 5.

Table 5: Data for robustness parameter

Conditions	**Variations**	**% Assay**	**Standard Deviation**	**% RSD**
Mobile phase flow rate (0.5 mL min-1)	0.4	92.61	2.03	1.926
	0.6	93.02	1.08	1.161
Column	25	87.17	1.13	1.301

temperature (30 ^{0}C)	35	84.99	0.93	1.100
Mobile phase ratio (70:30)	65:35	89.63	0.82	0.915
	75:25	84.28	0.46	0.549
Mobile phase pH (2.2)	2	90.24	1.85	1.908
	2.4	93.00	0.89	0.955

3.3.8 Analysis of dopamine in rat urine

By comparing the test and standard regions, the amount of dopamine hydrochloride in each injected solution was estimated and determined to be 97.39 ± 0.13 for dopamine hydrochloride. Table 6 summarises the findings The chromatogram for dopamine separated in rat urine shown in figure 5.

Figure 5: Chromatogram of dopamine in rat urine

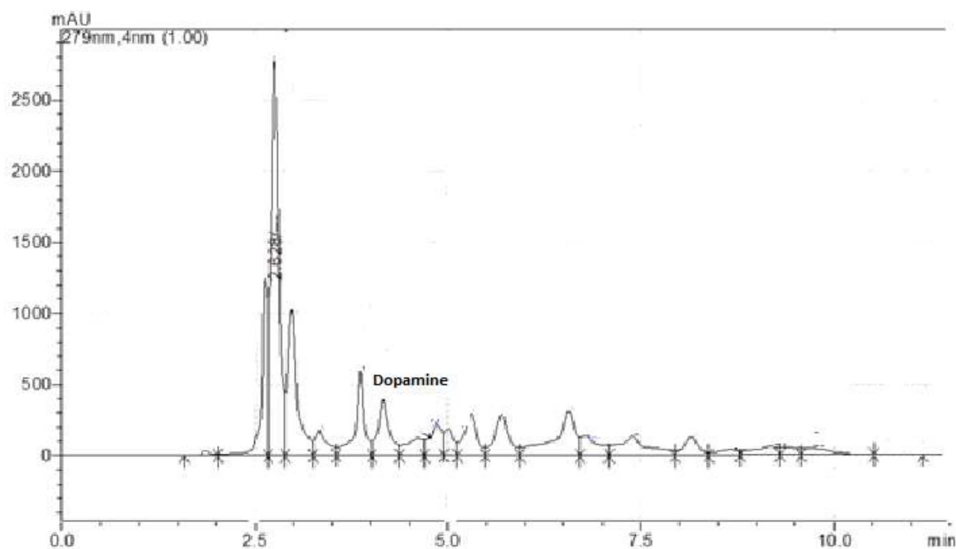


Table 6: Analysis of dopamine in rat urine (n=3)

Solution	Added amount (µg/mL)	Recovered amount (µg/mL)	% Assay mean ± SD
Dopamine in rat urine	40 µg mL-1	38.96	97.39 ± 0.13

4. DISCUSSION AND CONCLUSION

A successful HPLC technique for assessing dopamine hydrochloride has been devised. The analytical method had a good peak area and suitable parameters for system suitability, and it took only 4.1 minutes to run.

The ICH Guidelines were used to validate the analytical approach, which was determined to be linear, accurate, precise, and specific. The dopamine hydrochloride quantification method has been successfully employed in rat urine. The specificity and recovery for dopamine hydrochloride in the injected solution illustrate the applicability of this technique to biological fluids. As a result, the method can be used for daily quality control analysis of dopamine hydrochloride in urine samples. The proposed method is time consuming and the phosphoric buffer used in the mobile phase is compatible with chromatographic equipment.

5. FUNDING SOURCE

There was no financial support for the research from any agency.

6. CONFLICT OF INTEREST

The research was conducted as part of a PhD research. There is no potential for a conflict of interest.

7. AUTHORS CONTRIBUTIONS

Vrushali Bhalchim: Literature review, Conceptualization, Experimental work, Manuscript writing.

Vaishali Undale: Conceptualization, Critical reviews.

Sunil Shewale: Conceptualization, Data analysis and curation

Vrushali Neve: Manuscript drafting and editing.

Shweta Lembhe: Experimental work

Shital Satone: Experimental work

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