Method Development and Validation of Anabasine and Nornicotine in Human Plasma by LC-MS/MS

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This work was carried out in collaboration among all authors. Author PR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NS and ND managed the analyses of the study. Authors ND and MS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: For the Method development and validation of Anabasine and Nornicotine in human plasma LC-MS/MS technique is used.

Methodology: Operated in positive electron spray ionization which removes the hardness in ions. Metoprolol is used as an internal standard. Mass analyzers are used to determine the mass to charge ratio. Liquid chromatography separates mixture components in the basis of differences in affinity for stationary and mobile phase. It removes undesired impurities. It increases reproducibility, sensitivity, robustness, detection of low-level proteins. C18 Column (Phenomenex Luna C18, 5 μm, 50*4.6mm ID) is used to for high resolution and peak area. Calibration curve is constructed with the help of linear regression. During the entire process no instability is found.

Results: m/z values of Anabasine are 162.84(Q1), 134.68(Q3) and for Nornicotine are 148.90(Q1), 79.82(Q3). Back calculated curve is calculated at different concentrations from 1-1000ng/ml and their accuracy range from 97-105% for Anabasine and 93-108% for Nornicotine. In Anabasine and Nornicotine the precision and accuracy are calculated by Intra-batch and inter-batch. In the process
of multi-step preparation, consider n=6 for intra-batch and n=18 for inter-batch the values obtained are between the nominal concentrations i.e., 90-110%. For each analyte the coefficients of variation are below nominal criteria <15%. For Anabasine and Nornicotine the average recovery rate is 98.3% and 94.3%.

Keywords: Nornicotine; anabasine; LC-MS/MS; metoprolol; ESI; pooled human plasma.

1. INTRODUCTION

Nornicotine is also known as 3-pyrrolidin-2-ylpyridine. Its molecular formula is C₉H₁₀N₂. Nornicotine don't contain methyl group except that it is similar to nicotine [1] and in threatening and dispensation of tobacco [2] it acts as antecedent for the cancer-causing agent N-nitrosonornicotine. Nornicotine activates certain receptors in the brain mainly in neuronal communication which converts neurotransmitter binding into membrane electrical depolarization. It bounds to nicotine acetylcholine receptors which increases dopamine related activity in the brain in cortico-limbic ways. This results in allowing the cations of Na, Ca, K in multiple ways leads to conductance. It results in movement of cells membrane potential to a more positive value initiates the channel to open and close in responses to changes in the membrane potential of a cell and permits calcium to pass in to axon. Calcium arouses a small sac to move over plasma membrane and release dopamine into nerve impulse passes from one nerve cell to another nerve cell. The addictive properties of nornicotine were obtained from the receptors which are bound to dopamine [3]. In the adrenal medulla Nornicotine bounds to nicotinic acetylcholine in pheochromocytes. Voltage-gated calcium channels are activated due to binding opens the ion channel flowing sodium leads to depolarization of the cell. Calcium initiates the release of adrenaline into cytosol vesicles causes decrease in the diameter of blood vessels and increase in BP, Pulse and glucose levels.

Anabasine is also known as 3-(piperidin-2-yl)pyridine. Its molecular formula is C₁₀H₁₂N₂. It activates certain receptors in brain and triggers the neuromuscular transmission. It is vulnerable to temperature and dampness and is an unbalanced yellow liquid. It was seen in plant stem and virtual relation between tobacco plant [4]. It is an analog to nicotine [5,6]. It is used as an insect repellent in industrial areas. It is detected in tobacco smoke and in urine [7]. The action of acetyl cholinesterase is suppressed by cholinesterase inhibitor. With this crucial function chemicals get involved with acetyl cholinesterase leads to muscle contractions and eventually death.

According to literature survey for the assessment Nornicotine HPLC-UV [8], LC-MS [9,10] and Anabasine GC-MS [11,12], HPLC-MS [13], LC-MS [14-17] analytical methods were used. Our present work studied about the method development and validation of Anabasine and Nornicotine in human plasma by liquid chromatography and mass spectrometry in human plasma. Several parameters are validated such as linearity, recovery, precision and accuracy, specificity, stability.

![Fig. 1. Structures of Anabasine and Nornicotine](image)

2. METHODOLOGY

2.1 Chemicals and Reagents

Standard working conditions of Anabasine and Nornicotine was attained from Akshaya labs, India. Acetonitrile of HPLC grade by Merck, Ammonium Acetate AR grade obtained from Qualigens fine chemicals and water HPLC grade from Milli-Q RO system were used.

2.1.1 LC-MS/MS instrumentation and chromatographic conditions

Waters TQD LC-MS/MS is used to separate mixture components on the basis of differences in affinity for stationary and mobile phase and removes undesired impurities. LC setup consists of online DGU-20A3 soluble degasser, segment boiler CTO20A, LC20AD drives-2, SIL-HTc sampler, detectors, column. C18 (Phenomenex Luna C18, 5 μm, 50*4.6mm ID) column is used for better retention. LC-MS/MS instrument
Electron spray ionization which is used to overcome the problem of hard ionization. It should have sample of interest in solution and can use the inline separation along with the liquid chromatography. ESI produces multiple charged ions. LC-MS/MS operates in positive ion mode using at collision gas:20, cone gas:20, Desolvation gas:700, source temperature :150°C. Chromatographic conditions are mentioned below.

- **HPLC**: Waters acquity UPLC
- **Pump mode**: Binary gradient
- **Total flow**: 0.2 mL
- **Injection volume**: 10 µL
- **Mobile Phase A**: 0.1% Formic acid in water
- **Mobile Phase B**: 100% Acetonitrile

### 2.2 Optimization of MS Parameters

With the concentration of 500ng/mL multi-reaction monitoring setting were determined by using infusion of each analyte. Table 1 shows the MS parameters of Anabasine and Nornicotine.

### 2.3 Preparation of Stock Solution

Dissolve 100mg of Anabasine and Nornicotine by using Acetonitrile and water with 1:1 ratio to obtain a solution of 1mg/ml. solution should be stored at 8°C. The standard stock solution is kept at mobile phase from -2 ± 20 C.

### 2.4 Selection of HPLC Column

Initially C8 columns were tested and it results in poor separation and peak value. Factors such as mobile phase and PH values were also results in no improvement in chromatographic separation. Finally, C18 results in good resolution. LC which makes use of C18 capillary columns are used for their ability to achieve final separations. For these separated components passed from the column outlet into the flow cell, which is present in the detector. The most commonly used detector is uv detector.

### 2.5 Internal Standard Selection

By using the metoprolol as an internal standard positive mode analysis is carried out. Anabasine and Nornicotine needs neutral PH after extraction but Metoprolol requires acidic PH which is equally diluted with H₂O for supernate. It results in good resolution and elution.

### 3. RESULTS AND DISCUSSION

#### 3.1 Validation

Establish the acceptability criteria for each component of the analytical validation before the start of validation i.e., regulatory experiments, guidance documents and biological variation. Validation determines the accuracy, precision, sensitivity, linearity, specificity, matrix effects, carryover etc. pre-validation testing overlaps with method development. It needs blank matrix for best practice acceptability criteria such as no peak or peak area <20% of analyte LLOQ and <5% of internal standard. According to the FDA guidelines acceptability criteria of precision is <15% and bias<15%. At first determine this method is appropriate for concentration determination of target analytes. Start with the construction of calibration curve and the range should be 1-1000ng/ mL. Metoprolol is used as an internal standard.

#### 3.1.1 Construction of calibration curve

By using the same plasma on alternate days prepare the quantitation standards of five replicate sets and prepare the stock solution with 9 different concentration levels. And these are termed as intra-day and inter-day data. This results in the reproducibility and robustness. The main purpose is Quantified lowest level should be assessed. The peak area is calculated from the extracted ion to the internal standard. Linear response is obtained by regression.

### Chart 1. Gradient time programme

| Time (min.) | %B | Events          |
|------------|----|-----------------|
| 0.00       | 80 | Pump B conc.    |
| 1.50       | 80 | Pump B conc.    |
| 4.00       | 98 | Pump B conc.    |
| 4.01       | 80 | Pump B conc.    |
| 6.00       | 80 | Pump B conc.    |
Table 1. Anabasine and Nornicotine MS/MS conditions

| Parameter                        | Value                                      |
|----------------------------------|--------------------------------------------|
| Ionization type and polarity     | Electron spray ionization, +ve ion mode    |
| Ion source                       | Turbo spray                                |
| Scan type                        | MRM                                        |
| Q1 Resolution                    | Unit (1)                                   |
| Q3 Resolution                    | Unit (0.75)                                |
| Desolvation Temperature          | 450°C                                      |
| Source temperature               | 150°C                                      |
| Collision Gas:                   | 20                                         |
| Cone Gas:                        | 20                                         |
| Desolvation Gas:                 | 700                                        |

Fig. 2a. Product ion mass spectra of Anabasine in positive ionization mode

Fig. 2b. Product ion mass spectra of Nornicotine in positive ionization mode
Fig. 3. Representative example of LLOQ chromatogram for Anabasine and Nornicotine

Fig. 4. Representative example of LQC chromatogram for Anabasine and Nornicotine

Fig. 5. Representative example of MQC chromatogram for Anabasine and Nornicotine
### Table 2. MRM transition conditions

| Compound     | Mode of ionization | Q1 mass (m/z) | Q3 mass (m/z) | CE | Cone voltage |
|--------------|--------------------|---------------|---------------|----|--------------|
| Anabasine    | +ve                | 162.84        | 134.68        | 4  | 92           |
| Nornicotine  | +ve                | 148.90        | 79.82         | 22 | 32           |
| Metoprolol   | +ve                | 268.3         | 116.3         | 30 | 68           |

### Table 3. Anabasine back calculated curve data in human plasma

| Std conc. | Concentration (ng/mL) | Batch-1 | Batch-2 | Batch-3 | Mean | SD    | % CV  | %Accuracy |
|-----------|-----------------------|---------|---------|---------|------|-------|-------|-----------|
| 1         | 1.0                   | 0.99    | 1.01    | 1       | 1    | 0.01  | 1     | 100.0     |
| 2         | 1.76                  | 2.03    | 1.94    | 1.94    | 1.94 | 0.137477 | 7.086457 | 97.0      |
| 10        | 9.99                  | 9.93    | 10.33   | 9.99    | 9.99 | 0.215716 | 2.159318 | 99.9      |
| 50.01     | 49.87                 | 52.20   | 53.01   | 52.2    | 52.2 | 1.630164 | 3.122919 | 104.4     |
| 200.03    | 214.16                | 208.38  | 197.24  | 208.38  | 208.38 | 8.600333 | 4.127235 | 104.1     |
| 500.06    | 496.38                | 500.38  | 458.10  | 496.38  | 496.38 | 23.34151 | 4.702347 | 99.2      |
| 800.1     | 826.37                | 792.35  | 784.10  | 792.35  | 792.35 | 22.40601 | 2.827792 | 99.0      |
| 900       | 796.10                | 888.24  | 907.78  | 888.24  | 888.24 | 59.6434 | 6.714785 | 98.7      |
| 1000      | 1038.8                | 947.23  | 986.5   | 986.5   | 986.5 | 45.98623 | 4.661554 | 98.7      |

### Table 4. Nornicotine back calculated curve data in human plasma

| Std conc. | Concentration (ng/mL) | Batch-1 | Batch-2 | Batch-3 | Mean | SD    | % CV  | %Accuracy |
|-----------|-----------------------|---------|---------|---------|------|-------|-------|-----------|
| 1         | 0.9                   | 1.0     | 0.9     | 0.9     | 0.9  | 0.1   | 7.7   | 93.7      |
| 2         | 2.1                   | 1.9     | 2.4     | 2.1     | 2.1  | 0.2   | 11.3  | 106.5     |
| 10        | 9.4                   | 9.3     | 9.7     | 9.5     | 9.5  | 0.3   | 2.7   | 94.5      |
| 50.01     | 43.9                  | 49.0    | 46.7    | 46.5    | 46.5 | 2.6   | 5.6   | 93.0      |
| 200.03    | 228.1                 | 205.5   | 212.7   | 215.5   | 215.5 | 11.5  | 5.4   | 107.7     |
| 500.06    | 538.1                 | 494.5   | 507.4   | 507.4   | 507.4 | 26.7  | 5.3   | 101.5     |
| 800.1     | 766.7                 | 851.7   | 792.1   | 803.5   | 803.5 | 43.6  | 5.4   | 100.4     |
| 900       | 908.2                 | 905.3   | 904.2   | 905.9   | 905.9 | 2.1   | 0.2   | 100.7     |
| 1000      | 966.2                 | 968.7   | 981.8   | 972.2   | 972.2 | 8.4   | 0.9   | 97.2      |
Table 5. Precision and accuracy for Anabasine

| S.NO | Batch-1 | Batch-2 | Batch-3 |
|------|---------|---------|---------|
|      | LQC     | MQC     | HQC     | LQC     | MQC     | HQC     | LQC     | MQC     | HQC     |
| 1    | 3.27    | 573.35  | 844.75  | 2.98    | 566.73  | 848.07  | 3.02    | 597.46  | 870.47  |
| 2    | 3.89    | 553.67  | 840.38  | 3.06    | 575.67  | 850.89  | 3.04    | 587.46  | 870.47  |
| 3    | 3.26    | 547.99  | 874.89  | 2.93    | 596.06  | 837.77  | 3.25    | 563.43  | 805.62  |
| 4    | 2.96    | 519.6   | 793.54  | 3.46    | 593.84  | 826.7   | 3.34    | 507.95  | 878.78  |
| 5    | 3.61    | 517.41  | 828.66  | 2.95    | 594.14  | 835.19  | 3.72    | 563.48  | 846.46  |
| 6    | 3.59    | 535.38  | 832.12  | 3.61    | 597.87  | 845.17  | 3.52    | 519.61  | 844.23  |
| MEAN | 3.43    | 541.23  | 835.72  | 3.36    | 578.21  | 836.49  | 3.36    | 558.39  | 868.91  |
| SD   | 0.24    | 21.46   | 4.0     | 0.30    | 30.51   | 9.14    | 0.24    | 38.02   | 54.79   |
| %CV  | 6.8     | 3.2     | 1.1     | 7.1     | 6.3     | 6.3     | 6.3     | 6.3     | 6.3     |

Table 6. Precision and accuracy for Nornicotine

| S.NO | Batch-1 | Batch-2 | Batch-3 |
|------|---------|---------|---------|
|      | LQC     | MQC     | HQC     | LQC     | MQC     | HQC     | LQC     | MQC     | HQC     |
| 1    | 3.66    | 564.42  | 824.86  | 3.29    | 581.71  | 908.06  | 3.69    | 526.66  | 871.61  |
| 2    | 3.43    | 538.76  | 820.61  | 3.45    | 598.8   | 886.05  | 3.73    | 557.89  | 859.98  |
| 3    | 3.31    | 540.21  | 865.81  | 3.8     | 561.94  | 884.34  | 3.86    | 521.97  | 880.31  |
| 4    | 3.38    | 509.32  | 810.24  | 3.4     | 557.36  | 851.72  | 3.92    | 578.67  | 849.07  |
| 5    | 3.27    | 512.6   | 809.54  | 3.33    | 544.31  | 869.28  | 3.73    | 589.86  | 860.21  |
| 6    | 3.88    | 567.98  | 863.54  | 3.21    | 566.67  | 882.17  | 4.02    | 598.48  | 880.96  |
| MEAN | 3.49    | 538.88  | 832.43  | 3.41    | 568.47  | 880.27  | 3.83    | 562.26  | 867.02  |
| SD   | 0.24    | 24.76   | 25.87   | 0.21    | 19.23   | 18.77   | 0.13    | 32.41   | 12.73   |
| %CV  | 6.8     | 4.6     | 3.1     | 6.1     | 3.4     | 2.1     | 3.4     | 5.8     | 1.5     |

3.1.2 MRM transitions: Production determination

In MRM Q3 detects the product ions and scans the stream of ion fragments, which are emerging from the collision cell to generate a collision induced dissociation spectrum. The spectrum consists of multiple product ions and their respective precursor ions. Anabasine and Nornicotine is present in liquid form and ions are created by spraying the dilute solution of the analyte at atmospheric pressure. These ions are then accelerated towards the mass analyzer depending upon their mass to charge ratio. At each stage collision energies are differing.

3.2 Linearity

In order to determine the linearity, the stock solutions of Anabasine and Nornicotine where taken at five level concentrations ranges from 85% to 115%. In the same concentration tests were carried out for three successive days. By using least squares linear regression analysis peak area Vs concentration data was analyzed. For calibration curve coefficient of correlation was >0.99.

3.3 Accuracy and Precision

Evaluation of agreement between LC-MS/MS test results and true value is called Accuracy. In Anabasine and Nornicotine the precision and accuracy are calculated by Intra-batch and inter-batch. In the process of multi-step preparation, consider n=6 for intra-batch and n=18 for inter-batch the values obtained are between the nominal concentrations i.e., 90-110%. For each analyte the coefficients of variation are below nominal criteria <15%.

3.4 Sensitivity

Sensitivity is depicted by lower limit of quantitation. It is the lowest concentration that can be reproducibly measured. It determined by the imprecision i.e., <20% at LLOQ. It is determined by signal to noise ratio.

3.5 Recovery

For Anabasine and Nornicotine the average recovery rate is 98.3% and 94.3%. Recovery rate of Anabasine and Nornicotine in human plasma from LQC is 98.6% and 92.3% and MQC is 97.7% and 94.9% and HQC is 98.4% and 95.8%.
Table 7. Intra and Inter-run precision and Accuracy for Anabasine and Nornicotine

|          | **Concentration (ng/ml)** | Anabasine |          |          | Nornicotine |          |          |
|----------|---------------------------|-----------|----------|----------|-------------|----------|----------|
|          |                           | **LQC 3.5** | **MQC(550)** | **HQC (850)** | **LQC (3.5)** | **MQC (550)** | **HQC (850)** |
| **BATCH-1** |                           | 3.43      | 541.23      | 835.72      | 3.49        | 538.88       | 832.43      |
| (N=6)    |                           | 0.33      | 21.46       | 26.38       | 0.24        | 24.76        | 25.67       |
|          |                           | 9.6       | 4           | 3.2         | 6.8         | 4.6          | 3.1         |
|          |                           | 98.0      | 98.4        | 98.32       | 99.7        | 98.0         | 97.9        |
| **BATCH-2** |                           | 3.15      | 578.21      | 836.49      | 3.41        | 568.47       | 880.27      |
| (N=6)    |                           | 0.3       | 30.51       | 9.14        | 0.21        | 19.23        | 18.27       |
|          |                           | 9.57      | 5.3         | 1.1         | 6.1         | 3.4          | 2.1         |
|          |                           | 90.0      | 105.1       | 98.4        | 97.4        | 103.4        | 103.6       |
| **BATCH-3** |                           | 3.33      | 558.39      | 868.91      | 3.83        | 563.26       | 867.02      |
| (N=6)    |                           | 0.24      | 38.02       | 54.79       | 0.13        | 32.41        | 12.73       |
|          |                           | 7.14      | 6.8         | 6.3         | 3.4         | 5.8          | 1.5         |
|          |                           | 95.1      | 101.5       | 102.2       | 109.4       | 102.4        | 102.0       |
|          |                           | 3.3       | 559.3       | 847.0       | 3.6         | 556.9        | 859.9       |
| **INTER-BATCH** |                         | 0.1       | 18.5        | 18.9        | 0.2         | 15.8         | 24.7        |
| (N=18)   |                           | 4.3       | 3.3         | 2.2         | 6.2         | 2.8          | 2.9         |
|          |                           | 94.3      | 101.7       | 99.6        | 102.9       | 101.3        | 101.2       |

Table 8. Recovery data of Anabasine

|          | **LQC Area (Counts)** | **MQC Area (Counts)** | **HQC area (Counts)** |
|----------|------------------------|-----------------------|-----------------------|
| **Aqueous** |                         | **Extracted**        | **Extracted**        |
| **S.NO** | **LQC Area (Counts)** | **MQC Area (Counts)** | **HQC area (Counts)** |
| 1        | 29497                  | 28976                 | 876490               | 899345      | 1320233       | 1298436      |
| 2        | 29563                  | 29043                 | 897169               | 812376      | 1379846       | 1398754      |
| 3        | 29339                  | 28764                 | 895361               | 853582      | 1368995       | 1378653      |
| 4        | 29776                  | 29134                 | 864128               | 842165      | 1389142       | 1381342      |
| 5        | 29345                  | 28954                 | 877177               | 893472      | 1432822       | 1367399      |
| 6        | 28435                  | 28765                 | 867591               | 854312      | 1569104       | 1498760      |
| **MEAN AREA** |                         | **Extracted**        | **Extracted**        |
|          | 29326                  | 28939                 | 879653               | 859209      | 1410024       | 1387224      |
| **% RECOVERY** |                         | **Extracted**        | **Extracted**        |
|          | 98.6                   | 97.7                  | 98.4                 | 98.4        |

**AVG. %RECOVERY= 98.3; SD= 0.47; CV= 0.48**
Fig. 6. Representative example of HQC chromatogram for Anabasine and Nornicotine

| Table 9. Recovery data of Nornicotine |
|--------------------------------------|
| S.NO | LQC Area (COUNTS) | MQC Area (COUNTS) | MQC Area (Counts) |
|      | Aqueous Extracted | Aqueous Extracted | Aqueous Extracted |
| 1    | 42909 41347       | 3430600 3235670   | 5712799 5510976   |
| 2    | 43665 42786       | 3531124 3415892   | 5765827 5643221   |
| 3    | 43330 41890       | 3490938 3346789   | 5745948 5891203   |
| 4    | 48594 46786       | 3523590 3416754   | 5769703 5623419   |
| 5    | 48997 47064       | 3610759 3513424   | 5710852 5589762   |
| 6    | 48761 44360       | 3512160 3408754   | 5975611 5476591   |
| MEAN AREA | 46043 44039 | 3516529 3389547   | 5780123 5622529   |
| % RECOVERY | 92.3          | 94.9                  | 95.8                  |
| AVG. %RECOVERY= 94.3; SD= 1.82; CV= 1.93 |

| Table 10. Showing recovery data of Anabasine and Nornicotine |
|-------------------------------------------------------------|
| Analyte          | Concentration | AVG. RECOVERY | SD  | % CV |
|                  | LQC  MQC HQC  |              |     |      |
| Anabasine        | 98.6 97.7 98.4 | 98.3          | 0.47 | 0.48 |
| Nornicotine      | 92.3 94.9 95.9 | 94.3          | 1.82 | 1.93 |

3.6 Stability

Stability can be determined by three ways i.e., Freeze-thaw, auto-sampler and Bench-top stability. For n=6, LQC, MQC and HQC samples are validated by using Freezing and thawing for three cycles, and in Auto-sampler stability these values are validated by keeping them in auto-sampler for 24 hours at 4°C and in bench-top stability these samples are validated by keeping them at room temperature for 4hours. By comparing the obtained values with the fresh samples, the precision and accuracy are within the range and no instability is found during the entire process.

4. CONCLUSION

This method describes the development and validation of Anabasine and Nornicotine in human plasma by LC-MS/MS. Metoprolol is used as an internal standard and should be operated in positive ion mode. The average recovery of Anabasine is 98.3% and Nornicotine is 94.3%. For n=6 samples the stability is calculated, and no instability is found during the process. The sensitivity is determined by LLOQ. Linearity ranges from 85-115%.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.
CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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