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Validation of GC-MS Method for Determination of Nicotine and Cotinine in Plasma and Urine

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Abstract: ***Nicotine and cotinine are the major determinants of tobacco use and addiction. After smoking, traces of nicotine and cotinine can be found throughout all the body, even in blood and urine.This paper provided and validated a new gas chromatography- mass spectrometry (GC-MS) method for the determination of these nicotine and its major metabolite, cotinine. For sample preparation, the analytes and internal standard were extracted from alkalinized sample for both nicotine and cotinine. Linear calibration curves over a range of10-1000ngmL^{-1} of the analytes in urine and plasma were established with the linear correlation coefficients ($r^2 \geq 0.997$). Also peak quantification, shape and separation were excellent within this range. The limit of deduction for both nicotine and cotinine were obtained at the range of 0.3-0.8 ng mL^{-1}. The mean recoveries for nicotine and cotinine were 98.0 and 101.7%.***

Very quite satisfactory methods have been found for the analysis of nicotine and cotinine in plasmaand urine. This method included only one simple extraction step and gave good recoveries with small variations with a lower LOD value. Relative standard deviations of within-day and day-to-day assay results were less than 8.7%. Therefore, his method could be useful for routine monitoring of nicotine and cotinine exposure, using a sample amount as low as 1 mL of human plasma or urine.

Keywords: Nicotinine, cotinine, GC-MS, plasma, urine

I. INTRODUCTION

Nicotine is only one of the ingredients in cigarettes. The tobacco products such as cigarettes are made from a blend of different types of tobacco leaves, and then sugar and other flavoring are added. Nicotine is a poisonous alkaloid that contains carbon and nitrogen. The origin of nicotine is Nicotine Tabacum. A cigarette is a well-structured nicotine dispensing device that proves to be deadly when it is smoked regularly. Nicotine, a smoked cigarette, will reach the brain in as little as 7 seconds after it is inhaled. It moves fast and affects the brain, central nervous system, hypothalamus and pituitary gland. Nicotine's primary metabolites are cotinine and nicotine-N-oxide. Cotinine is commonly used as a biomarker to determine active and passive smokers[1].

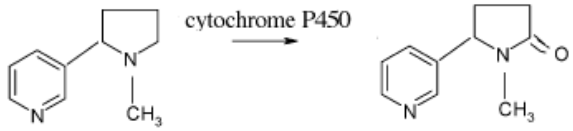

Fig. 1 Nicotine transformation into cotinine.

Nicotine has important cardiovascular, endocrine and metabolic effects. Metabolic effects of nicotine are related to effects on serum lipids and body weight. Nicotine useis effective for the increase of the metabolic rate and, induces lipolysis and releases free fatty acids into the plasma. Endocrine effects of nicotine is associated with thatch increase and cortisol release[2].Nicotine has also effects on cardiovascular physiology, and increases blood pressure and force of concentration of the heart[3].

Due to serious health consequences resulted from nicotine, methods for the determination of nicotine and its metabolites in biological samples are needed. Several methods including radioimmunoassay [4], gas chromatography [5], or gas chromatography coupled to mass spectrometry [6], high-performance liquid chromatography [7], or liquid chromatography tandem-mass spectrometry [8]were published. These methods have some limitations in sensitivity and reproducibility because of the volatility of nicotine.

The half life of nicotine is averagely 2 hours [9]. Cotininie, due to its long half life (16-20 hours), is commonly used as a marker of nicotine intake in studies [10]. The nicotine is detectable for about 1 to 3 days in plasma, 15 to 20 days in urine. Cotinine stays about

1 to 10 days in plasma and more longer in urine. The actual smoking status is determined by the level of cotinine in body fluids but age, levels of estrogen, food intake, renal impaired patients, time of smoking affect the stay of nicotine in the body [3].
In this study,a sensitive and reproducible gas chromatographic method was established for the support of clinical research studies, by modifying an extraction procedure reported for nicotine and cotinine in plasma by Moriya et. al. [11]. The purpose of the study was to obtain a rapid, simple and inexpensive method for the simultaneous determination of nicotine and cotinine in plasma and urine.

II. EXPERIMENTAL

A. Standards, Reagents and Instrumentation

Nicotinine, cotinine and diphenylamine were acquired from Sigma (St Louis, USA). Acetic acid, asetonitrile, methanol and hexan Sigma (St Louis, USA)were in HPLC grade. GC-MS analyses were performed on HP6890 GC coupled with HP5973 mass spectrometer detector. The column was fused-silica capillary, HP-5MS column (30 m x 0.25 mm I.D. x 0.25 µm film thickness).

B. Chromatographic Conditions

GC-MS analyses were performed with mass spectrometer detector. Helium was used as carrier gas at a constant flow 1.5 ml min-1. One micro liter injection volume using splitless mode was programmed from 70 to 230°C (1 min hold) at rate of 25°C min-1. Post run was set at 310°C for 6 min. Total run time was 6.9 min. The interface temperature was set at 280°C. Selection ion monitoring (SIM) mode was used in analysis. Operation of the MSD was in the electron impact (EI) mode at 70 eV, 230°C. The major ions peaks using the scan mode (50-180 a.m.u) were m/z 84 and 162 for nicotine; m/z 98 and 176 for cotinine; and m/z 169 and 168 for internal standard, diphenylamine. Quantitation ions were m/z 84 for nicotine; m/z 98 for cotinine; m/z 169 for internal standart. A 100 ms dwell time was used for all ions.

C. ExtractionProcedure of Nicotine and Cotinine from Urine and Plasma

1 mL of plasma or urine mixed with 20 µL of diphenylamine (10 mg mL^{-1} in methanol) as an internal standard and 1 mL of 1 M carbonate buffer (pH 9.5) was placed in a 20 mL glass test-tube. The mixture was mildly extracted with 4 mL of dichloromethane for 10 min using a mechanical shaker. Organic phase was transferred to a new disposable test tube. The organic phase vigorously back-extracted with 1 mL of 0.1 N HCl after 30 sec vortex mix. The resulting aqueous phase was transferred to a new disposable test tube and mixed with 1 mL carbonate buffer. The mixture was re-extracted after vigorous 30 sec vortex mixing with 4 mL dichloromethane. The organic phase was mixed with 20 µL of isoamyl alcohol and dichloromethane was evaporated under a gentle stream of nitrogen in heating block at 35°C. About 10 µL aliquot of the remaining isoamyl alcohol solution was then injected into the gas chromatograph.

D. Validation of the GC-MS Method

Calibration curve were constructed on peak ratio of analyte/internal standard versus concentrations using linear regression.Spiked human urine and serum standards were prepared from the known concentrations of the analytes in conjunction with the internal standards to create calibration curves. The verification program includes linearity, dedection limit (LOD), sensitivity and accuracy studies of cotinine in plasma and urine. Three different concentrations of nicotine and cotinine (25, 100 and 1000 ng mL^{-1}) were analyzed to determine accuracy and precision of the assay.

III. RESULTSAND DISCUSSION

The most important metabolite of nicotine in most mammalian species is cotinine. In humans, about 70-80% nicotine is converted to cotinine[12].Due to the long half life of cotinine, it is used as biomarkers of daily intake in smokers and passive smokers. There is a high correlation between concentrations of cotinine measured in plasma and urine, and measurements on any of these fluids can be used as a marker of nicotine uptake.

Extraction of the nicotine and cotinine from plasma and urine was carried out using liquid-liquid extraction. The pH content (acid/alkaline balance) of the smoke determined how much nicotine was absorbed through the mouth when it was smoked. Also the absorption of nicotine into biological membranes depended on pH.Generally, in more alkaline tobacco, the more nicotine was absorbed in the mouth [13]. In the light of this information, a carbonate buffer (pH 9.5)was used at the extraction stage.
The retention times for nicotine, cotinine and ISTD were 4.65, 6.45 and 6.05 min, respectively.There were no substances in extracts of urine and plasma that produced interfering peaks at the retention time of nicotine, cotinine and internal standart. Total ion

chromatograms of GC-MS analysiswere shown in Fig. 2.These analysis were made by standard addiction method. The peaks of nicotine, cotinine and internal standard were symmetric, and the separation of analytes from other compounds in plasma and urine was very good.

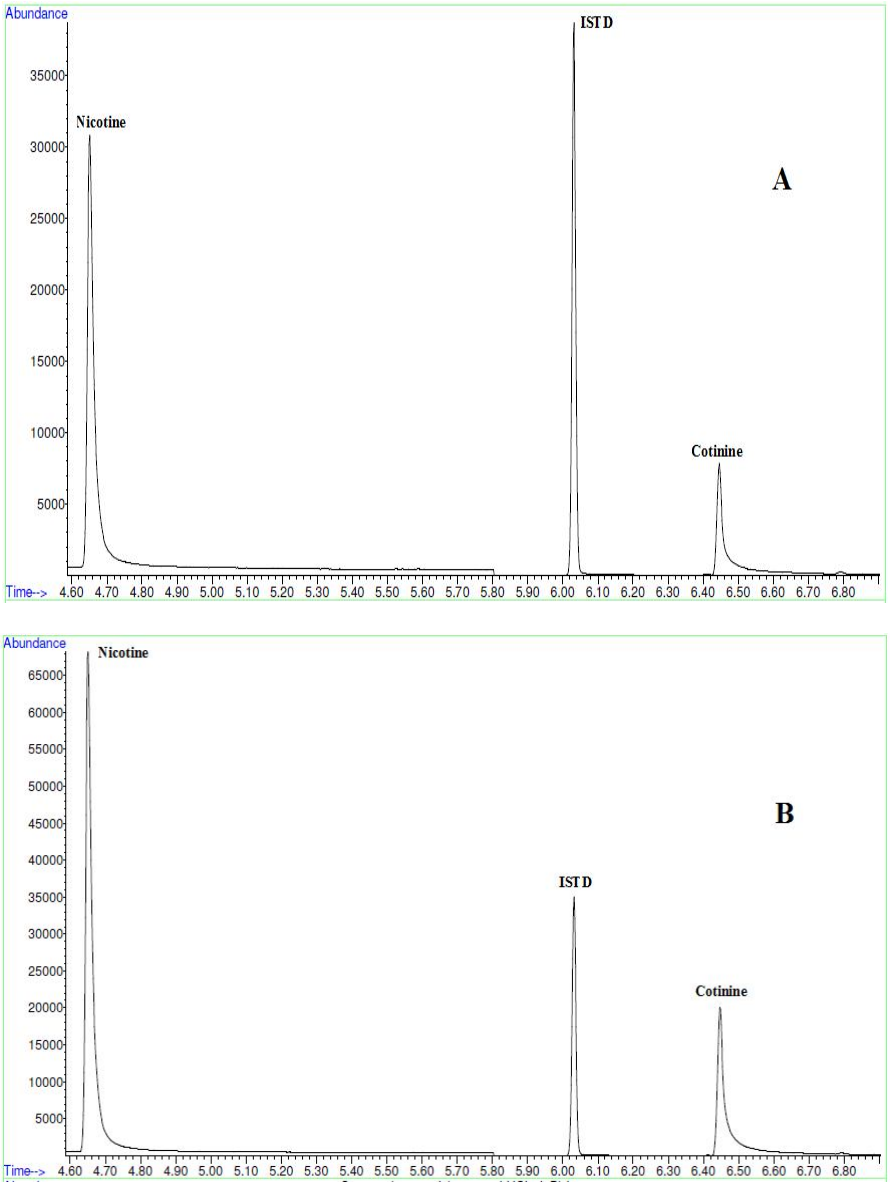

Fig. 2Ion chromatograms for standard addicion analytes in plasma (A) and urine (B) extract.

A sensitive method for determination of nicotinine and cotinine in human urine and plasma was developed in our laboratory. In the SIM mode of GC-MS, the molecular ion of m/z 84 for cotinine, m/z 98 for cotinine and m/z 169 for internal standard were the quantification ions.

Limit of dedection (LOD), accuracy and precision were determined by replicate analysis of urine and plasma spiked with nicotine and cotinine over the expected concentraiton ranges. LOD was 0.6 and 0.8 ng mL^{-1} for nicotine; 0.5 and 0.3 ng mL^{-1} for cotinine in plasma and urine. Limits were defined by a minimum signal to ratio of 3. The assay was linear rangingfrom 1 to 1000ng mL^{-1} for nicotine and cotinine in plasma; from 1 to 5000 ng mL^{-1} in urine. Good accuracy and precision were obtained from nicotine and cotinine at the concentrations of 25-100-1000 ng mL^{-1}. The coefficient of variation was less then %8.7 for each concentration. Validation data for GC-MS analysis of nicotine and cotinine was shown in Table 1. and Table 2.

Table 1**.** Validation data for GC-MS analysis of nicotine.

Parameters	Plasma	Urine

LOD	0.8	0.6
Linearity	1-1000 ng mL^{-1}	1-5000 ng mL^{-1}
	(9 points)	(11 points)
	r^2=0.998	r^2=0.998
Accuracy		
Target (n=3)		
25 ng mL^{-1}	23.8±0.6	24.6±0.4
100 ng mL^{-1}	103±4.8	102±3.0
1000 ng mL^{-1}	1041±36.3	1031±34.1
Within-run precision (%CV, n=3)		
25 ng mL^{-1}	2.5	1.6
100 ng mL^{-1}	4.7	2.9
1000 ng mL^{-1}	6.1	3.3
Between-run precision (%CV, n=3)		
25 ng mL^{-1}	5.2	3.4
100 ng mL^{-1}	7.1	2.5
1000 ng mL^{-1}	5.1	8.7

Table 2. Validation data for GC-MS analysis of cotinine.

Parameters	Plasma	Urine
LOD	0.5	0.3
Linearity	1-1000 ng mL^{-1}	1-5000 ng mL^{-1}
	(9 points)	(11 points)
	r^2=0.997	r^2=0.998
Accuracy		
Target (n=3)		
25 ng mL^{-1}	22.9±0.7	24.1±0.3
100 ng mL^{-1}	104±5.1	104±4.7
1000 ng mL^{-1}	1052±21.3	1038±49.1
Within-run precision (%CV, n=3)		
25 ng mL^{-1}	3.1	1.2
100 ng mL^{-1}	4.9	4.5
1000 ng mL^{-1}	2.0	4.7
Between-run precision (%CV, n=3)		
25 ng mL^{-1}	8.1	3.3
100 ng mL^{-1}	3.7	5.2
1000 ng mL^{-1}	6.9	7.9

Even if the highest nicotine exposure on humans occured after tobacco smoke, the level of exposure increaseed during the day. Nicotine was taken intermittently but could not be quickly removed from the body and accumulated in the body [14]. Therefore, when applying the method, we have developed, to actual plasma and urine specimens,it was important to to look at the plasma and urine specimens collected within 24 hours.

IV. CONCLUSION

A high thruput method for simultaneous quantification of plasma and urinary nicotine and cotinine was developed and validated. The method is rapid, sensitive, accurate, convenient and simple. The extraction of the analytes was carried out with the use of very little amount of solvents. The GC–MS analysis was carried out in 6.9 min with no interferences.The results indicated that nicotine and cotinine level serve as a useful biomarker for tobacco exposure. Hence, it is applicable for routine assessment and monitoring of active and passive smoking and exposure to environmental tobacco smoke.

A. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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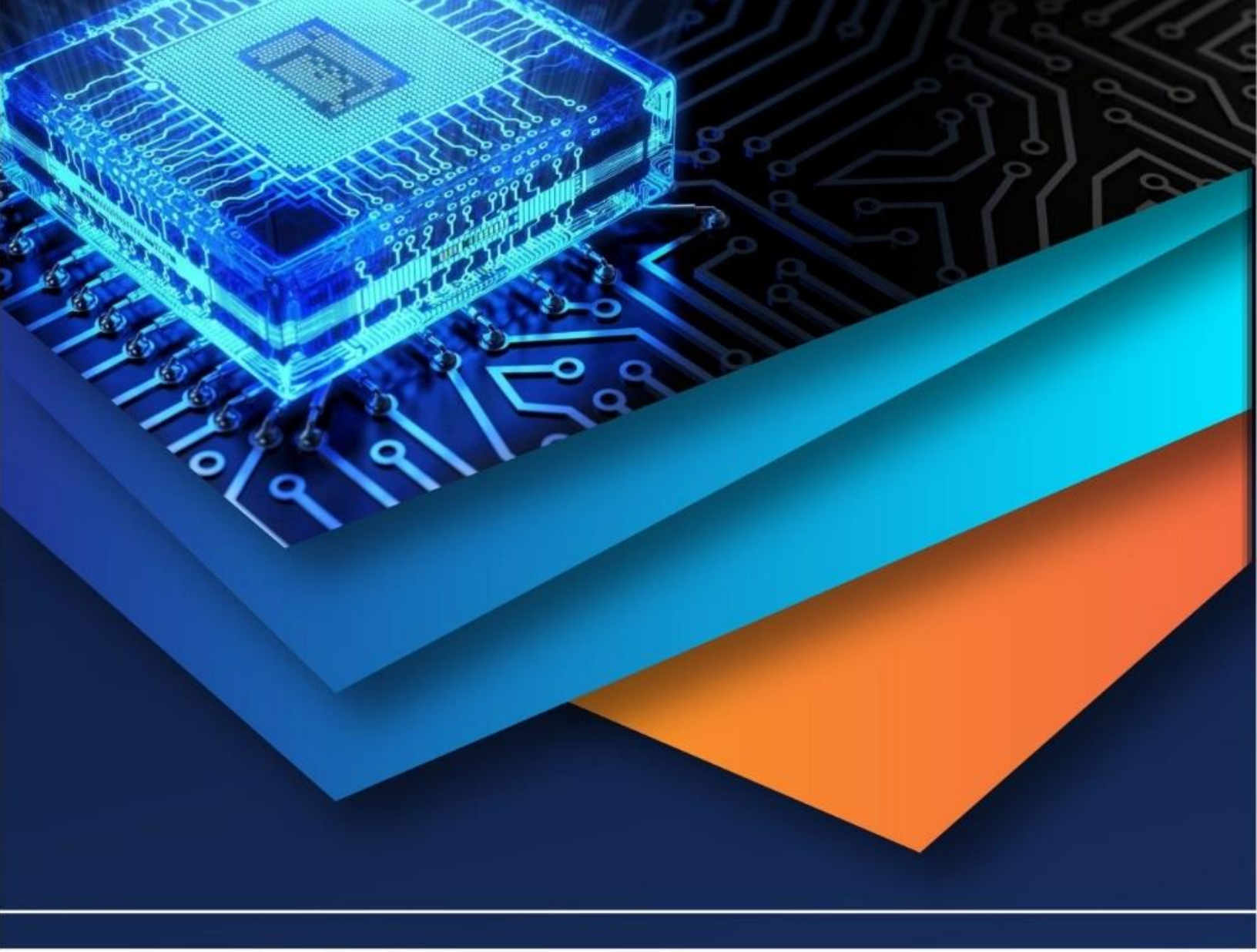

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