ORIGINAL ARTICLE

A Novel Validation of Analysis Method for Determining Nicotine Levels in Human Blood Plasma (*In vitro*) High-Pressure Liquid Chromatography Ultraviolet Detector

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ABSTRACT

The necessity of nicotine analysis in blood plasma is increasing along with the increased number of smokers and nicotine poisoning cases. One of the analytical methods for nicotine is using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detector because it has been commonly owned by instance in Indonesia. To guarantee accuracy, an analytical method can be used, and it must be validated. This research was the purpose of finding out the validity of the nicotine analysis method in human blood plasma (*in vitro*) using HPLC with UV detection. Blood plasma samples remained treated with centrifugation procedure by protein denaturation method using acetonitrile. The compounds were analyzed using methanol and buffer acetate 0.01 M (pH 5) 85:15 v/v as a mobile phase on an octadecylsilane column 250 mm, with UV detection at 254 and 260 nm, and flow rate 0.6 mL/minute. Parameter of analytical methods that were validated includes selectivity, accuracy, precision, repeatability, linearity, limit of detection (LoD), limit of quantification (LoQ), and system suitability. According to the result, the selectivity was 2.479, repeatability expressed by its variation coefficient = 0.701%, linearity at range 5–22 µg/mL expressed by coefficient correlation (r) = 0.996. Based on the chromatogram's area under a curve, the LoD value was found 2.021 µg/mL, LoQ value was 6.737 µg/mL, the accurate percentage was 112.49 to 114.12%, and precision (% CV) was 2.15 to 3.95%. The system suitability from retention time and chromatogram's area under curve showed % CV 0.70 and 1.64%. According to the experiment result, all parameters meet the requirements of validation criteria.

Keywords: HPLC, Nicotine, Plasma, Validation.

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INTRODUCTION

Nicotine is an alkaloid that has high addictive and toxic properties. It can be found in all parts of tobacco plants (*Nicotiana tobacum*).[1] Nicotine 1-methyl-2-(3-pyrrolidine) is a colorless or pale yellow, becomes brown when exposed to light, and easily dissolves in alcohol.[2] Nicotine concentration in postmortem blood is 11 to 600 µg/mL. Some cases of poisoning consequences of nicotine consumption, either intentionally or unintentionally, can cause death, such as, the consumption of oral worming drugs, poisoning of pesticides containing nicotine, and the use of nicotine together with other drugs.[3] This increases the need for toxicological analysis in clinical laboratories, especially the analysis of determining levels of nicotine in the blood by a fast, effective, and sensitive tool.

As a first step before conducting drug analysis *in vivo*, it is necessary to do drug analysis in human blood plasma *in vitro*. Determination of nicotine levels in the blood *in vitro* can be done by several methods, one of which can be determined by HPLC. To ensure that the *in vitro* analysis method meets the requirements for grade determination and can provide accurate and reliable results, it must first be validated against the analysis method used.[4] There are several validation parameters of the analysis method, including selectivity, linearity, precision, accuracy, LoD, and LoQ.[5] So far, several studies have been carried out to analyze nicotine levels including, nicotine in hair with HPLC-UV detectors and mass spectrometry (MS) HPLC,[6,7] nicotine in toenails with gas chromatography-mass spectrometry (GC-MS),[8] nicotine in urine using KCKT/electron impact/mass spectroscopy (EI-SM/SM),[9] and nicotine in plasma by the solid-phase extraction (SPE) method, using high performance liquid chromatography,chemical inoization-mass spectroscopy (HPLC/APCI-MS).[10]

In this study, validation of the nicotine analysis method in human blood plasma *in vitro* using the HPLC protein

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deposition method with a UV detector so that it can be used as an alternative for determining nicotine levels. HPLC is a tool that can separate quickly, efficiently, and has high sensitivity and is already owned by many agencies in Indonesia, and most of them use a UV detector.[11,12] The use of protein deposition techniques can be done because the method is quite simple and fast, and can be used for complex biological liquid matrices.[5] Based on the complex nature of blood plasma, this protein deposition technique is seen as an effective extraction method in determining nicotine levels in human blood plasma.[13]

MATERIALS AND METHODS

Material

Nicotine hydrogen tartrate (Sigma), all chemical reagents with degree p.a: methanol p.a (Jt. Baker), acetate buffer pH 5, acetonitrile (Jt. Baker), aquabidest (PT. Ikapharmindo Putramas), and human blood plasma (PMI Bandung).

Tools

A set of HPLC (Shimadzu LC-10 ATVP) equipped with UV SPD-10 AV detectors, Shimadzu SCL-A System Controller auto-injector, LiChroCART C-18 100 RP column with 250 mm column length and particle size of 10 µm, a set of UV-Vis spectrophotometer (analytical Jena, Specord 200), pH meter (Ohmmeter), ultrasonic bath (NEY 1510), analytical scales (Sartorius) sensitivity of 0.1 mg, vortex mixer, centrifugation (Hettich EBA 20), tubes centrifugation, vacuum filters along with 0.4 to 0.45 µm porous filters, and glassware commonly used in the research laboratory.

Determination of Maximum Wavelength Measurement

Nicotine was weighed about 5 mg, put into a 10 mL volumetric flask, then dissolved and diluted using aquabidest to the mark so that a standard solution was obtained with a concentration of about 500 µg/mL. The nicotine standard solution was then scanned using an ultraviolet spectrophotometer at a wavelength of 250 to 270 nm, to obtain the absorption spectrum and the maximum wavelength of nicotine.

Determination of Nicotine Molar Extensions

The standard nicotine solution with a concentration of 500 µg/mL was diluted gradually with distilled water until three final concentrations were obtained in µM units, *viz.*, 97.32, 151.38, and 216.26 µM. The three solutions were then analyzed with a spectrophotometer, and the absorption at the maximum wavelength of nicotine was read, and the molar extinction value was determined using the equation:

$$\varepsilon = \frac{A}{b\,C}$$

Note: A = Molar absorption ($M^{-1}.cm^{-1}$)
b = Cuvette thickness (cm)
C = Concentration of nicotine (molar)

Optimization of HPLC Conditions

Optimization of HPLC conditions is carried out by making changes to the composition of the mobile phase to obtain the optimum conditions for analyzing the determination of nicotine levels. Experiments were carried out with various variations of the concentration of the mobile phase and flow rate using the LiChroCART column measured at wavelengths of 254 and 260 nm. The HPLC conditions used in the optimization stage are:

Column: LiChroCART C18, the particle size of 10 µm, column length of 250 mm
Mobile phase: methanol: acetate buffer pH 5 (80:20; 85:15 v/v)
Detection: UV detectors 254 and 260 nm
Flow rate: 0.6 mL/minute
Injection volume: 20 µL

A standard nicotine solution with a concentration of 100 µg/mL is injected, as much as, 20 µL (auto-injector) into the HPLC tool with a mobile phase composition at the same flow rate, then the retention time of the nicotine produced, is then used for the next analysis stage.

Pretreatment of Blood Plasma Samples

Pretreatment was carried out on blood plasma samples before analyzing using HPLC, including spiking with nicotine and extraction using protein deposition methods.

Spiking Blood Plasma

The 1,000 µg/mL nicotine solution was diluted with aquabidest in a 10 mL volumetric flask gradually, until a final concentration of 50, 80, 110, 160, and 220 µg/mL was obtained.

Extraction by Protein Deposition

The protein precipitation extraction method is carried out by adding 0.5 mL of nicotine sample solution to 450 µL blood plasma in a centrifugation tube. The nicotine solution in blood plasma was vortex for 10 seconds, and 1 mL of acetonitrile was added. The solution was vortexed again for 30 seconds, then centrifuged for 3 minutes at 3,000 rpm. The analyte obtained from the extraction was put into a 2 mL vial bottle, and then, injected into HPLC as much as 20 µL.[1]

Efficiency of Extraction (Recovery Absolute)

Absolute recovery or extraction efficiency is obtained by comparing the area of the plasma chromatogram containing nicotine with a concentration of 11 and 22 µg/mL to the area of the aquabidest chromatogram containing nicotine in the same concentration as the plasma sample with the equation:

$$\%\text{recovery} = \left(\frac{\text{peak area in plasma chromatogram}}{\text{peak area mobile phase chromatogram}}\right) \times 100\%$$

Validation of the HPLC Nicotine Analysis Method

To ensure that the analysis method has fulfilled the requirements and can provide good results, validation is performed on the parameters of selectivity, repeatability, linearity, precision, accuracy, LoD, LoQ, and system suitability test.

Selectivity

Selectivity is expressed by the value of resolution or separability (Rs). Values that meet the criteria are > 1.5. The resolution value is seen from the nicotine chromatogram resulting from HPLC separation, in which there are no overlapping peaks, even though the two substances in one derivative.[5]

Repeatability

The concentration of nicotine solution was 11 µg/mL in plasma, then extracted using the protein deposition method. A total of 20 µL of the extracted analytes was injected into the HPLC device at optimum conditions. The experiment was repeated six times, then the %CV was calculated.[5]

Preparation of a Standard Curve

Five series of nicotine concentrations of 5, 8, 11, 16, and 22 µg/mL in plasma (at each nicotine concentration) were prepared by making serial dilutions of stock of nicotine raw solution with human blood plasma, which does not contain analytes. Furthermore, the standard solution of nicotine in blood plasma is extracted using the protein deposition method. A total of 20 µL of the extracted analytes was injected into the HPLC device at optimum conditions. Linearity determination is done by three repetitions. The calibration curve obtained is used to determine sample levels. The linear regression equation is described as the relationship between nicotine concentration and chromatogram area, and then the correlation coefficient is calculated.[14]

Detection Limits (LoD) and Quantitation Limits (LoQ)

The detection limit value and the nicotine analysis quantitation limit are calculated statistically through a linear regression line from the calibration curve with the equation y = ax + b, and the standard blank deviation (Sb), which is the same as the residual standard deviation (Sy/x).[14]

LoD is stated by:

$$LoD = \frac{3S_b}{a}$$

Information:

Sb = Standard deviation of analytical responses from blanks;

a = Slope

The Sb value is obtained from the equation:

$$S_b = S_{y/x} = \left\{ \frac{\sum_i (Y_i - \overline{Y})^2}{n-2} \right\}^{\frac{1}{2}}$$

LoQ measured by an equation:

$$LoQ = \frac{10S_b}{a}$$

Sb = Standard deviation of analytical responses from blanks;

a = Slope[12]

Accuracy and Precision

Accuracy and precision obtained by setting the levels of two samples each of three repetitions (n = 3). Sample concentrations made outside the standard curve include low concentrations of 6 and high 20 µg/mL. Calibration curves with the best correlation coefficient are used to determine nicotine levels in the sample. Precision can be seen from the CV value (%) with the equation**:**

$$\%CV = \frac{SD}{X} \times 100\%$$

Note: CV = Coefficient of variation;

SD = Standard deviation;

X = mean

Average percent accuracy is obtained by looking at the closeness of the results of the sample to the nominal value.

Recovery is calculated by the equation:

$$\%\text{recovery} = \left[\frac{CT}{CA} \right] \times 100\%$$

CT is the concentration of nicotine measured, and CA is the amount of nicotine that is administered (nominal concentration) into the human blood plasma.

System Suitability Test

The system suitability test is carried out on a nicotine concentration of 11 µg/mL in human blood plasma, then extracted using the method of control. CT is the concentration of nicotine measured, and CA is the amount of nicotine that is administered (nominal concentration) into the human blood plasma six times (n = 6). From the chromatogram obtained, it was determined the repeatability of the standard solution measurement method expressed by % CV of the retention time and chromatogram area.

RESULTS AND DISCUSSION

Determination of Research Conditions

Nicotine has a chromophore group that can absorb ultraviolet radiation. Therefore, nicotine can be analyzed using a UV-vis detector. The level of nicotine in the blood is small enough to be analyzed by HPLC. The polar nature of nicotine allows nicotine to be analyzed using an inverted phase, where the stationary phase is more non-polar than the mobile phase.[2] In this study, the reverse phase with the mobile phase of methanol:acetate buffer pH 5 is used, which is the result of optimization of the HPLC condition using column C18 and UV-vis detector.

Determination of Maximum Wavelength

In determining the maximum wavelength of nicotine in the aquabidest solvent, a maximum wavelength of 260 nm is obtained. The results of scanning with a spectrophotometer can be seen in Figure 1.

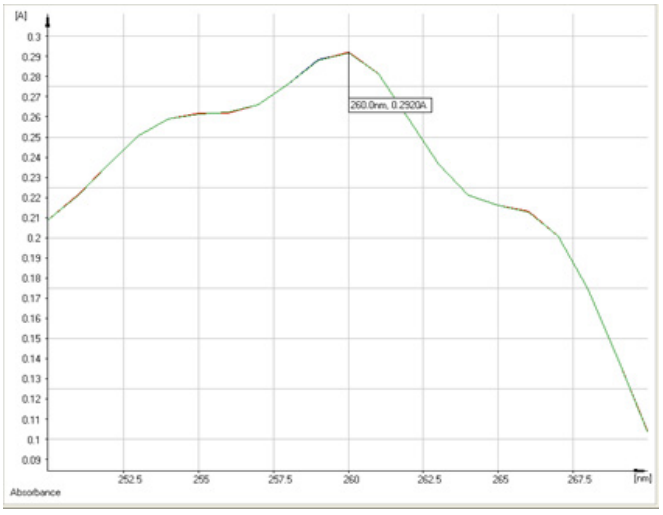

Figure 1: Nicotine spectrum with a maximum wavelength of 260 nm

In this study, the detection of wavelengths of 254 and 260 nm was carried out. Based on the literature, nicotine has a wavelength of 254 and 259 nm (Jackson, 1986), while the measurement results using UV-vis spectrophotometry, the maximum wavelength of nicotine is 260 nm.

The Result of Determining the Molar Extinction Value (e) of Nicotine

To determine the value of nicotine molar extension, measurements were made using three variations of concentration, *viz*. 97.32, 151.38, and 216.26 µM in the aquabidest solvent at a wavelength of 260 nm. The nicotine molar extinction value was calculated by comparing the absorption of the nicotine molar absorptivity value of the cuvette thickness and the measured nicotine concentration. Data on the molar extension values of the experimental results are presented in Table 1.

The measurement results show that nicotine has an average molar extension value of 3,049.12. This molar extinction value can be used to see whether nicotine can be analyzed using a UV detector or not. The minimum limit that can be used is 1,000,[14] so that nicotine can still be detected using a UV detector.

HPLC Condition Optimization Results

Optimization of HPLC conditions was carried out on chromatographic parameters, including resolution, retention time, and mobile phase flow rates from various variations of the composition. The resulting resolution value must be > 1.5.[5] Resolution value > 1.5 indicates that the two peaks are perfectly separated (Table 2). The resolution value obtained in this study was 2.479. Retention time will determine the analysis time needed, the faster the retention time, the better it will be because the faster the analysis. Flow rate affects the width of the peak, the smaller the flow rate, the peak produced will be wider, but the separation will be better.

In this study, the composition of the mobile phase of methanol:acetate buffer pH 5 (85:15) was chosen because this composition produced sharper peaks with a retention time of 0.6 mL/min for better separation between the peak nicotine impurity peak of blood plasma. The results of determining the HPLC conditions for nicotine analysis are as follows:

Column: LiChroCART C18, the particle size of 10 µm, column length of 250 mm
Mobile phase: methanol:acetate buffer pH 5 (85:15 v/v)
Detection: UV detectors 254 and 260 nm
Flow rate: 0.6 mL/minute
Injection volume: 20 µL

Table 1: Results of molar exctinction (e) nicotine at 260 nm

n	*Concentration (M)*	*A*	*Molar exctintion e ($M^{-1}.cm^{-1}$)*
1	0.00009732	0.292	3,000.41
2	0.00015138	0.4574	3,021.54
3	0.00021626	0.6759	3,123.4
$\bar{X}$			3,049.12

Table 2: Variations in the composition of mobile phases and flow rate in the determination of optimum nicotine HPLC conditions

Mobile phase composition (methanol:acetate buffer pH 5)	*Flow rate (mL/minute)*
80:20	0.6
85:15	0.6

Recovery Extraction Results

This extraction recovery value was obtained by comparing the chromatogram area of nicotine samples concentrations of 11 and 22 µg/mL in blood plasma extracted by protein deposition method using acetonitrile against nicotine samples in water, which were also extracted with acetonitrile. The sample is injected into the HPLC and three times repeated (n = 3). Based on the analysis results, % recovery extraction for both nicotine sample concentrations meets the requirements because the values are in the range of 80 to 120%.[14] Data recovery% extraction of experimental results is presented in Table 3.

The Results of the Analysis Method Validation

The validation of the analysis methods included parameters of selectivity, repeatability, linearity, LoD, LoQ, accuracy, precision, and system suitability test.

Selectivity

The selectivity test is done by calculating the resolution value of the peak of the chromatogram of the sample and the peak of the other chromatogram, which is separate (non-overlapping). This selectivity value illustrates the separation between the two peak chromatograms, where the resolution value obtained from the analysis results is 2.479, with a retention time of 7.025. This value meets the validation.

Repeatability

The repeatability test was performed by injecting a nicotine concentration of 11 µg/mL into HPLC and repeated six times (n = 6). Then, the retention time and chromatogram area are seen to calculate % CV of each parameter.[15]

Based on the repeatability test results (Table 4), % CV for retention time is 0.701, and for chromatogram, the area is 1.639. This shows the results of the repeatability test analysis meet the requirements, i.e., CV < 2%.[14]

Linearity

Linearity test is performed to determine the response of an analytical method to the concentration of the sample in the form of a calibration curve in which, the parameters used are correlation coefficients, which will express the linear relationship of the calibration curve. The standard curves were made from five variations of concentrations, *viz.*, 5, 8, 11, 16, and 22 µg/mL, and measurements were made three times (n = 3).[16,17]

Table 3: Results of nicotine extraction recovery 11 and 22 µg/mL based on AUC (n = 3)

Recovery nicotine (%) base on (AUC)			
n	*Nicotine 11 µg/mL (%)*	*n*	*Nicotine 22 µg/mL (%)*
1	99.74	1	100.32
2	100.76	2	99.71
3	100.5	3	99.86
$\bar{X}$	100.28	$\bar{X}$	99.96

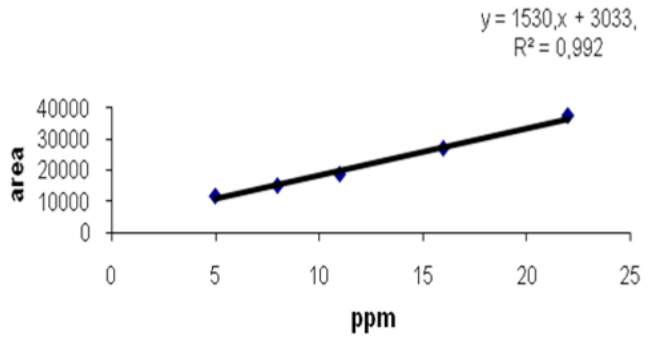

Figure 2: Nicotine calibration curves based on AUC

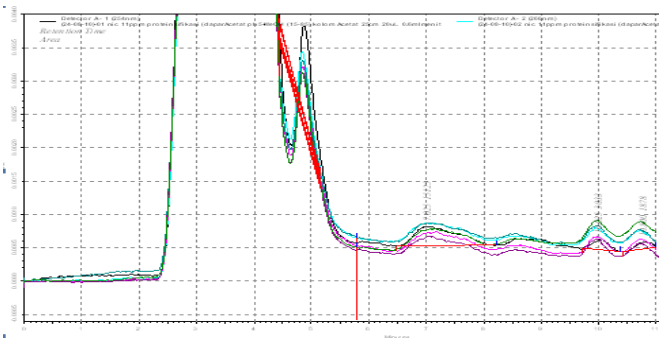

Figure 3: Chromatogram overlay system suitability of nicotine

Based on the calculation results of the linear line equation, the correlation coefficient (r) of 0.996 is obtained with the straight-line equation Y = 1,530.30x + 3,033.62 (Table 5). The r value meets the linearity requirements for a valid analysis method >0.995 (Figure 2).[5]

Boundary Test Results LoD and LoQ

The LoD and LoQ values are obtained from the calculation of the nicotine calibration curve to the chromatogram area, by using the curve that has the best correlation coefficient (r). The equation used is y = 1,530.30x + 3,033.62, so the LoD value of the area is 2,021 µg/mL, and the LoQ of the area is 6,737 µg/mL. The value obtained can detect levels of nicotine in the blood.

Accuracy and Precision

The accuracy seen results are based on % recovery for accuracy where the range of accuracy allowed for an analysis method in a biological matrix is 80 to 120% and CV for precision giving a value <10%.[14]

Based on the analysis results, precision, and accuracy meet the validation criteria. Where the nicotine concentrations of 6 and 20 µg/mL have precision with CV values of 3.95 and 2.15%. As for the accuracy parameters, both concentrations have recovery% 114.12 and 112.49%. Data from the calculation of precision and accuracy are presented in Table 6.

System Suitability Test

This system suitability test is conducted to find out that the system used can run effectively, and the parameter used is % CV, which must meet the requirements, i.e., CV <2% for biological fluids.[5]

Table 4: Results of nicotine 11 µg/mL in plasma (n = 6) based on retention time and AUC

Concenteration (µg/mL)	*Retention time (minute)*	*AUC of nicotine*
11	7.033	14,659
	7.33	15,001
	7.025	15,125
	6.95	15,107
	6.942	15,192
	6.933	15,407
$\bar{X}$	6.986	15,081.83
CV %	0.701	1.639

Table 5: Results of linearity nicotine base on AUC (n = 3)

Replikasi	*b*	*a*	*r*
1	1,331.15	1,661.65	0.982
2	3,033.62	1,530.3	0.996
3	–43.94	1,739.64	0.978

Note: b = intercept, a = slope, and r = cooffecient correlation

Table 6: Results of calculation of accuracy and precision of nicotine samples based on AUC

		Nominal concentration	
	Replication	*6 µg/mL*	*20 µg/mL*
AUC	1	13,990	37,660
	2	13,285	38,080
	3	13,261	36,643
Concentration (µg/mL)	1	7.16	22.63
	2	6.7	22.9
	3	6.68	21.96
$\bar{X}$		6.85	22.5
CV %		3.95	2.15
Recovery (%)	1	119.33	113.14
	2	111.65	114.51
	3	111.39	109.81
$\bar{X}$		114.12	112.49

Note: Concentration results obtained from the standard curve equation y = 1530.30x + 3033.62; r = 0.996

Table 7: System suitability test results for nicotine analysis method with a concentration of 10 µg/mL in blood plasma (n = 6)

Parameters	*CV (%)*
Retention time nicotine	0.7
AUC nicotine	1.639

Based on the research results that can be seen in Table 7, this system has met the system suitability test because it has a % CV value of 0.7 for retention time and 1.639 for chromatogram area (Figure 3).

CONCLUSION

From the results of the study, it can be concluded that the optimum HPLC conditions for nicotine analysis in human blood plasma (*in vitro*) using the protein deposition method by HPLC-UV detectors are using a mobile phase of methanol:acetate buffer pH 5 (85:15) with a flow rate of 0.6 mL/minute and wavelength measurements of 254 and

260 nm. For the range of 5 to 22 µg/mL, the parameters of selectivity, repeatability, linearity, precision, accuracy, and suitability of the system meet the validation criteria. In this study, the LoD value was 2,021 µg/mL, and the LoQ value was 6,737 µg/mL.

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