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Determination of Nicotine in Libyan Smokers and Nonsmokers' Urine samples by Ultraviolet-Visible Spectrophotometry

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Abstract This study was aimed to determine the nicotine in Libyan smokers and nonsmokers 'urine. Twenty urine samples are collected from smokers and nonsmokers. Each volunteer was required to complete a questionnaire before providing the urine sample. The samples were prepared by one-step extraction method for the determination of nicotine in human Urine using Ultraviolet-Visible Spectrophotometer. The criteria and factors taken into consideration for this evaluation and validation include the linearity, precision, accuracy, limit of detection, and limit of quantitation. The results of nicotine concentrations in male smokers' urine were in the range of 0.360–2.644 µg/ml with an average of 1.185 µg/ml. Whereas its concentrations in non-smokers'urine were in the range of 0.355-2.914 µg/ml with an average of 0.873 µg/ml. Statistical analysis show that the nicotine concentrations were significant difference in the smoker samples in contrast with the nonsmoker samples using UV-VIS spectrophotometric methods.
Keywords: Cigarettes, Extraction, Nicotine, Smoking, Urine; UV-Vis Spectrophotometer.

تقدير النيكوتين في عينات بول مدخنين و غير مدخنين ليبين باستخدام مطيافية الأشعة فوق البنفسجية والمرئية

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الملخص تهدف هذه الدراسة لتقدير النيكوتين في 20 عينة من البول مأخوذة من عشرة مدخنين وعشرة غير مدخنين في ليبيا. كان على كل متطوع استكمال استبيان قبل اخذ عينة البول. تم تقدير النيكوتين من خلال عملية استخلاص بمرحلة واحدة سريعة، رخيصة، عالية الأداء، وذلك باستخدام تقنية التحليل الطيفي في منطقة الأشعة المرئية وفوق البنفسجية، وقد تم تطويرها والتحقق منها لهذا الغرض. تشمل المعايير والعوامل التي أخذت في الاعتبار لهذا التقييم والتحقق: الخطية والدقة والحساسية وحدود الكشف وحدود الكميات. تراوحت تركيزات النيكوتين في عينات البول من المدخنين من 0.360 إلى 2.644 ميكروجرام / مل، وبمتوسط 1.185 ميكروجرام / مل. من ناحية أخرى، تراوحت تركيزات النيكوتين لدى غير المدخنين من 0.355 إلى 2.914 ميكروجرام / مل، وبمتوسط 0.873 ميكروغرام / مل. تبين التحاليل الإحصائية أن تركيزات النيكوتين كانت كبيرة في عينات المدخنين على عكس عينات غير المدخنين.
الكلمات المفتاحية: السجائر، الاستخلاص، النيكوتين، التدخين، البول، مطيافية الاشعة المرئية والفوق بنفسجية.

Introduction

Cigarette smoking is the leading cause of mortality and morbidity in our society [1], the leading cause of preventable disease and death in world [2] , Each year, tobacco deaths in the United States are estimated to be higher than those from motor vehicle accidents, suicide, fires, homicide, AIDS, alcohol, heroin and cocaine combined [3]. People often commence smoking at the age of 16-17 years, and adolescent males tend to continue smoking for another 16 years and females for 20 years [4]. Every day, approximately 3000 teenagers and children become regular tobacco users [5]. Cigarette smoking is an unnecessary habit that causes significant health and economic problems among smokers and non-smokers. As these problems are better recognized, the movement to ban smoking is gathering momentum [6-7]. Some research studies that have focused on adolescent smoking and its relation to several individual and family backgrounds, have discovered that white people are more likely to engage in smoking in comparison to other racial groups [8-10]. Furthermore, it is more common for non-smokers to come from two-parent families or families in which the parents have higher levels of education [9]. Smoking has been strongly implicated as a risk factor for chronic obstructive pulmonary disease, cancer and atherosclerosis, etc. [11-12]. The World Health Organization predicts that tobacco deaths in India may exceed 1.5 million annually by 2020 [13-14]. The highly toxic chemical in tobacco alkaloids is nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine present in the leaves of Nicotiana tabacum [15-16]. Nicotine is only one of the several thousands of compounds identified in tobacco, many of which contribute to the flavor, aroma, and physiological effects. Nicotine is a tracer for environmental tobacco smoke (ETS) due to the fact that it specifies the tobacco [17]. In addition, it is a chemical that is commonly used as a natural insecticide, as well

as being a highly addictive drug [18]. Assessment of nicotine metabolism and disposition has become an integral part of nicotine dependency treatment programs. Cotinine is the major primary metabolite of nicotine [19-20], and it accumulates in the body with regular smoking. Nicotine and cotinine appear to be metabolized by the same liver enzyme [20]. Nicotine were determined in human body fluids (e.g., urine and blood) using different technique such as GC, TLC, HPLC and spectrophotometry [21-25]. In our study, certain modifications were made for the purpose of the isolation and determination of nicotine in urine in smokers, constituting liquid–liquid extraction with binary solvents [21-23] to get better detection limit, linearity over high range, recovery, and no interference peaks. The extraction method used is more rapid and simple compared with other extraction methods [24]. Another advantage of this method is that it utilizes a single extraction step with 5-10 ml of a solvent mixture. The analyses were developed and validated using UV-Visible spectrophotmeter. This study was aimed to estimate the concentration of nicotine in smokers and nonsmokers' urine samples using UV-Visible spectrophotometer because of its simplicity, availability, cost compared to other methods.

Material and Methods

1. Chemicals and reagents:

All chemicals, analytical standards, reagents, and solvents used throughout this study were analytical grade and highly pure. Nicotine was purchased from (Fluka) with purity of Assay ≥ 99% (for research and development use only). Methanol (SPECTRO) was purchased from (Sigma-Aldrich) with purity 99.9 % Assay (GC). Also, other chemicals and solvents were used including dichlormethane (Riedel-Dehaen AG Seelze Hannover) with purity 99.5 %; potassium dihydrogen phosphate (KH_2PO_4) (Riedel-Dehaen AG Seelze Hannover), with purity 99%); diethylether (Sigma-Aldrich) with purity 99.9 % inhibitor-free; sodium hydroxide (NaOH) from (Riedel-Dehaen AG Seelze Hannover); hydrochloric acid (HCl) (Merck).

2. Preparation of standard solutions:

Standard nicotine solution: 100 mg in 100 ml (1 mg/ml) solution was prepared. After that the desired standards solution were prepared by appropriate dilution of the stock. (5, 10, 15, 20 and 25 µg/ml). The solution of Potassium dihydrogen phosphate (KH_2PO_4): (0.2973 g) of salt was dissolved in one litre or (0.5946 g) of KH_2PO_4 in two litres. This standard solution was used with its pH modified by means of drop wise addition of ortho-phosphoric acid (pH ≈ 3.2). Sodium hydroxide (5M) solution is prepared by dissolving 20 g of NaOH in 100 ml of H_2O to make 5M of the solution. Also 0.25M of hydrochloric acid was prepared.

3. Instrumentation:

The UV-Visible Spectrophotometer system: UV-VIS Spectrophotometer (BECKMAN COULTER DU 800) was used.

4. Standard Solutions (Calibration Curve):

Calibration standards in the range (5-25 µg/ml) were prepared by serial dilution from the stock solution of nicotine and the calibration curve for it as shown in Figure (1). Figure (2) display the spectrums of different concentrations of nicotine (5-25 µg/ml) at 258 nm.

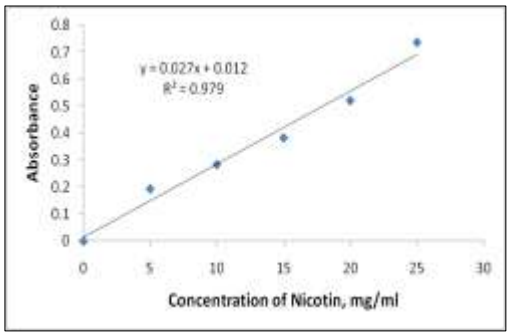

Fig. 1: Calibration curve for standard solutions of nicotine, expressed on a linear scale

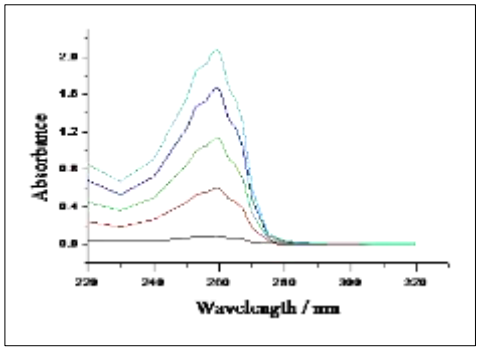

Fig. 2: Spectrums of different concentrations of nicotine by UV-Vis Spectrophotometer.

5. Sample collection:

The samples were collected at the Medical Laboratory of the Clinic of Omar Al-Mukhtar University, El-Beida in Libya. A total of twenty samples were taken, ten from male smokers, eight from male non-smokers and two from female non-smokers. All samples were taken at the same time. The detailed content of each volunteer was tailored according to the answers in their individual questionnaires. The collected data were classified on the basis of smokers' urine (male) and nonsmokers' urine (male/female) and the data are shown in Tables 1 and 2, respectively. Note: all the urine samples were collected and transported immediately to the laboratory and kept at ‾ 80 °C until analysis.

Table 1: Samples collection from male smokers' urine.

S. No.	Age/Year	Smoking Period /year	cigarette brands	Amount smoked/Daily	Time/min
1	40	20	Milano	20	5
2	50	30	Milano	20	5
3	22	15	Eagle	20	15
4	42	20	Onis White	20	10
5	18	8	Platinum	30	15
6	36	17	Oris Blue	20	10
7	48	25	Oris Blue	20	3
8	48	25	D.G	20	3
9	50	30	Onis White	8	60
10	21	14	Oris Blue	2	5

S. No. = Sample Number; Time/min = After Smoking

Table 2: Samples collection from male and female nonsmokers' urine.

S. No.	Volunteer Age /Year	Volunteer Gender
1	32	Female(pregnant)
2	33	Male
3	60	Male
4	22	Male
5	18	Male
6	45	Female
7	13	Male
8	9	Male
9	16	Male
10	21	Male

6. Extraction of Nicotine:

The extraction procedure was carried out according to those described in the literature, with minor modifications [21-23] at room temperature and neutral pH.

Procedure: 200 µl of 5 M NaOH was added to 0.5 ml of the sample and mix rapidly at 2800 rpm for 2 minutes. And 6 ml of dichloromethane-diethyl ether mixture (1:1) was added and mix again at 2800 rpm. The organic layer was separated and put it in the centrifuge at 3000 rpm for 3 minutes, then transfered to a new glass tube containing 40 µl of 0.25M HCl and then evaporate at 35 °C in bath water, and the evaporated fraction was dissolved in 2 ml of solution containing 0.2973 g of KH_2PO_4, 180 ml of methanol and 820 ml of distilled water. The samples were analyzed by UV-Visible Spectrophotometer.

Results and Discussion

This paragraph explains the results obtained in this study, as well as highlighting the efficiency of the methods used, together with the instrumentation. The results indicate that the nicotine level in the smoker's urine were in the range of 0.360 – 2.644 µg/ml with an average of 1.185 µg/ml. These results are shown in table (3), While the nicotine levels in the non-smokers'urine, were in the range of 0.355-2.914 µg/ml with an average of 0.873 µg/ml. These results are shown in table (4). According to the detailed results shown in table (3) and table (4), there was a significant difference in nicotine concentrations between smokers and non-smokers at a 95% confidence level. The nicotine level in nonsmokers' urine samples in some cases even higher than obtained in smokers' urine samples, due to the passive smoking, but the average concentration of nicotine in smokers' urine was greater than that in non-smokers urine. The obtained values of nicotine amounts in our study were within the safe limits [21-25].

Study the stability of nicotine in water and buffer by UV-VIS Spectrophotometer :

We have studied the stability of nicotine in water and in buffer phosphate solution by UV-VIS Spectrophotometer at 258 nm, to make sure the stability of the solpe in the calibartion curve during our measurements and calculations. The table (5 and 6) and the figure (3) show the absorbances at 258 nm of standards solutions of nicotine for five different series of concentrations, from these results we can confirm that the nicotine is stable in buffer more than in water during three days.

Table 3: Concentration of nicotine in male smokers' urine, (n=3)

Sample No.	Age/year	Collection time/min	Concentration of nicotine/ppm (µg/ml) in 0.5 ml of Urine
1	40	5	2.080
2	50	5	1.943
3	22	15	0.444
4	42	10	0.622
5	18	15	1.229
6	36	10	0.893
7	48	3	1.196
8	48	3	2.644
9	50	60	0.441
10	21	5	0.360
		Average	1.185
		SD	0.795

Table 4: Concentration of nicotine in male and female nonsmokers' urine, (n=3).

Sample No.	Age /year	Gender	Concentration of nicotine/ppm (µg/ml) in 0.5 ml of Urine
1	32	Female (pregnant)	0.355
2	33	Male	0.547
3	60	Male	2.914
4	22	Male	1.346
5	18	Male	0.720
6	45	Female	0.442
7	13	Male	0.513
8	9	Male	0.561
9	16	Male	0.736
10	21	Male	0.593
		Average	0.873
		SD	0.767

Table 5: Absorbances at 258 nm of standards solutions of nicotine for different serails of concentrations in water

Days	Absorbance at 258 nm				
	Concentration of Standared Nicotin Solution /ppm				
	5	10	15	20	25
1	0.0819	0.601	1.1389	1.668	2.0871
2	0.5027	1.0177	1.5633	2.091	2.5217
3	0.5183	1.0363	1.5552	2.0545	2.4846

Statistic study:

1. Linearity:

Examination of calibration curves was conducted by computing a linear least-squares regression analysis on the plot of the absorbances of nicotine versus concentration over the range 5-25 µg/ml, with correlation coefficients (R^2) being consistently greater than 0.979. (see table (7)).

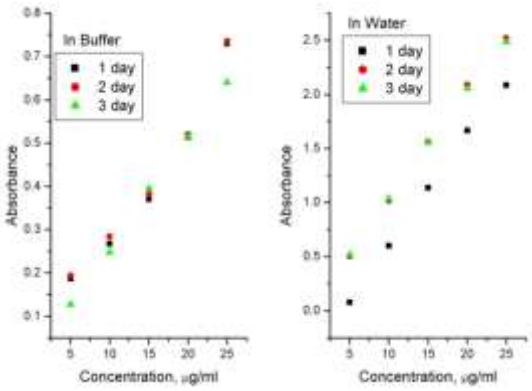

Fig. 3: Stability of different concentrations of nicotine in water and buffer.

Table 6: Absorbances at 258 nm of standards solutions of nicotine for different serails of concentrations in buffer phosphate solution.

Days	Absorbance at 258 nm				
	Concentration of Standared Nicotin Solution				
	5	10	15	20	25
1	0.1870	0.2675	0.3703	0.5145	0.7306
2	0.1936	0.2838	0.3822	0.5207	0.736
3	0.1308	0.2547	0.4048	0.5286	0.6563

Linearity of the technique was appreciated by successive dilutions of high concentration nicotine sample. Limit of detection and quantitation were determined, as well as precision and confidence limit for the mean.

Table 7: Standard solutions of nicotine of different concentrations at 258 nm.

Concentration of Nicotin µg/ml	Absorbance ar 258 nm
5	0.1936
10	0.2838
15	0.3822
20	0.5207
25	0.736
Intercept	0.012
Slope	0.027
Sy/x	0.0465
LOD	0.455
LOQ	1.519
R^2	0.979

2. Limit of detection (LOD) :

Limit of detection (LOD) is defined as the concentration of analyte required to give a signal equal to three times the standard deviation of the blank. The LOD was calculated using the following equation:

$$LOD = \frac{3 \cdot s_{y/x}}{b}$$

where *S* is the average of the standard deviation SDyx of the peak ratio (peak area of analyte/ peak area of external standard), and *b* is the average of the slope of a calibration curve. In the presented study, the limit of detection (LOD) value for nicotine in Urine samples using UV-Visible spectrophotometer was 0.455 µg/ml.

3. Limit of quantitation (LOQ) :

Limit of quantitation (LOQ) is defined as the concentration of analyte required to give a signal equal to ten times the standard deviation of the blank.. The LOD was calculated using the following equation:

$$LOQ = \frac{10 \cdot s_{y/x}}{b}$$

The limit of quantitation (LOQ) value for nicotine in Urine sample by UV-Visible Spectrophotometer was determined to be 1.519 µg/ml.

4. Accuracy and precision :

Accuracy is expressed as percent relative error (% R.E.). Precision is expressed as percent relative standard deviation (% RSD).

Accuracy (% R.E.) = 8.217 % .

Precision (% RSD) = 7.76 % .

5. Confidence Limit (or Interval) for the Mean:

This is the limit (above and below) around x that μ must lie, with a given degree of certainly (or probability or confidence level).

$$x_t = \overline{x} \pm \frac{ts}{\sqrt{n}}$$

In our study, the Confidence Limit for the Mean for nicotine in smokers'urine samples using UV-Visible spectrophotometer was x_t= 1.185 ± 0.0313 ; whereas its value for nicotine in non-smokers'Urine samples was x_t= 0.873 ± 0.0302.

Conclusion

The modified methods used in this study are applicable and reliable for the determination of nicotine in urine using UV-Visible Spectrophotometer. The concentrations of nicotine in Urine were less than expected among a lot of people, but the average of the concentrations of nicotine in the male smokers'Urine samples (1.185 μg/ml) were higher than their concentrations in male and female non-smokers's Urine samples (0.873 μg/ml). The extraction method used in this study provided a high efficiency. This method has good results regarding LOD, LOQ, correlation coefficient, %R.E. and %RSD.

The results obtained in the Figure (3) and table (5 and 6) showed that the nicotine was stable in buffer phosphate other than water during three days.

We advise the other researchers to study other method to examine of nicotine and cotinine concentrations and their metabolisms in serum and urine samples to complete our study.

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