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METHOD DEVELOPMENT AND VALIDATION OF METANEPHRINE AND NORMETANEPHRINE ON LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT:
Catecholamines and its metabolites play significant physiological roles in the human body. Paraganglioma and pheochromocytoma are rare tumors of an adrenal region that affect the catecholamines and their metabolites (metanephrine (MN) and normetanephrine (NMN)) concentration. Given the significance of these metabolites for diagnosing, managing, and monitoring adrenal tumors, a method has been developed and validated using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). This method is specifically designed for quantification of MN and NMN in urine. The objective of developing and validating this analytical approach is to provide physicians with a reliable tool for assisting in the management of adrenal tumors.
For sample extraction, 2-aminoethyl-diphenylborinate and ethyl acetate were used. The organic layer was separated and dried under nitrogen at 50-60°C and reconstituted with mobile phase. The chromatographic separation was done using the Agilent Eclipse C-18 column with two mobile phases: (A) 5% Methanol in 0.1% Formic acid and (B) 100% Methanol in an isocratic flow. The run times for MN and NMN were 1.259 and 1.268 min, respectively. The validated analytical measuring range (AMR) was 7.8-1000 ng/mL with lower limits of quantification (LLOQ) 7.8 ng/mL for MN and NMN, and the recovery was 95.11% to 105.6% for MN and 95.87% to 104.78% for NMN. The method showed an excellent correlation (r2) of 0.997 for MN and 0.998 for NMN. The method was found steady after inter-day and intra-day analysis with coefficients of variation (CV) <3% and accuracy <6%. This validated method can be used for metabolite screening in clinical and research settings.

Keywords: Liquid chromatography-mass spectrometry, metanephrine, normetanephrine, Pheochromocytoma.

1. Introduction

Pheochromocytoma and paraganglioma are rare tumors arising from the adrenal medulla's chromaffin cells and sympathetic ganglia, present in adrenal glands [1]. These tumors release catecholamines (dopamine, epinephrine, and norepinephrine) from the intra-adrenal and extra-adrenal parts of the gland. In general, the term pheochromocytoma is used for both adrenal pheochromocytoma and catecholamine-secreting paragangliomas. However, the distinction between both is critical because of differences in the risk of malignancy and testing [2]. Catecholamines include dopamine, adrenaline, and noradrenaline. These chemicals are produced by chromaffin cells of the adrenal medulla and secreted by nerve tissues and the brain [3]. These are responsible for sympathetic and parasympathetic responses in the body, including emotional and physical stress. Its metabolites, metanephrine (MN) and normetanephrine (NMN), control the immunity regulation and various physiological processes in the body [4].

The concentration of these metabolites is analyzed to diagnose rare neuroendocrine tumors (pheochromocytoma and paraganglioma) [5]. Patients affected by pheochromocytoma show symptoms of headache, sweating, and tachycardia, and when not diagnosed properly, it can lead to hypertension and fatal cardiovascular diseases [6, 1].

Annual prevalence of pheochromocytoma internationally is 2–8 people out of 1 million, and in Pakistan 0.08 out of 1 million population is affected every year [7, 8]; however, these numbers are underestimated as 50% of pheochromocytomas are diagnosed during an autopsy. Although these tumors can exist in any age and affect both genders equally, the highest occurrence has been noted in the 40-50 age group [6, 3]. Catecholamine-secreting tumors are mostly sporadic in nature, but 40% are familial and bilateral.

Quantification of catecholamines (dopamine, epinephrine, and norepinephrine) has been widely used to screen pheochromocytomas. The episodic nature of catecholamine's secretion makes them a poor marker of pheochromocytoma compared to metanephrines as being a metabolite, their concentration is constant [4, 9]. Catecholamines (dopamine, epinephrine, and norepinephrine) produced in adrenal or extra-adrenal tissues undergo *O*-methylation catalyzed by catechol-*O*-methyltransferase (COMT) enzyme and are converted into metanephrine (MN) and normetanephrine (NMN) as collectively called metanephrines (MNs) (Figure I). MNs may exist in free or conjugated form. The conjugation is done by an enzyme sulfotransferase in the gastrointestinal tract.

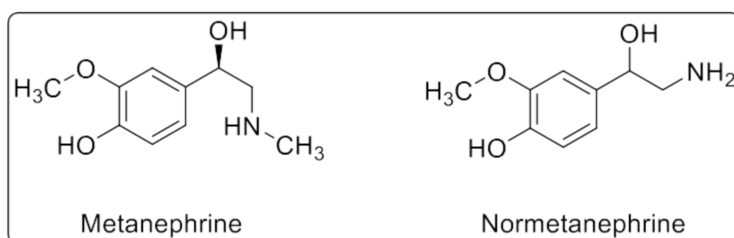


Figure I: Chemical Structures of Metanephrine (MN) and Normetanephrine (NMN).

The catalysis of free MNs is done by an enzyme monoamine oxidase (MAO) and converted into vanillylmandelic acid (VMA). This VMA and other conjugated MNs are eliminated through the kidneys. These metabolites have better specificity and sensitivity for the diagnosis of pheochromocytoma. MNs exist in both urine and blood but are different in nature. In plasma, MNs are found in free form, while both free and conjugated forms co-exist in urine [10]. The 2014 Endocrine Society Clinical Practice Guideline with a level A quality of evidence by the GRADE system recommends that the measurement of plasma-free or urinary fractionated MNs should be done in the initial biochemical testing with no preference for one over the other [1].

The quantification of total MNs can be complicated due to errors in analytical steps, such as the acid hydrolysis deconjugation step. With the advancement in measurement techniques and methods, free MNs in plasma or urine are measured with high sensitivity and minimal interference for the diagnosis of Pheochromocytoma [4]. Plasma and urine both are used for sample collection. Urine has been recommended as an alternative specimen due to the difficulty in collecting plasma samples and the physiological constraint that prevents blood collection in the supine position [8, 11].

Analytical methods including colorimetric, immunoassay, High Performance Liquid Chromatography (HPLC) with electrochemical detection or HPLC with fluorescence detection [1,10] and Gas Chromatography-Mass Spectrometry (GC-MS) [12] have been reported for the detection of MN and NMN. Advances in the instrumentation of clinical chemical laboratories have led to a transition for the detection of MN and NMN from the classical fluorometric and spectrophotometric techniques to more modern methods such as high-performance liquid chromatography (HPLC) and immunoassays.

These advancements have notably enhanced both the analytical sensitivity and specificity in the field of clinical chemistry for the diagnosis of Pheochromocytoma. [13]. Out of these previously reported methods commercially available enzyme-linked immunoassays (EIAs) are cost-effective and easy to handle but contributed to poor diagnostic sensitivity of only 74.1% [1]. However, Liquid chromatography with mass spectrometry has been the most widely reported and recommended [1] analytical technique for the analysis of MN and NMN due to the reason that the detection ability of the MS is to quantify and identify compounds by associating their retention times with structural information.

For instance, Gabler J *et al.* [14] used LC-MS/MS to analyze MN and NMN in urine samples of patients who suffered from pheochromocytoma and paraganglioma after extracting the metabolites in urine by solid phase extraction (SPE) and found the linear assay in the range of 0.2 to 27.4 µmol/L for MN and 0.3 to 14.6 µmol/L for NMN. Also showed that the intra-day and inter-day precision were <5% for MN and <10% for NMN. Another study used the same LC-MS/MS method for the quantification of urinary MN and NMN in pediatric patients [15]. Similarly, the LC-MS/MS method was used by Woo HI *et al.* for the estimation of catecholamines and their metabolites in urine followed by SPE and found the linear assay in the range of 3.5–2466 µg/L for MN and 3.7–2569 µg/L for NMN.

The coefficients of variation (CV) in their developed method were less than 10% with respect to imprecision [16]. Reports show that the quantification of MN and NMN in urine samples by LC-MS/MS is comparatively better than other methods. Lefeuvre S *et al.* compared the LC-HRMS method with a liquid chromatography assay with electrochemical detection (LC-EC) and showed that the LC-HRMS method offers significant advantages compared to LC-EC with good sensitivity, an unambiguous analyte determination and high sample throughput. For instance, Lenders *et al.* [10] compared LC-MS/MS with HPLC with electrochemical detection and found that LC-MS/MS had higher sensitivity and specificity. Likewise, Dirk Weismann *et al.* [17] compared LC-MS/MS with an enzyme immunoassay and found that LC-MS/MS had better accuracy and precision.

The importance of LC-MS/MS in clinical practice cannot be overemphasized, as it is a highly sensitive and specific method for the quantification of urinary MN and NMN. Accurate measurement of urinary MN and NMN is crucial for the diagnosis, monitoring, and managing the patients who suffer from these tumors. Hence, this study was conducted to develop and validate a sensitive and specific LC-MS/MS method with simple sample preparation for the simultaneous quantitative determination of MN and NMN within two minutes for effective detection in urine

samples with high sensitivity and minimal interference for the timely diagnosis and appropriate management of Pheochromocytoma patients.

2. Materials and Methods

2.1. Standards and Chemicals

Urinary based Catecholamine Mix 2 (MN and NMN) standard (1 mL vial, Product number: C-110) having concentration 1 mg/mL and (±)-Metanephrine-d3 hydrochloride solution (1mL vial, Product number: M-148) having concentration 100 µg/mL; were purchased from Sigma-Aldrich (Cerilliant, Supelco Inc.). Blank urine (MN and NMN free urine) (50 mL vial, Product number: SAE0074) was purchased from Sigma-Aldrich. NANO-pure water system (Millipore apparatus, Merck, Germany) was used for deionized water. Methanol (LC-MS grade), acetonitrile (LC-MS grade), ethyl acetate, formic acid, NH_3 solution, and 2-aminoethyl-diphenylborinate were ≥99.995% pure and bought from Sigma Aldrich.

2.2. Solutions, internal standards, and controls

90:10 v/v solution of water: methanol was prepared by adding 90 mL water and 10 mL methanol in a 100 mL volumetric flask. 0.25% NH_4OH solution in a 100 mL volumetric flask was prepared by adding 780.125 µL of NH_3 solution. 2 g/L solution of 2-aminoethyl-diphenylborinate was prepared by adding 0.2 grams of 2-aminoethyl-diphenylborinate in 100 mL volumetric flask along with 780.125 µL of NH_3 Solution and making up the volume with 90:10 v/v solution of water: methanol. Dilute and shoot Solution: 100% Methanol: 100% Acetonitrile: 0.1% Formic acid (2:2:1) solution in water was prepared and placed at 15 to 25ºC for two years. Mobile Phase A (5% methanol in 0.1% formic acid): 1L of formic acid was added in 1000 ml of water. 50 mL of this solution was removed, 50 mL of 100% methanol was added to it, and it was stored at 25ºC for one month. Mobile Phase B was 100% methanol and was placed at 25ºC for one month.

2.3. Preparation of calibrators

Internal standards of 1 µg and 0.1 µg/mL were prepared by adding 10 µL of 100 µg/mL and 1 mL of the 1 µg/mL from a stock solution of deuterated standards, respectively, in a 10 mL flask and qs the volume with methanol. Stock standard solutions A (10 µg/mL), B (1 µg/mL), and C (0.5 µg/mL) were prepared by adding 100 µL of 1.0 mg/mL, 1000 µL of the 10 µg/mL and 500 µL of the 10 µg/mL of standards metabolites ((±)- (MN) and (±)- (NMN)) respectively in 10 mL flask and was qs to volume with methanol. For the calibration curve, (10 µg/mL), (1 µg/mL), and (0.5 µg/mL) of metabolite solution were taken into 16 x 125 mm screw cap test tubes (Table I). To eradicate the solvent effect, all calibrators were dried out by using nitrogen before adding 1 mL of blank urine.

Table I: Illustrated the final concentration of the solution after the addition of blank urine

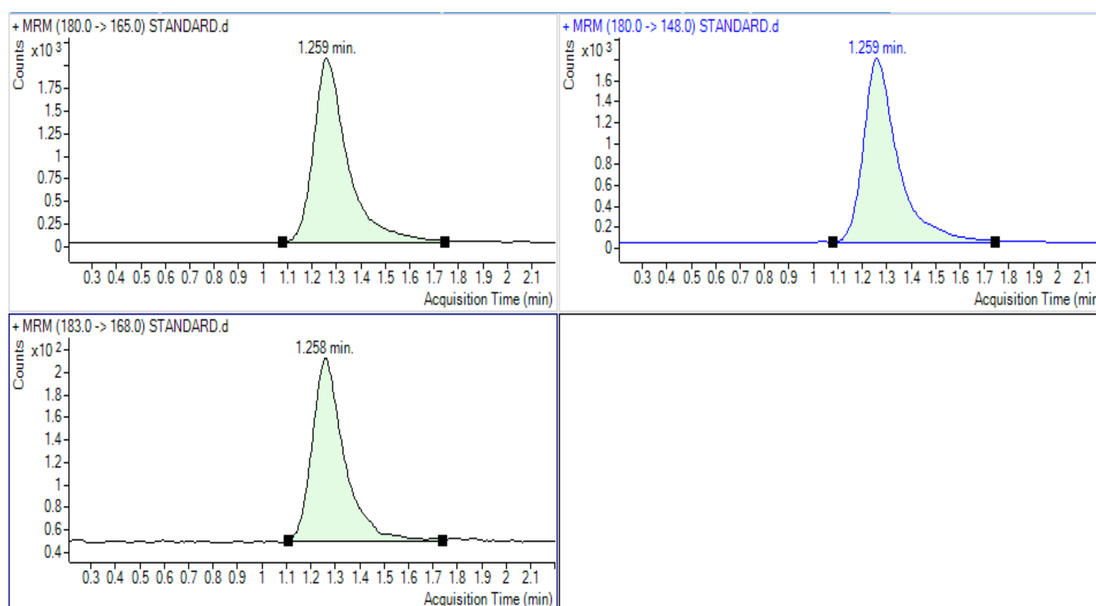
Stock solution (A) of 10 µg/mL(µL)	Stock solution (B) of 1 µg/mL(µL)	Stock solution (C) of 0.5 µg/mL(µL)	Final concentration of Metanephrines (ng/mL)
100			1000
50			500
	250		250
	125		125
		125	62.5
		62.5	31.25
		31.25	15.625
		15.625	7.8125

2.4. LC-MS/MS conditions

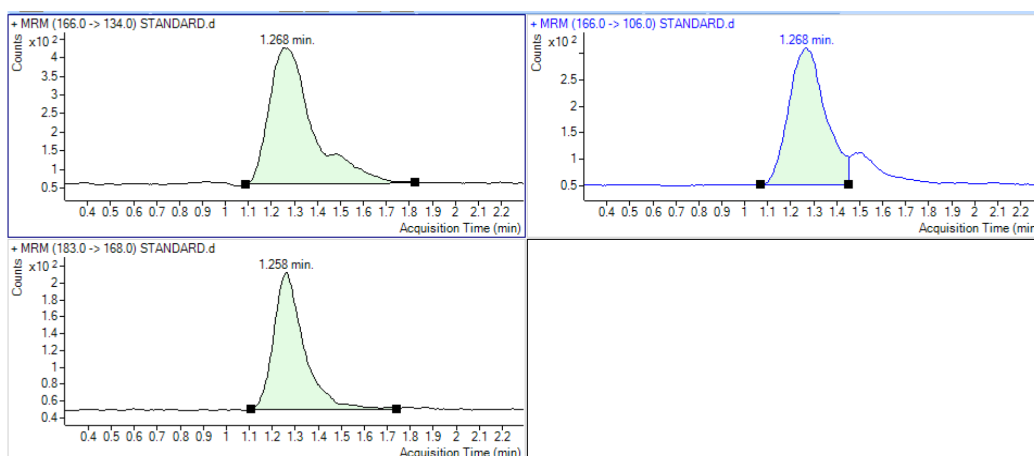
The system included HPLC Agilent 1260 infinity series, Agilent 6460 Triple Quad QQQ MS, and Eclipse C-18 Column (2.1 × 75 mm 2.7 µm) from Agilent with inline filter and frit (0.2 µm 2.1 mm) kept at 30°C. Mobile phases A and B were of 5% methanol in 0.1% formic acid and 100% methanol, respectively. The isocratic flow was maintained for 2.5 min, with 60% of mobile phase A and 40% of mobile phase B. The injection volume was 5 µL. Electrospray Ionization (ESI) in positive mode was used as a source, operated in multiple reaction monitoring (MRM) mode with 4000 V Capillary voltage, 350°C temperature, and 55 Psi nebulizer gas pressure. In ESI, nitrogen gas was used for desolvation with 9 L/min of flow rate at 350°C. An electrical signal produced was processed by Mass Hunter Software and displayed as a chromatogram on the screen. The mass transitions, dwell time, fragmentor voltage, and collision energy (CE) data for the analytes are shown in Table II. The chromatograms of metabolites (MN and NMN) with internal standards are shown in Figure II.

Table II: Mass transitions and instrumental settings in a mass spectrometer.

Compound	IS	Precursor ion	Product Ion	Dwell time	Fragmentor(V)	CE (V)
(±)-Metanephrine-d3	Yes	183	168	70	110	15
(±)-Metanephrine	No	180	165	70	110	15
(±)-Metanephrine	No	180	148	70	110	15
(±)-Normetanephrine	No	166	134	70	110	15
(±)-Normetanephrine	No	166	106	70	110	15

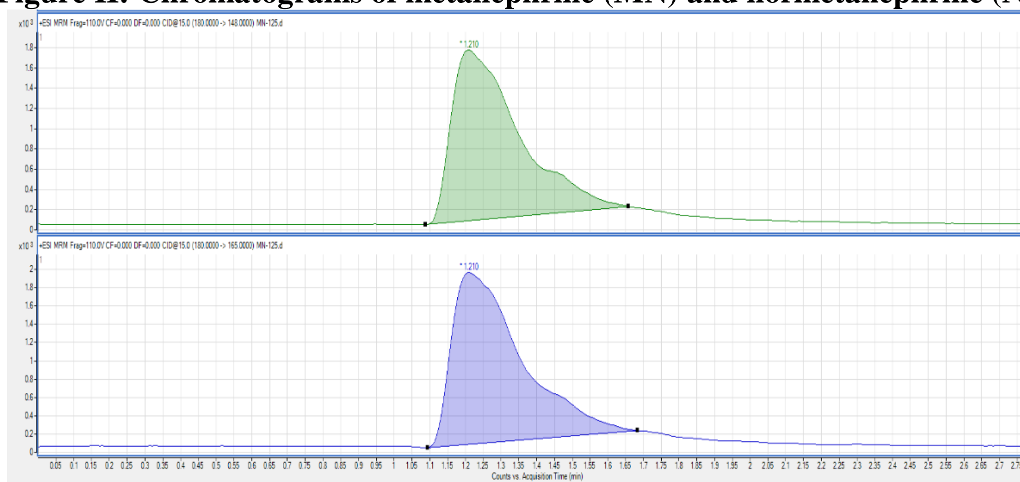


a) Metanephrine (MN)

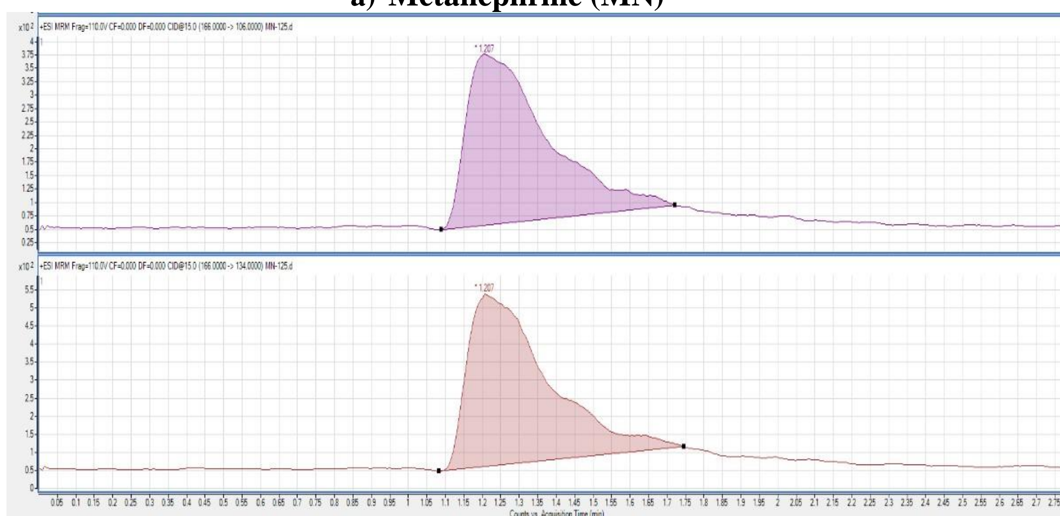


b) Normetanephrine (NMN)

Figure II: Chromatograms of metanephrine (MN) and normetanephrine (NMN).

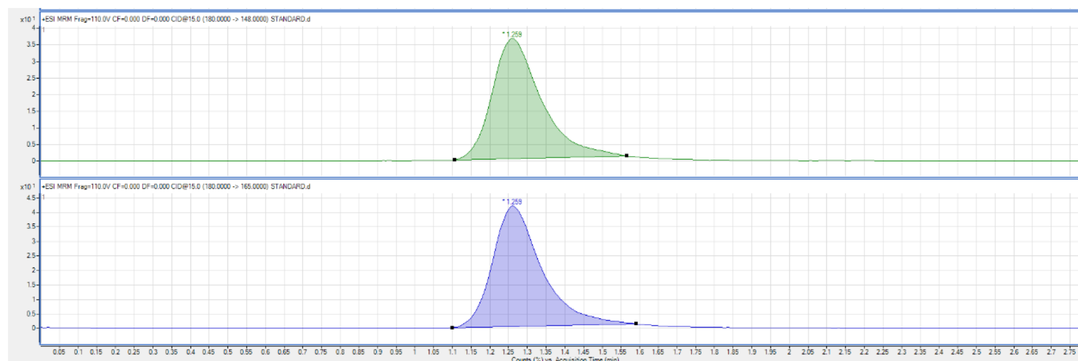


a) Metanephrine (MN)

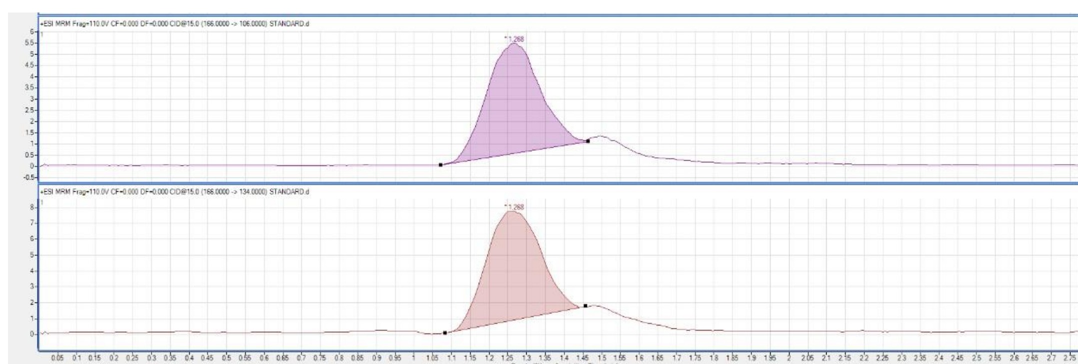


Normetanephrine (NMN)

Figure III (a). Chromatogram of metanephrine (MN) and normetanephrine (NMN) with mobilephase 0.1% formic acid in methanol and 0.1% formic acid in acetonitrile shows poor peak resolution of analytes.



a) **Metanephrine (MN)**



b) **Normetanephrine (NMN)**

Figure III (b): Chromatogram of metanephrine (MN) and normetanephrine (NMN)with mobile phase 5% methanol in 0.1% formic acid and 100% methanol shows the best peak resolution ofanalytes.

2.5. Method Validation

The method was validated in accordance with US Food and Drug Administration (FDA) guidelines [18, 19]. The method validation was done by testing linearity (calibration curve) with recovery, LLOQ, AMR, sensitivity, precision, accuracy, selectivity, carryover, stability, and dilution effects. For linearity, blank urine was spiked with varying concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 ng/mL of metabolites in triplicate. Quality Control (QC) samples with different concentrations 7.8125 ng/mL lower limit of quantitation (LLOQ), 31.25 ng/mL Low(L) (3 times LLOQ), 500 ng/mL Medium (M), and 1000 ng/mL High (H) samples with six copies were analyzed for inter and intra-day precision on three different days. The acceptable criteria for method validation were accuracy within ±15% (-0.93- 5.6%) at each level except at the LOQ ±20% of (CV), and the ratio of mass transitions was ±30% [9]. The analysis of metabolite-free urine (blank) and standards with the same MN and NMN concentration were spiked for comparison of MN and NMN peak areas.

2.6. Sample extraction

For method assessment, de-identified residual aliquots of 24-hour urine were used by evaluating the distribution of the metabolites (MN, NMN). The urine sample was preserved at 20°C with a small amount of boric acid as a preservative. Calibrators, control, and case samples were taken in 16 x 125 mm tubes. 1.0mL specimens/blank urine, 100µL of the 0.1µg/mL of internal standard, and 1.5mL of 2g/L solution of 2-aminoethyl-diphenylborinate were added in labeled tubes and vortex for a short period (30 to 60 seconds). In it, 5 mL of extracting solvent (ethyl acetate) was added, vortexed at 30 RPM, and then centrifuged at 3500 RPM both for 15 min. Upper organic layers were separated and

evaporated till dry at 50-60°C under nitrogen. Samples were reconstituted in 200µL of dilute and shoot solution and run on LC/MS/MS for analysis (Figure IV).

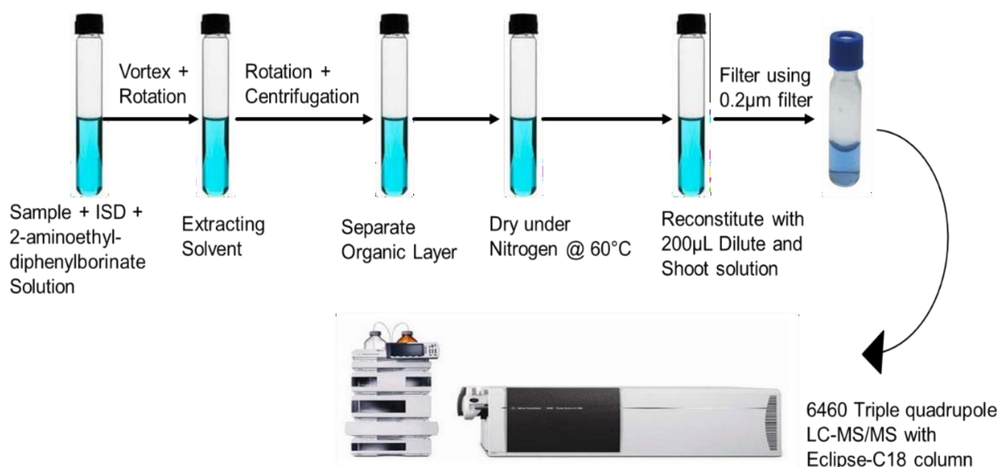


Figure IV: Flow chart illustrating the sample preparation for LC-MS/MS analysis.

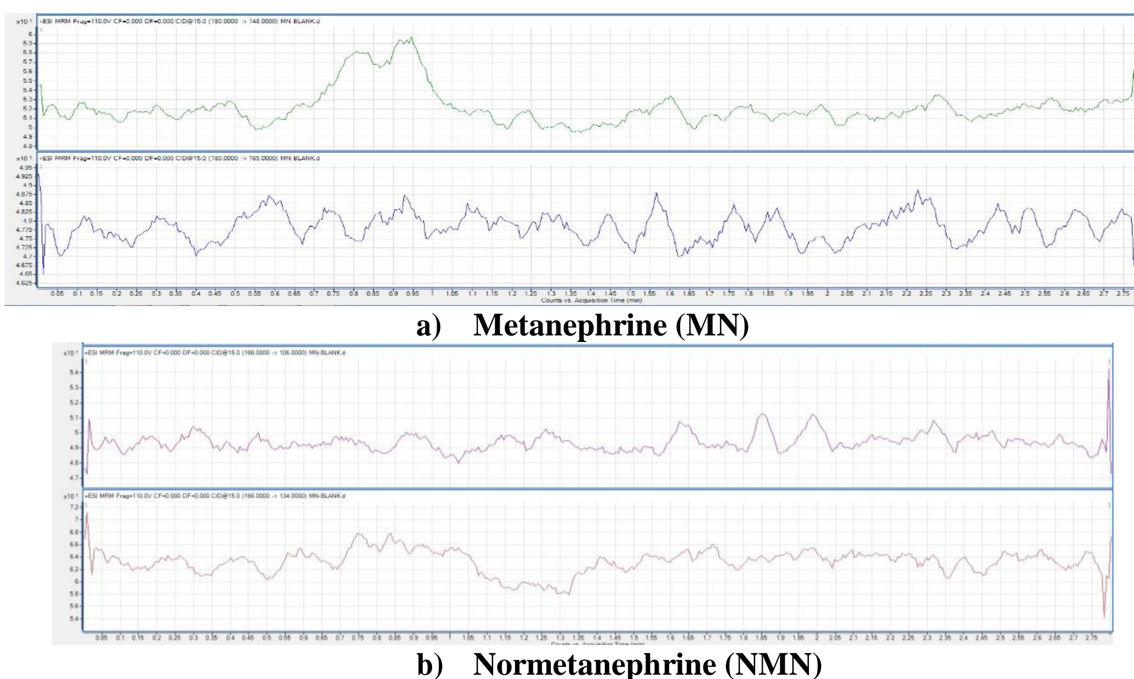
3. Results

3.1. Method Validation

Guidelines of the United States Food and Drug Administration (FDA) were used to validate this LC-MS/MS technique. Parameters on which validation was assessed included selectivity, linearity, precision, accuracy, recovery, matrix effect, stability, and carryover.

3.1.1. Selectivity:

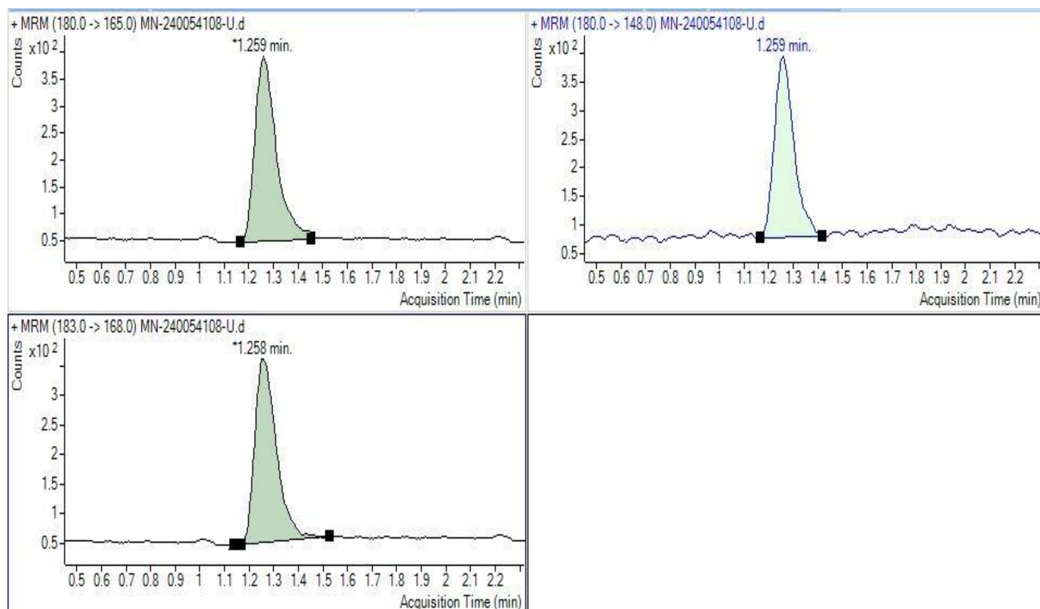
The selectivity of the method was assessed by using blank urine and zero calibrators. It illustrated no interference of internal and external reagents on the retention time of metabolites. The results were in ±20% of LLOQ, as shown in Figure V (a) and (b). MN and NMN completely resolved without any interference and showed good chromatograms in a real urine sample. As for selectivity, blank urine was only free for MN, and NMN was used that rule out the chances of interference with other compounds present in real urine samples (Figure VI).



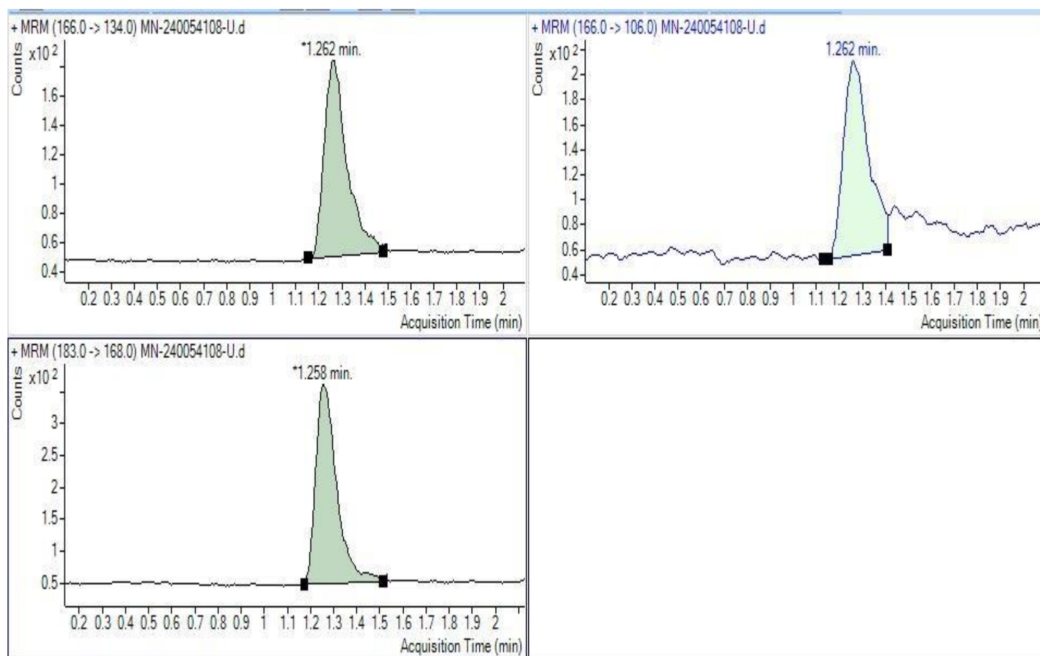
a) **Metanephrine (MN)**

b) **Normetanephrine (NMN)**

Figure V: Blank chromatogram shows no interference of peaks.



a) Metanephrine (MN)

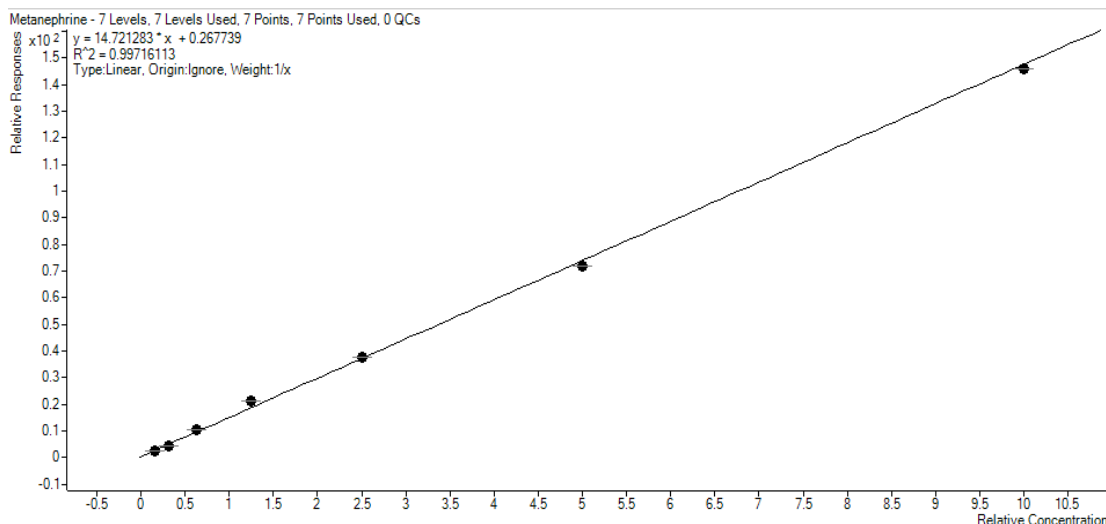


b) Normetanephrine (NMN)

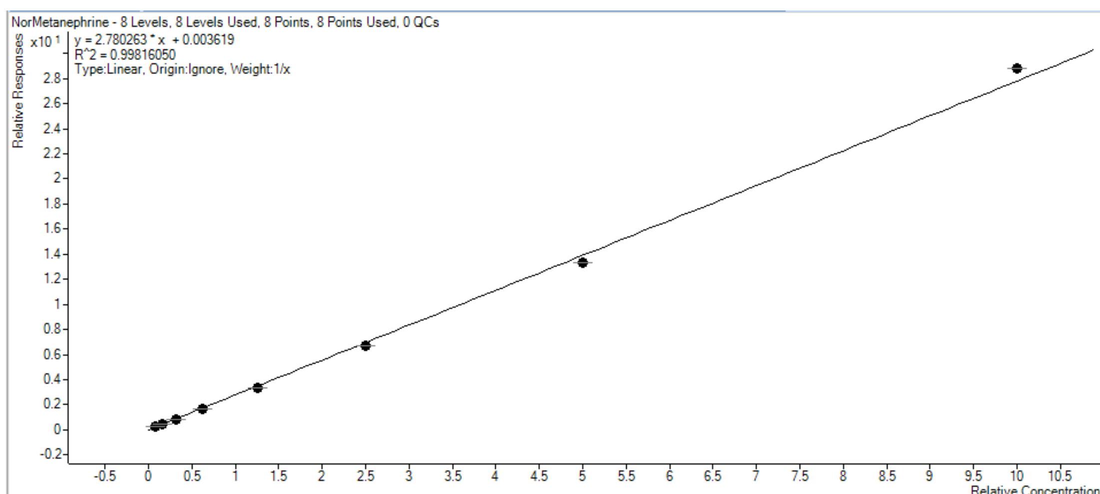
Figure VI: Chromatograms of real urine sample a) Metanephrine and b) Normetanephrine.

3.1.2. Linearity:

Linear regression values were determined by analyzing eight standards of metabolites ranging from 7.8 to 1000 ng/mL for checking the linearity of the method. The value of the correlation coefficient (r^2) was 0.997 for MN and 0.998 for NMN, which shows the linearity of the developed method for respective ranges of standards. A linear calibration curve in the form of y = ax + b with a weighing factor of 1/x was automatically obtained by Mass Hunter Software, as shown in Figure VII. The parameters of the calibration curves for MN and NMN and the corresponding regression coefficients are summarized in Table III.



a) Metanephrine (MN)



b) Normetanephrine (NMN)

Figure VII: Calibration Curves of a) Metanephrine (MN) and b) Normetanephrine (NMN) with1/x weighing factor.

Table III: Linear Regression (With Weight Factor 1/x) Data of Metanephrine (MN) and Normetanephrine (NMN)

Sr no.	Compound	Linearity Range (ng/mL)	LLOQ Precision (%CV)	LLOQ Accuracy (%Bias)	Calibration Parameters	
					Regression equation	r^2
1	Metanephrine	7.8 – 1000	1.90	5.6	$y = 14.72 * x + 0.26$	0.9971
2	Normetanephrine	7.8 – 1000	1.21	4.78	$y = 2.78 * x + 0.003$	0.9981

3.1.3. Precision and Accuracy:

To evaluate the precision and accuracy of the method, six replicates of quality control (QC) samples at three concentration levels (low, medium, and high) were employed on three different days. The intra-day and inter-day precision and accuracy values were within the acceptable range (Table IV).

Table IV: Accuracy and Precision of Metanephrine (MN) and Normetanephrine (NMN)

Sample	Nominal value (ng/mL)	Accuracy		Intra-day and inter-day precision			
		Average (ng/mL)	Bias (%)	Mean (ng/ mL) Intra-day	Intra-day CV (%) n=10	Mean (ng/mL) Inter-day	Inter-day CV (%) n= 15
Metanephrine (MN)							
LLOQ	7.8	8.25	5.6	8.30	1.90	8.24	1.38
Low	31.25	29.72	-4.89	29.14	1.37	29.24	2.32
Mid	500	485.15	-2.97	484.73	0.16	486.3	0.102
High	1000	990.7	-0.93	991.64	0.11	991.5	0.093
Normetanephrine (NMN)							
LLOQ	7.8	8.18	4.78	8.20	1.21	8.36	1.36
Low	31.25	30.53	-2.28	31.7	0.41	32.7	0.48
Mid	500	479.3	-4.13	479.6	0.04	482	0.32
High	1000	1037.3	3.72	1037	0.15	1036.2	0.185

3.1.4. Recovery and Matrix Effect:

For recovery and matrix effect, QC samples spiked with known amounts of metabolites (MN and NMN in urine). The results showed that the mean recovery was 95.11% to 105.6% for MN and 95.87% to 104.78% for NMN, respectively, and the matrix effect was within the acceptable range. The concentrations-to-evaluate and acceptance criteria were ±20%.

3.1.5. Stability:

To assess the method's stability, QC samples were stored under different storage conditions. The QC samples were analyzed under various storage conditions to evaluate the method's stability. The results showed that MN and NMN were stable under conditions including freeze-thaw cycles, short-term storage, and long-term storage.

3.1.6. Carryover:

The carryover of the method was evaluated by injecting a blank sample after the highest concentration QC sample. No significant carryover effect for either MN or NMN was observed. Response in blank was not exceeded ±20% of LLOQ and was minimized by post-run (Figure V).

4. Discussion:

The advancement in analytical methods for the quantification of metabolites in biological samples is a crucial step in clinical and research studies. In the case of urinary MN and NMN, these compounds are biomarkers for certain diseases such as pheochromocytoma and paraganglioma, a rare tumor of the adrenal gland that can cause high blood pressure, headache and cardiovascular disease [20, 21]. We devised a method using the LC-MS/MS technique for the detection of MN and NMN in urine samples at the same time, which is not yet available in Pakistan. This method's application on real samples resulted in the maximum detection of metabolites (MN and NMN).

For this study, urine samples were collected in 24 h duration; although it has been said that the detection of MN and NMN in plasma showed more sensitivity and specificity but, it has some disadvantages, such as requiring the patient to be in a supine position and sample collection carried

out by an indwelling cannula for 30 minutes [22]. Patients that show a low prevalence of the disease displayed satisfactory results from urine analysis; furthermore, urine samples are not affected by the patient's emotional condition and lying position which was an added advantage [23]. For the extraction step, we used 2-aminoethyl-diphenylborinate and ethyl acetate. This was, by far, the most challenging part of our study as we were initially trying to achieve extraction with in-house available reagents and methods, but reliable output was not achieved.

However, the extraction method mentioned above, albeit time-consuming, gave us the best reproducible results. 2-aminoethyl-diphenylborinate was used because of its known ability to derivatize catecholamines. This capability enhances the chromatographic separation and sensitivity of these compounds in LC-MS/MS analysis. The stability of this compound assists in providing reliable and reproducible results during the analysis of urine samples over time. Its stability is what offers an advantage over other derivatization techniques. Ethyl acetate is reliable as an efficient extractor for extracting polar and non-polar compounds. This ensures a comprehensive extraction of MN and NMN from urine samples. It is also quite volatile and is easily evaporated, leaving behind the extracted analytes in a concentrated form suitable for subsequent analysis. If we compare our extraction method with other alternative methods, such as Liquid-Liquid Extraction (LLE), we saw from the literature that the use of ethyl acetate is often preferred over traditional LLE methods due to its simplicity, lower cost and reduced use of hazardous organic solvents [24].

While experimenting with various compositions of the mobile phase, the first composition of the mobile phasecomprising0.1% formic acid in methanol and 0.1% formic acid in acetonitrile showed poor peak resolution of analytes (Figure III (a) We achieved the best resolution by using another combination of solvents as the mobile phase, which comprised 5% methanol in 0.1% formic acid and 100% methanol in an isocratic flow with a ratio of 60:40 (v/v) (Figure III (b). We observed that this composition exhibited much better polarity-based compatibility for the separation of analytes, leading to sharp and more symmetrical peaks.

For chromatographic separation, two reversed-phase columns, one is Eclipse C-18 Column having 75mm length, 2.1mm internal diameter, and 2.7µm particle size, and the other is Poroshel 120 EC-C18 column having 75mm length, 2.1mm internal diameter, and 2.7µm particle size were used for the separation of analytes. The Eclipse C-18 (2.1 × 75 mm 2.7 µm) column yielded well-defined sharp peaks of both standards and sample analytes. The metabolites ((±)- (MN) and (±)- (NMN)) in the standard and sample were simultaneously eluted at 1.259 and 1.268 minutes, respectively, using these optimized chromatographic conditions with a decreased run time of 2.5 minutes. (Figure II)
A different range of column incubation temperatures (25, 30, 35, 40, 45, and 50°C) was used to study the effect of temperature on the separation efficiency of the column. Among various ranges of temperatures, the most optimal separation was observed at 30°C with 0.5 mL/min flow rate and 10 µL injection volume.

As shown in Table II, the MS parameters were also optimized. ESI source was operated in multiple reaction monitoring (MRM) mode by adjusting the Capillary voltage to 4000 V temperature 350°C and nebulizer gas pressure at 55 Psi. The flow rate of nitrogen gas in ESI for desolvation was optimized at 9 L/min at 350°C.

We tested the stability of our method by analyzing QC samples after placing them in various storage conditions, such as short-term and long-term and also processing them through repeated freeze-thaw cycles. To assess the method's stability, QC samples were stored under different storage conditions. Our method showed accurate and stable results under all the conditions mentioned above.

Good linearity was achieved by checking eight standards of metabolites of different concentrations. The assay was linear within the analytical concentration range, which was adequate for precise

measurement of all analytes in real patient samples. (Figure VI) The precision and accuracy of our technique were also determined on multiple days by testing six replicates of different concentrations of QC samples. The intra-day, as well as inter-day precision and accuracy values were within the permissible range (Table IV).

After going through the above-discussed process and observing the accuracy of our final results, we may conclude that the validated method demonstrates its appropriateness for the analysis of urinary catecholamines and metanephrines at levels that hold clinical relevance and significance. However, we do suggest large case-control studies be conducted consisting of patients who are suffering from pheochromocytoma and paraganglioma to establish better predictability and accuracy of our devised method.

5. Conclusion:

An LC-MS/MS method was developed and validated for the quantification of MN and NMN in human urine, which is precise, selective, and sensitive. A simple and rapid LLE technique with 2-aminoethyl-diphenylborinate and ethyl acetate was implemented. LC-MS/MS assay was defined by excellent linearity, accuracy and precision for the diagnosis of patients who are suffering from pheochromocytoma and paraganglioma. Optimized conditions of the method exhibited that this method is practical, precise and robust for the analysis of catecholamines and metanephrines in urine at clinically significant levels.

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Ethical Approval

The study was conducted after getting ethical approval from the Institutional Review Board of the Institute, Rawalpindi, reference number *PhD/READ-IRB/23/2187*.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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