978-602-9420-29-6

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Muhammadiyah Tobacco Control Center

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PROCEEDING

conference and exhibition

Addressing Tobacco Problems in Developing Countries

- Economic Impact of Tobacco Use
- Social Determinants of Tobacco Use and Demand Reduction Interventions
- Culture, Employment and Agriculture: Between Tobacco Myth and Reality
- Tobacco Use and Health
- Youth, Cigarettes, and Drugs

Wednesday-Thursday, December, 5-6, 2012

University of Muhammadiyah Yogyakarta

conference and exhibition

Addressing Tobacco Problems In Developing Countries

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Abstract

ANALYSIS OF RATIO 3-HYDROXYCOTININE: COTININE: PHENOTYPING STUDY OF CYTOCHROME P450 2A6 ON INDONESIAN SMOKER

Christine Patramurti

ABSTRACT

A reversed-phase liquid chromatography with ultraviolet detection is described for the determination of cotinine, 3-hydroxycotinine and also 3-hydroxycotinine/cotinine ratio. A mobile phase consisted of a mixture ammonium acetate:methanol (50:50). Using a C8-type column endcapping, a good separation was achieved in 8 min. The wavelength of UV detector was set at 260 nm. With acetanilide as internal standard, human urine samples were cleaned up by liquid-liquid extraction. The regression equations obtained from both the standard were linear between 1.0 - 5.0 μg/mL for 3-hydroxycotinine and between 2.0-10.0 μg/mL ($r > 0.999$). This method has been successfully applied to the determination of urinary 3-hydroxycotinine and cotinine content and also for determining 3-hydroxycotinine/cotinine ratio in several Javanese smokers from Indonesia.

Keywords: 3-hydroxycotinine/cotinine ratio, HPLC, human urine

Full Paper

ANALYSIS OF RATIO 3-HYDROXYCOTININE: COTININE: PHENOTYPING STUDY OF CYTOCHROME P450 2A6 ON INDONESIAN SMOKER

Christine Patramurti

I. INTRODUCTION

Background

Nicotine is considered as the most responsible for the onset of pharmacologic response contained in cigarettes. This compound that can be found in cigarette smoke, is also the largest chemicals contained in tobacco (Karaconji, 2005). When someone smoke a cigarette, on average about 1 to 1.5 mg of nicotine is absorbed systemically during smoking, accompanied by the release of adrenaline and blocking the hormone insulin (Hukkanen, *et al.,* 2005). Nicotine is not carcinogenic compound, but in the case of lung cancer this compound may stimulate cancer cell proliferation (Puliyappadamba, *et* al., 2010).

Nicotine is metabolized extensively by the liver enzyme CYP2A6, primarily to cotinine, about 70 to 80% of nicotine is metabolized via the cotinine pathway in humans by the CYP2A6 through C-oxidation mechanism. Cotinine is itself metabolized by CYP2A6 to 3'-hydroxycotinine (3-HC). The ratio of metabolite to parent (i.e., 3-HC:cotinine) would be expected to reflect CYP2A6 activity. The ratio of the enzyme product, 3-HC, to the precursor, cotinine, would be expected to reflect CYP2A6 activity and could serve as a marker for nicotine metabolism rate (Benowitz *et al.*, 2003). Nicotine metabolism by CYP2A6 varies across ethnicity/race and is hypothesized to affect smoking behavior. The 3-hydroxycotinine/cotinine ratio has been studied as predictor of response to pharmacotherapy (Derby *et al.*, 2008).

Determination 3-hydroxycotinine/cotinine ratio in this study was run using the *High Perfomance Liquid Chromatography* (HPLC). HPLC method was chosen because it can be used for simultaneous multicomponent analysis.

Indonesia is one country in Asia with the level of cigarette consumption ranked 3 in the world. This condition shows that public awareness of the dangers of cigarette in Indonesia is still low, therefore research on nicotine metabolism and enzyme CYP2A6 phenotypes among smokers on, in this region is very needed.

Problems

Based on the background that has prepared the above, the problems that arise in the study were:

1. How much the levels of nicotine metabolites in the urine, cotinine and 3-hydroxy cotinine, in the smoker subjects during the study period?
2. How much the ratio of 3-hydroxycotinine and cotinine the smoker subjects during the study period?

Research Objectives

Based on the problems mentioned above, this study aims to:

1. Determining the levels of nicotine metabolites in the urine, cotinine and 3-hydroxy cotinine, on the subjects of Indonesia's smoker ethnic Javanese.
2. Determining the ratio of 3-hydroxycotinine/cotinine on the subjects of Indonesia's smoker ethnic Javanese that were examined during the study period.

Outcomes Research

Research outcomes expected from the results of this research are:

1. Provide methods and procedures for multicomponent analysis of nicotine metabolites, primarily cotinine and 3-hydroxycotinine, which can be applied in the urine sample.
2. As one of the reference to the possibility of the presence of the enzyme CYP2A6 polymorphisms form in Indonesia.

Uses Program

1. Provide input on strategic prevention of various diseases caused by nicotine and other compounds that the metabolic pathway were mediated by the enzyme CYP2A6.
2. Optimizing support for government efforts to create a society to stop smoking.

II. REVIEW THE LITERATURE

Nicotine

Nicotine (Fig. 1) is a natural ingredient in tobacco leaves. It is the principal tobacco alkaloid occurring to the extent of about 1.5% by weight in commercial cigarette tobacco and comprising about 95% of the total alkaloid content. An average tobacco rod contains 10 to 14 mg of nicotine and on average about 1 to 1.5 mg of nicotine is absorbed systemically during smoking (Hukkanen *et al.,* 2005). This compound has the chemical name 3 -(1-methyl-2-pirrolidinil)-pyridine; l-methyl-2-(3-pyridyl)-pirrolidin, or pyridyl-α-N-metil-pirrolidin. Molecular weight of nicotine is 162.23 g/mol; C 74.03%, H 8.70% and N 17.27%, has the empirical formula $C_{10}H_{14}N_2$. Here is the molecular structure of nicotine:

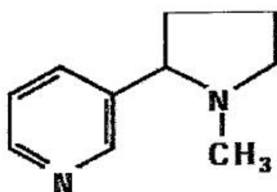

Figure 1. The molecular structure of nicotine

Nicotine is extensively metabolized to a number of metabolites (Fig. 2) by the liver. Six primary metabolites of nicotine have been identified. Quantitatively, the most important metabolite of nicotine in most mammalian species is the lactam derivative cotinine. In humans, about 70 to 80% of nicotine is converted to cotinine. This transformation involves two steps. The first is mediated by a CYP2A6 system to produce nicotine-1(5)-iminium ion, which is in equilibrium with 5-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehyde oxidase (Hukkanen *et al.,* 2005). Cotinine is itself metabolized by CYP2A6 to 3'-hydroxycotinine

(3-HC). The ratio of metabolite to parent (i.e., 3-HC:cotinine) would be expected to reflect CYP2A6 activity. The ratio of the enzyme product, 3-HC, to the precursor, cotinine, would be expected to reflect CYP2A6 activity and could serve as a marker for nicotine metabolism rate (Benowitz *et al.*, 2003). Nicotine metabolism by CYP2A6 varies across ethnicity/race and is hypothesized to affect smoking behavior. The 3- hydroxycotinine/cotinine ratio has been studied as predictor of response to pharmacotherapy (Derby *et al.*, 2008).

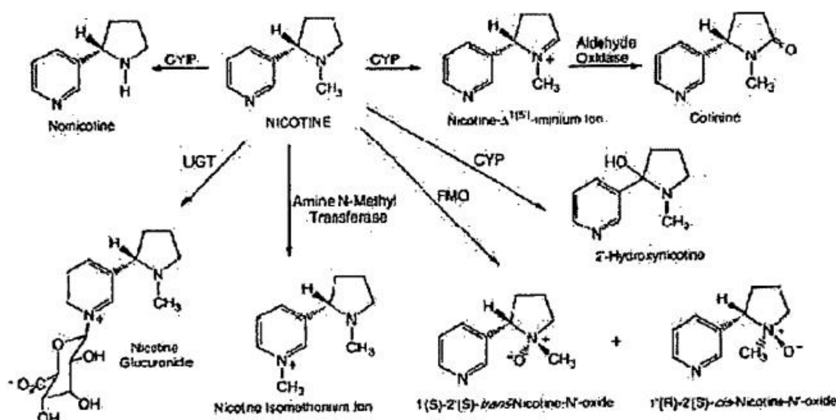

Figure 2. Nicotine metabolism pathways in the liver (Hukkanen, *et* al., 2005).

Cytochrome P450 2A6 (CYP2A6)

The CYP2A6 enzyme metabolises substrates that can be categorised into toxic (procarcinogens, promutagens and other toxins) and pharmaceutical agents (drugs). The substrates are usually detoxicated, but some are also activated. Such drugs as coumarin, halothane, losigamone, letrozole and SM-12502 are substrates of CYP2A6. This enzyme is also able to activate several carcinogenic compounds found in cigarette smoke, ie compounds derived N-nitrosamines, such as 4 - (metilnitrosamino) -1 - (3-pyridyl)-1-butanone (NNK). Carcinogenic compounds can be toxic effect once activated by enzymes in the body, therefore the presence of enzymes capable of activating carcinogenic compounds is very important (Figure 3) (Kamataki, 2006).

The polymorphisms of CYP2A6 have reported by Fernandez and Gonzalez (1995), there are three forms of the CYP2A6 allele: CYP2A6*1 *(wild-type),* CYP2A6*2 (variant 1), and CYP2A6*3 (variant 2) . The presence of the enzyme CYP2A6 polymorphisms suggests that the behavior of a smoker to smoke cigarettes per day is strongly influenced by the shape of the enzyme CYP2A6 polymorphisms. A smoker who only has the form monozygotic CYP2A6*1 allele, in a day will spend more than the cigarette smokers who have heterozygous alleles form, especially in the form of inactive alleles.

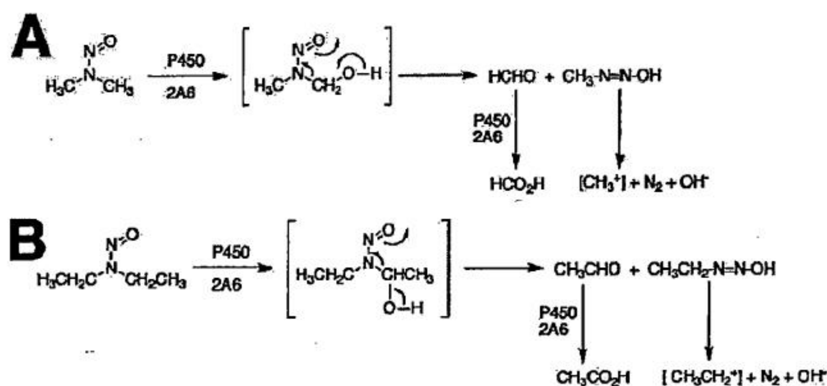

Figure 3. Activation of the enzyme CYP2A6 nitrosamine compounds to form diazohidrosida, a *DNA-alkylating agents* (Chowdhury *et* al, 2010)

In the case of lung cancer, the form of the enzyme CYP2A6 polymorphism is thought to be an important factor in increasing the risk of cancer cells. Theoretically, the absence of CYP2A6 may decrease the risk of lung cancer. According to the hypothesis Pianezza *et al.* (1998), individuals who have CYP2A6 enzyme in an inactive form recommended not to smoke, it is related to the ability of CYP2A6 to metabolize nicotine. On the other hand a number of carcinogenic compounds found in cigarette smoke and *4 - (methylnitrosamino) -4 - (3-pyridyl) butanal* (NNA) is activated by CYP2A6, and therefore the absence of this enzyme can reduce the risk of lung cancer (Oscarson, M., 2001). Several other studies have also reported a significant relationship between decreased activity of CYP2A6 enzyme with multiple cases of colorectal cancer and pancreatic cancer (Kadlubar *et*al., 2009).

Variations of the CYP2A6 polimorfi is influenced by genetic factors. One of the specific substrate that can be used to determine the CYP2A6 phenotype is nicotine. Levels cotinine undetectable in plasma after 2 hours of administration of nicotine in people with genotype CYP2A6*4/CYP2A6*7. Metabolism of nicotine in people who have experienced a decrease alelCYP2A6*9 (Yamanaka *et al.,* 2004). Shape polimorfi CYP2A6 enzyme is found in many Asian people, which is inactive allele frequencies so high that individual variation on the metabolism of nicotine to be larger. According to Nakajima and Yokoi (2005) form of CYP2A6 polymorphisms in Asian populations are CYP2A6*4 (11-20%), CYP2A6*7 (4-7%), and CYP2A6*9 (20%).

III. MATERIALS AND METHOD

Reagents

Cotinine (99% pure ; Sigma Chemical Co., St. Louis), 3-hydroxycotinine (CAS 34834-67-8, 99% pure; Santa Cruz Biotechnology Inc.), "HPLC-grade" ammonium acetate and methanol from *E Merck*, potassium hydroxide (reagent grade), chloroform (reagent grade) and etanol (reagent grade) from J. T. Baker Chemical Co., Phillipsburg, NJ; aquabidest.

Instrumentation

To obtain the ultraviolet absorption spectra, we used a UV mini-1240 scanning spectrophotometer (Shimadzu Instruments). The LC-2010HT Compact HPLC (Shimadzu Instruments). We used

a 25 x 4.6 cm Premier C8 column column, 120A particle size, from Shimadzu. Micropipette; centrifuges; refrigerator (-20°C), a set of glassware commonly used in the labo ratory analysis.

HPLC instrumentation

The conditions of HPLC systems used were as follows:

Column	: Premier C8 (Shimadzu 250 × 4.6 mm, 120A)
UV detector	: 260 nm
Injection volume	: 20 mL
Mobile phase	: Methanol: Ammonium Acetate 5 mM (50: 50).
Flow rate	: 0.8 mL / min

Procedures

Subjects and sample collection

Ten samples were collected from javanese Indonesian smoker who smoked cigarettes in the number of 1-10 cigarettes. Urine samples were taken from the each volunteer after they filled out a questionnaire. The questionnaire included, the age, the time of the sample collection, and the number of cigarettes smoked.

Urine samples were stored at -20 °C for a maximum o f 2 weeks before delivery. Frozen samples were shipped by express mail to the laboratory. Delivery lasted; 4h and allowed for melting of samples. On arrival, samples were kept at 5 °C for a maximum of 2 days before analysis.

Sample preparation

1.0 mL aliquot of urine sample, with 10μL of acetanilide (100μg/mL) as internal standard, was thoroughly mixed with 500 μL of 1 N potasium hy droxide. These mixture was extracted using 2 mL solvent kloroforom/ethanol (9:1, v/v) for 5 times. The organic phase obtained re-extracted using 5 ml of 0.1 N KOH 2 times. Organic phase was then evaporated, the residue obtained was dissolved in 1.0 mL methanol 30%. 20 μL of this solution is inje cted into the HPLC for analysis.

Standard solutions

Stock solution of cotinine, 3-hydroxycotinine and internal standard solution acetanilide (1000μg/mL) was obtained in methanol and the intermediet standart (100μg/mL) solution was obtained by diluting approp riate volume of stock solution with methanol 30%.

Calibration curves

Caliberation cuvers were prepared by processing various concentrations, i.e. 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml of working standard of 3-hydroxycotinine and 2.0, 4.0, 6.0, 8.0 and 10.0 μg/ ml of working standard co tinine prepared by diluting intermediet standards in methanol 30%. Twenty microliter from each concentration was injected into HPLC. The peak rasio areas of nicotine/acetanilide and cotinine/acetanilide were plotted versus the original concentrations and evaluated by linear least square regression analysis.

Validation of analytical methods

Some aspects are measured in the validation of the method in this study is the selectivity, linearity, accuracy, and precision.

Results Analysis

In accordance with the objectives and procedures over the data obtained were analyzed as follows:

1. Cotinine and 3-hydroxycotinine levels in the urine of subjects was determined using the caliberation curve.
2. CYP2A6 enzyme activity in metabolizing nicotine from each subject is determined by calculating the ratio 3-hydroxycotinine/cotinine.

PROGRAM IMPLEMENTATION

Time and Place of Execution

This research was conducted in the laboratory of Chemical Analysis Instrument Faculty of Pharmacy, University of Sanata Dharma Yogyakarta and started in July and October 2012. Time allocation is effectively used by researchers for each month is 2 weeks. This is due to the limitations of HPLC instruments held by the laboratory, so the researchers had to take turns with other researchers in using the tool.

Stages of Implemen

No.	Acivity	Month							
		July		Agt		Sept		Oct	
1	HPLC Method Optimation								
2	Urine Prepairation Optimation								
3	HPLC Method Validation								
4	Determining Cotinin and 3-hydroxycotinine in urin								
5	Analisis Data								
7	Penyusunan Laporan								

Design and Realization of Cost

No.	Component	Amount	Price
A	Bahan habis pakai		
1	Cotinine	250 mg	2100000
2	3-Hydroxycotinine	10 mg	4818000
3	KaOH	100 g	266250
4	Chloroform	500 mL	400000
5	Ethanol	100 mL	57400
6	Methanol	10 Lt	1123000
7	Aquabidest	10 Lt	200000
8	Acetanilide	10 g	47550
9	Ammonium Acetate	100 g	145650
	Sub total		**9157850**
B	Volunteer Fee	5	250000
C	Laboratorium Fee	3 bulan	500000
D	Report		500000
	Grand total		**10407850**

V. RESULTS AND DISCUSSION

Determination 3-hydroxycotinine/cotinine ratio in this study performed using reversed-phase HPLC method with UV detector at 260 nm. Determination of the value of the observation wavelength HPLC systems is due both analytes (cotinine and 3-hydroxycotinine) were analyzed as well as the internal standard (acetanilide) used in the analysis of absorbing the UV wavelengths. Here is the spectrum of the three compounds:

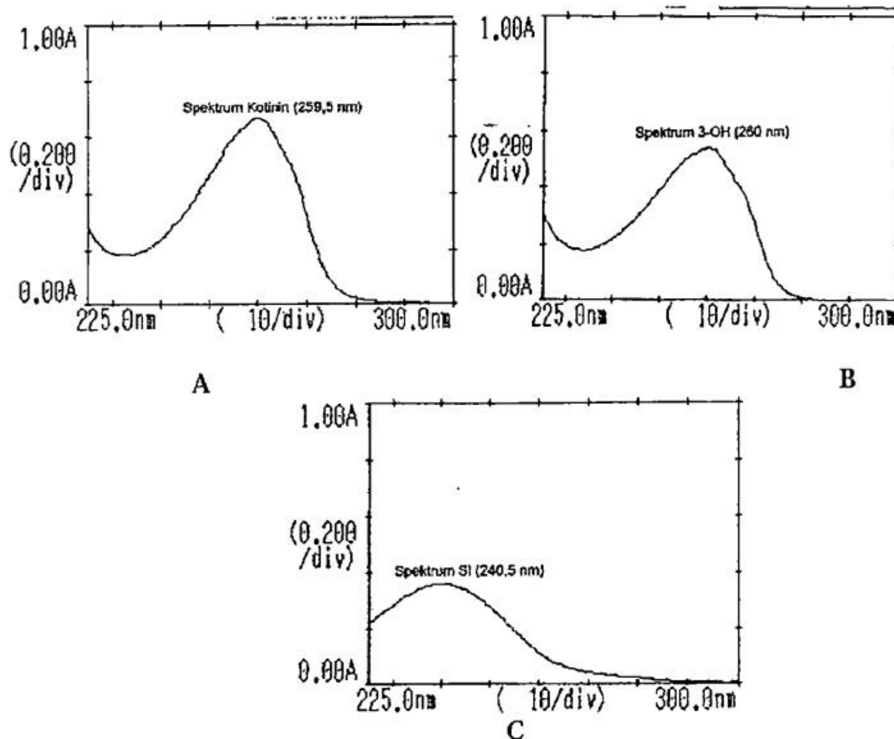

Figure 4. Cotinine spectrum (A), 3-hydroxycotinine (B) and acetanilide (C)

In Figure 4 above shows that both the analyte to be analyzed have optimum absorption at a wavelength of 260 nm, and therefore the subsequent analysis wavelength was used as the observation wavelength used in the HPLC system.

Chromatographic system used in this study is a reversed-phase chromatography with column C8 stationary phase and mobile phase a mixture of methanol:ammonium acetate 5mM (50:50). In this system the two analytes were analyzed and internal standards separate completely. Here are the chromatogram separation of three compounds using the system:

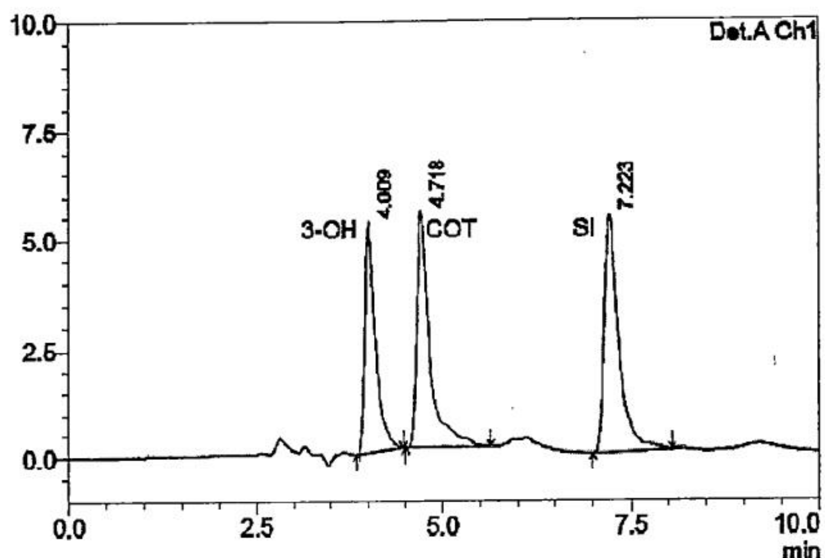

Figure 5. Kromatorgam raw mixture of compounds: 3-hydroxycotinine, cotinine and acetanilide using the optimum HPLC system (mobile phase a mixture of methanol: ammonium acetate 5 mM in 50:50 ratio, flow rate of 0.8 mL / min)

All three compounds, 3-hydroxycotinine, cotinine and acetanilide, using chromatography system separate completely. This is indicated by a resolution of each compound to another compound. Good separation indicated by resolution of > 2.0. The resolution of the 3-hydroxycotinine and cotinine is 2.67, while the value of the acetanilide and cotinine resolution is 8.836. The resolution also showed selectivity of the method used, where the resolution value > 2.0 indicates that each compound can be separated well with other compounds and the response of each compound is not affected by other compounds.

Biological samples analyzed in this study is urine from smokers. Preparation is a very important step in the analysis before the sample solution is analyzed using HPLC method. In this research, the preparation of urine performed using liquid-liquid extraction method. All three compounds were analyzed soluble in chloroform and ethanol, chloroform therefore be used as an extracting solvent three compounds. To improve the solubility of the compound in an organic solvent, ethanol is added into chloroform. The comparison between chloroform and ethanol is 9:1. In the first phase extraction, besides the three compounds analyzed several other organic compounds also soluble in the organic phase. Some compounds that might exist in the organic phase are organic acids and neutral organic compounds. The presence of organic compounds in the organic phase would interfere the analysis, therefore, the second extraction was done using 0.1 N KOH. The chromatogram of urine blank from subject who did not smoke:

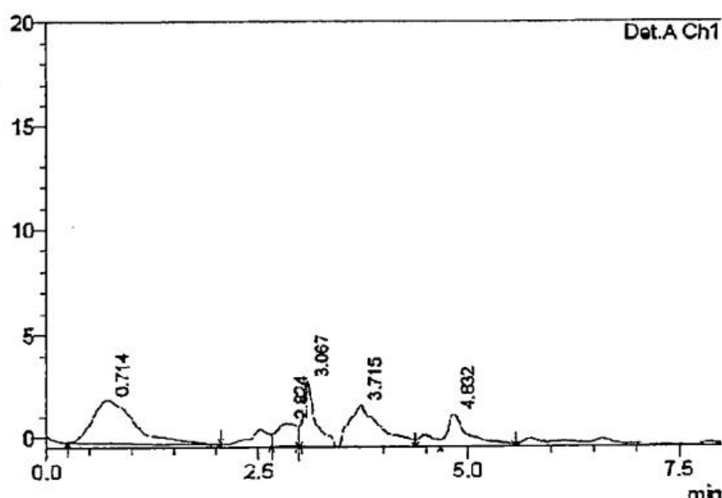

Figure 6. Chromatograms of urin blank

Figure 6 above shows that the urine blank chromatogram, in the third until the seventh minute, several small peaks may still interfere the analysis. To reaffirm that the preparation is optimal, the technique performed spiking blank urine using all three compounds were analyzed.

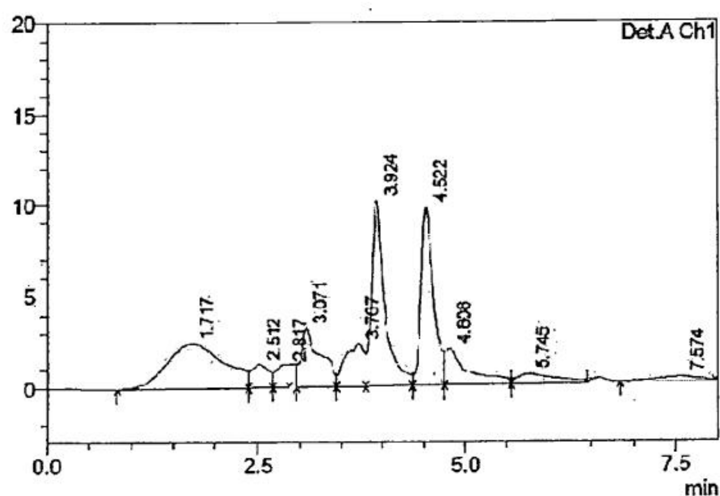

Figure 7. Chromatograms of blank urine that you get an addition to the standard 3-hydroxycotinine compound (A) and cotinine (B)

Based on the figure 7 above can be ensured that the preparation of the urine using liquid-liquid extraction techniques can produce good sample solution, because the several peaks in the urine blank did not interfere with the response of the three compounds were analyzed.

The validity of the HPLC method used in this study was assessed by some, namely:

1. Selectivity

   Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Selectivity parameters can be seen from the value of the resolution. In this study, the value of the third resolution compound is > 2.0, therefore the methods used have met good selectivity parameters.

2. Linearity

Linearity of an analytical method demonstrated the ability of a method to obtain test results proportional to the analyte concentration and is expressed by the correlation coefficient (r). Caliberation curve for 3-hydroxycotinine is $Y = 0.7726 X - 0.0088$ with $r = 0.9998$. Caliberation curve for cotinine is $Y = 0.4361 X - 0.0200$ with $r = 0.9998$. Correlation coefficient values both analytes > 0.999 therefore these methods can produce good caliberation curve and linearity qualify.

3. Precision

The ***precision*** of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

Table 1. Precision of 3-hydroxycotinine and cotinine

No.	Analyte	Mean (µg/mL)	CV (%)
1	3-Hydroxycotinine	0,48	5,86
		1,67	3,75
		3,46	3,08
2	Cotinine	1,12	1,76
		4,16	2,51
		7,17	0,99

4. Accuracy

The ***accuracy*** of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.

Table 2. Accuracy of 3-Hydroxycotinine and cotinine

No.	Analyte	True value (µg/mL)	Accuracy (%)
1	3-Hydroxycotinine	1,02	(46,86 ± 2,75)
		3,07	(54,28± 2,03)
		5,12	(67,62 ± 2,09)
2	Cotinine	2,00	(55,77 ± 0,98)
		5,99	(69,51± 1,75)
		9,99	(71,75 ± 0,71)

Based on the validity of the parameters analyzed, the HPLC method used for the analysis of both analytes qualify as a valid method; so this method can be used for analysis of 3-hydroxycotinine and cotinine in urine samples. Here is a chromatogram of urine samples from smokers subjects:

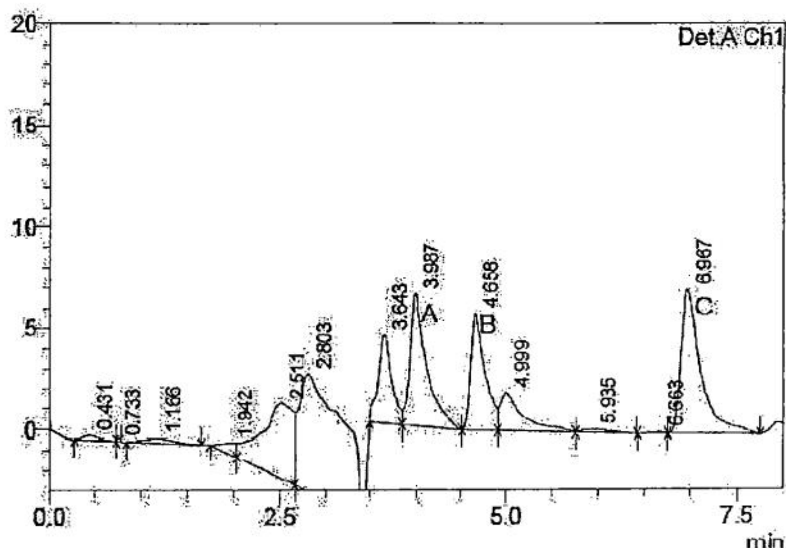

Figure 8. Chromatograms of urine sample of smoker: (A) 3-hydroxycotinine, (B) cotinine and (C) acetanilide.

In figure 8 above shows that urin sample was containing 3-hydroxycotinine and cotinine. This is indicated by the appearance of a peak at 3.9 minutes which is the culmination of a 3-hydroxycotinine and peak at 4.6 minutes which is the culmination of cotinine. The levels of both analyte and 3-hydroxycotinine/cotinine ratio test on each subject are as follows:

Table 3. Value 3-hydroxycotinine levels (3-OH), cotinine (Cot) and the ratio of smoker subjects 3-OH/Cot

No. Subject	C 3-OH (µg/mL)	C Cot (µg/mL)	C 3-OH /C Cot
1.	1,06	1,47	0,72
2.	1,4	1,52	0,92
3.	1,34	1,55	0,86
4.	0,22	0,24	0,92
5.	0,84	0,51	1,65
Mean	0,97	1,06	1,01
SD	0,48	0,63	0,36
CV	49,05	59,68	35,98

The subjects who participated in this study were drawn from active smokers who smoked cigarettes as much as 1-10 stems per day. Levels of 3-hydroxycotinine values ranged from 0.22 to 1.4, while the value cotinine levels ranged from 0.24 to 1.55. One of the test subjects had a grade 3-hydroxycotinine and cotinine very small, this leads to a very large variation in the data above. Large variations from both levels of this compound in urine is indicated by the value of % CV of each compound. The occurrence of large variations may be influenced by the amount of intake of nicotine into the body of each subject test through cigarettes smoked or the possibility of the presence of inactive enzyme CYP2A6 alleles, such as CYP2A6 or CYP2A6 * 4 * 9 of the test subjects.

3-hydroxycotinine/cotinine ratio may reflect the CYP2A6 enzyme activity of each subject test. According to Table 3, the variation of the ratio 3-hydroxycotinine/cotinine also quite high. This indicates that there is a difference in the activity of the enzyme CYP2A6 metabolizes into 3-hydroxycotinine cotinine. In the early stages of the CYP2A6 enzyme phenotype studies, this data can be used as input possibility polimorfi CYP2A6 enzyme in Indonesian society, especially ethnic Javanese.

IV. CONCLUSIONS AND RECOMMENDATIONS

Conclusion

1. A method using HPLC with UV detector for the analysis of 3-hydroxycotinine and cotinine with good selectivity has been developed. The method is simple, precise and accurate with a sample prepared by liquid-liquid extraction. The method has been successfully applied to determine the urinary 3-hydroxycotinine and cotinine concentrations of five smokers. Levels of 3-hydroxycotinine and cotinine in urine samples from the test subjects were analyzed in this study have a large variation.
2. CYP2A6 enzyme activity as indicated by the value of the ratio 3-hydroxycotinine/cotinine of test subjects have considerable variation.

Suggestion

Research conducted a preliminary stage of research CYP2A6 enzyme polymorphism identification studies in Indonesia, some further research to do to complete this research could consider the following:

1. Adding variation smoker group test subjects, namely:
   Group I : cigarette smokers who smoked 1-10 cigarettes/day Group II : cigarette smokers who smoked 11-20 cigarettes/day Group III : cigarette smokers who smoked 21-30 cigarettes/day.
2. Increasing the number of test subjects for each group.
3. Perform genotyping studies to the subjects, to further reinforce the identification of the CYP2A6 enzyme polymorphism of the subjects were studied.

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