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Voltammetry Detection of Cotinine in Urine Sample

Dissertation submitted in partial fulfillment for the degree of Bachelor of Science (Health) in Forensic Science

Noor Hasfalina Binti Razali

School Of Health Sciences
Universiti Sains Malaysia
Health Campus
16150, Kubang Kerian, Kelantan
Malaysia
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CERTIFICATE

This is certificate that the dissertation entitled

Voltammetry Detection of Cotinine in Urine Sample

Is the bonafide record of research work done by:

NOOR HASFALINA BINTI RAZALI

During the period of 16th December 2007 to 30th April 2008 under my supervision.

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ABSTRACT

Cotinine is a major metabolite of nicotine. Exposure to nicotine is measured by analyzing the cotinine level in the blood, saliva, or urine. Currents methods for determination of cotinine are Gas Chromatography – Mass Spectroscopy (GC-MS) and High Performance Liquid Chromatography (HPLC). In this study, the determination of cotinine in urine sample was performed by Differential Pulse Adsorptive Stripping Analysis (DPAdSV) for the first time.. The urine sample was filtered with filter paper and diluted in 25 mL deionized water. 1 mL sample from the solution was spiked in 9 ml supporting electrolyte, Britton Robinson Buffer pH 7 and determined using optimum parameters such as initial potential, E_i at -1.2, end potential, E_f at -1.82, scan rate at 0.30 V/s, deposition potential 0 V, equilibration time 30 seconds and deposition time 0 V. The validation of methodology was described by obtaining regression equation for its linearity by plotting the calibration curve of peak height, ip versus series concentration of cotinine standard solution. A linear graph with regression equation of $\hat{Y} = 10.922x + 2.066$, correlation coefficient of 0.991, standard deviation of ± 0.938, sensitivity of 10.922 uA/ppm and limit of detection of 0.258 ppm was obtained. 10 smoker urine samples were analyzed. Eight samples including the non-smoker urine sample showed the presence of cotinine in different concentration level with a range of 0.31 ppm to 29.15 ppm. Cotinine is not detected in the two remaining urine samples.

INTRODUCTION

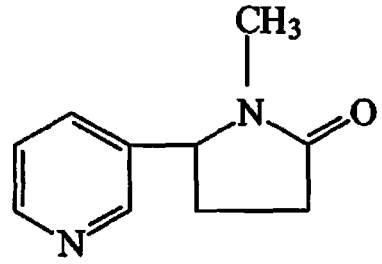
1.1. COTININE

Tobacco smoking is one of the bad habits that present among the people. Many medical and epidemiological studies shown that tobacco smoke have health hazard effect to the direct and passive smokers. Nicotine in tobacco is one of the chemical compounds that give hazardous effect to the smokers. Approximately 86% of the nicotine in human (smoker or non smoker) absorbed from tobacco smoke will metabolize to cotinine. (Piotr Kowalski,Marcin Marszall et al. 2007). Cotinine MedicineNet.com designed that cotinine is major metabolite of nicotine.

In children or non smoker people, exposure to nicotine is measured by analyzing the cotinine level in the blood, saliva, or urine. Its can be determined in different biological fluid in several days after the exposures, because of its long elimination half- life (15 – 20h). Since nicotine is highly specific to tobacco smoke, serum cotinine levels indicates that exposure of tobacco smoke and its toxic constituents. Cotinine assays provide an objective quantitative measure that is more reliable than smoking histories or counting the number of cigarettes smoked per day. (T. Welerowicz, K. Sliwka, B. Buszewski, 2007)

The Centers for Disease Control and Prevention (CDC) identified, the person will expose to cotinine when he or she is smoker or chew tobacco, secondhand tobacco smoke and are involved in tobacco production. (www.cdc.gov/tobacco/)

Cotinine, which it's structure shown in figure 1, is a chemical that made by the body from nicotine, which is found in cigarette smoke. Since cotinine is made only from nicotine, and since it enters the body with cigarette smoke, its measurements can relatively show how much cigarette smoke enters into the body. Nicotine replacement medications produce cotinine just as tobacco does. Substance that remains in body fluids after nicotine has been used, is considered proof of recent nicotine use. It is currently being studied for its possible contribution to a range of oral diseases. Drug tests can detect cotinine in the blood, urine, or saliva.



Cotinine

Figure 1: (S)-1-Methyl-5-(3-pyridinyl)-2-pyrrolidinone.

Cotinine typically remains in the blood between 48 and 96 hours. Nicotine's half-life is about 30 minutes. Timing of sampling relative to the last cigarette smoked may cause for some fluctuation of cotinine levels in steady smokers. For smokers with a fairly constant smoking pattern, cotinine levels reach a steady state, varying only by 15%-20% over the course of the day. Levels should be lowest in the morning as there is typically minimal exposure to

cigarette smoke overnight. Once a sample is collected, metabolism ceases and cotinine levels should not change; however, saliva or urine left at room temperature for extended periods may experience degradation of cotinine due to bacterial contamination (www.fbr.org).

Cotinine in serum, levels <10 ng/mL are considered to be consistent with no active smoking. Values of 10 ng/mL to 100 ng/mL are associated with light smoking or moderate passive exposure, and levels above 300 ng/mL are seen in heavy smokers - more than 20 cigarettes a day. In urine, values between 11 ng/mL and 30 ng/mL may be associated with light smoking or passive exposure, and levels in active smokers typically reach 500 ng/mL or more. The level of cotinine in the blood is proportionate to the amount of exposure to tobacco smoke, so it is a valuable indicator of tobacco smoke exposure, including secondary smoke (www.fbr.gov).

Nicotine is rapidly metabolized and has a short half-life, but cotinine is metabolized and eliminated at a much lower rate. Because of the resulting increase with time in the cotinine to nicotine ratio in the body, including in the brain, it is of interest to examine the effect of cotinine on nicotine-induced changes. Cotinine assays provide an objective quantitative measure that is more reliable than smoking histories or counting the number of cigarettes smoked per day. Cotinine also permits the measurement of exposure to second-hand smoke (passive smoking). Cotinine is not responsible for the lower nicotine clearance observed in smokers. Our data suggest that the pharmacokinetics of low-dose cotinine in nonsmokers do not differ from those of high-dose in smokers, and

therefore cotinine levels can be used quantitatively in environmental tobacco exposure. The longer half-life of cotinine derived from nicotine suggests that slow release of nicotine from tissues is responsible for the apparent long half-life of cotinine in nonsmokers exposed to environmental tobacco smoke. (*Shoshana Zevin, Peyton Jacob III and Neal Benowitz 2001*)

There is some research being done on the effects of cotinine on memory and cognition. Some studies have suggested that cotinine (as well as nicotine) improves memory and prevents neuron death. For this reason it has been studied for effectiveness in treating schizophrenia, Alzheimer's and Parkinson's diseases. There is research, however, which also suggests that nicotine and cotinine contribute to Alzheimer's disease in other ways which counter and maybe even negate the possible positive effects they might have. (*www.wikipedia.com*)

1.2. VOLTAMMETRY

Voltammetric methods of analysis involve the application of a potential (E) to an electrode, and measuring the current (i) that flows through electrochemical cell. The applied potential is varied and usually monitored over a period of time (t). All voltammetric technique is described as some function of E, i and t. they are considered active technique, because the applied potential forces a change in the concentration of an electroactive species at the electrode surface by electrochemically reducing or oxidizing process. One type of electrochemical cell used in voltammetry is the 3-electrode cell. This cell consists of working,

reference and counter electrodes. The potential is applied between the working and reference electrodes, and the current flow is measured between the working and counter electrodes. The working electrode provides the surface for electron transfer to occur for the system under investigation. Many different voltammetric techniques have been developed based on this current-voltage relationship, and have proven to be extremely valuable tools for analyzing trace metals in solutions, determining complexation in organic and inorganic systems, studying kinetics and diffusion.

This method applying a variable potential difference between references electrode (e.g. Ag/AgCl) and a working electrode at which a redox reaction is performed. As the potential at the working electrode reaches a value where the species present in solution is either reduced or oxidized, there is an increase of current in the circuit. To avoid flow of current through the references electrode, a third electrode (auxillary) made of inert material or carbon and a support electrolyte are used to create a conducting medium. Its also can be classified as microanalysis technique which only a small proportion of the solution is ever modified by the process occurring at the electrode

Redox reaction describes all chemical reaction in which atoms have their oxidation number changes. It is comes from two concepts of reduction and oxidation. It can be explained by the examples below:

- Oxidation describes the loss of electrons by molecule, atom or ion
- Reduction describes the loss of electrons by molecule, atom or ion

Advantages of voltammetry according to Metrohm, are

- Can be used to determine components in solution that can be electrochemically oxidized or reduced and also organic, non organic or ions sample.
- Excellent sensitivity low detection limits (ppb or ppt level)
- Low running cost because no intensive laboratory infrastructure
- Alternative and complementary method to AAS/ICP

(Methrom Voltammetry Seminar Book 2007)

1.3. ELECTRODE

1.3.1 WORKING ELECTRODE

The voltammetry strongly influenced by the material of the working electrode. Working electrode is of various geometrics and materials, ranging from small Hg drops to flat Pt disks. The working electrode should provide high to signal to noise characteristics, as well as reproducible response. Thus its selection depends primarily on the redox behavior of the target analyte and the background current over the potential region. Mercury is useful because it displays a wide negative potential range, its surface readily generated by producing the new drop or film, and metal ion can be reversibly reduced into it. The other commonly used electrode materials are gold, platinum and glassy carbon. (Samuel P. Kounaves 2005)

Mercury is very attractive choice material because it has a high hydrogen overvoltage, and possesse highly reproducible, readily renewable, smooth

surface and produces amalgam with metal. There several types of mercury electrode and the most used are dropping mercury electrode (DME), the hanging mercury electrode (HMDE) and the stationary mercury drop electrode (SMDE).

HMDE: Hanging mercury drop electrode is a popular Stationary mercury drops are displaced from a reservoir through a vertical capillary (mechanical extrusion)

SMDE: Stationary mercury drop electrode – uses a valve to obtain a static mercury drop on the capillary tip, which is removed by a drop knocker.

DME: mercury flow by the gravity through the capillary at the steady rate, emerging from its tip as continuously growing drops. Mercury reservoir completely filled with mercury, air eliminated

1.3.2 REFERENCES ELECTRODE

Reference electrode such as Ag/AgCl provides stable reproducible potential, against which potential of working electrode (WE) is compared. Its must provides a reversible half- reaction and be constant over time and be easy to assembles and maintain.

1.3.3 COUNTER ELECTRODE

Counter electrode is used to avoid current flows through the references electrode (Pt). in most voltammetric techniques the analytical reaction at electrode surface occur over very short time periods and rarely produce any appreciable changes in bulk concentrations.

1.4. SUPPORTING ELECTROLYTES

Electrochemical measurements are commonly use medium that consist of solvent containing a supporting electrolyte. The choice of the solvent is based on the solubility of the analyte and its redox activity, and also by its properties. Purpose of the electrolyte are to increase the conductivity, to adjust the pH and obtain the suitable peak, complexes the analyte, decrease the resistance of solution, eliminate alectro migration effects, and maintain a constants ionic strength. The solvents should not react with the analyte and should not undergo the electrochemical reaction over a wide potential range (Joseph Wang 2000)

LITERATURE REVIEW

In forensic cases usually urine was taken as one of the biological sample. In most autopsy cases, analysis for nicotine and cotinine, is helpful for not only determining possibility of nicotine poisoning but also evaluating life style factors and smoking habits. For example, severe depression was reported in people who were nicotine dependant and heavy smoking occurred in schizophrenic patients (Fumio Moriya, Yoshiaki Hashimoto, 2004).

According to T. Welerowicz, K. Sliwka, B. Buszewki (2007), presence of cotinine in urine or biological fluid as a specific, sensitive marker of intake of nicotine as a result of human smoking. Measurement of cotinine enable estimation of amount exposure to tobacco smoke for both smoker and non smoker. Data from the 1988–1991 National Health and Nutrition Examination Survey data found that 87.9% of nonsmokers had detectable concentrations of serum cotinine (Mark D. Kellogg et al 2001). Cotinine has a long half-life, low plasma protein binding and dose independent disposition kinetics - a good marker for estimating both active and passive exposure to tobacco smoke. (Behera Digambar, Uppal Rajan, Majumdar Sidharath 2004).

A simple, sensitive, and rapid gas chromatographic–mass spectrometric method is described for the simultaneous detection and quantitation of nicotine and its metabolite, cotinine, in urine and serum. The detection limit of the assay was 0.16 ng/ml for both nicotine and cotinine. The limit of quantitation for each analyte was 1.25 ng/ ml (Hutchinson James et al 1997). Gas chromatography–

mass spectrometric (GC–MS) quantitative method for nicotine and cotinine levels in the indoor air and in urine was developed, simple and rapid (Monica Culeaa, et. al 2004).

According to Ho-Sang Shin, Jin-Gu Kim, Yoon-Jeong Shin and Sun Ha Jee (2002), gas chromatography – mass spectroscopy is a method which is quite satisfactory for the simultaneous analysis of nicotine and cotinine in various biological samples. The peaks have good chromatographic properties and a very sensitive response for the electron ionization – mass spectrometry (EI-MS). The one-step extraction of these compounds from biological samples also gave relatively high with small variations and a range of method detection limits of 0.2–1.0 ng/ ml. The developed method is simple, convenient, and it also easily used by relatively inexperienced personnel. It was applied to urine, plasma and saliva from smokers or passive smokers. The results from their experiment among 301 non-smoker and 40 smoker was show that through the accurate determination of cotinine in saliva, the risk of lung cancer and heart disease can be predicted.

The GC–MS method presented for determination of nicotine and cotinine concentrations in urine and air is simple and rapid. The method was validated and gave good linearity, precision, accuracy and limit of detection. The method validation gave the following values: 27% RSD precision and 32% RSD for accuracy. Choosing an internal standard of the same chemical class of compounds or better, a stable isotope labelled nicotine could further improve the validation parameters. They found that the urinary cotinine determination seems

to be a more important measure than the air nicotine determination. The time declared for the duration of exposure to nicotine as low, medium or high, or the number of cigarettes smoked indoors, could be sufficient for an estimation of indoor air nicotine. Any association with teeth, hair, blood or saliva cotinine levels could be important especially for Environmental Tobacco Smoke (ETS) exposure studies on children (Monica Culea, Onuc Cozar, Simona Nicoara and Ra˘zvan Podea, 2004)

According to Harald W. A. Teeuwen, Ronald J. W. Aalders & Jacques M. Van Rossum (1988), a rapid and sensitive capillary gas-chromatographic method with nitrogen-sensitive detection has a satisfactory accuracy over the range of concentrations of both amines encountered in active smokers, and has also been successful in the analysis of the urine samples of passive smokers. Its lower limit of sensitivity is 0.2 ng of nicotine and 0.5 ng of cotinine per ml of plasma or saliva or per 100ml of urine. They found that the beneficial characteristics of the presented method were achieved by the combination of solid phase extraction of 0.1-1.0 ml of fluid specimens, capillary column gas chromatography with splitles's injection and nitrogen sensitive detection, and the use of separate, structurally analogous compounds as internal standards for nicotine. The specificity and the sensitivity of the nitrogen-phosphorous detector for compounds containing atoms of these elements are very suitable for an accurate estimation of even small concentrations of nicotine and cotinine in biological fluids.

One-step liquid–liquid extraction (LLE) procedure of the trace nicotine and cotinine in human urine combined with analysis of the extract by gas chromatography–mass spectrometry-selected ion monitoring. A simple and reproducible procedure for the simultaneous analysis of nicotine and its metabolite and a quick turnaround time were targeted (Ho-Sang Shina et al 2001). According to Dwalne A. Machacek and Nal-Slang Jiang (2007), the specific, sensitive method for quantifying it in plasma and saliva is by reversed-phase paired-ion liquid chromatography. Its provides and improved sensitivity and increased sample capacity

Children's Hospital,Boaton developed an assay to detect and quantify cotinine levels in blood plasma. They used High Performance Liquid Chromatography (HPLC) to detect presence of cotinine. Advantages of HPLC are inexpensive, sensitive (low limit of quantitation), and has high throughput capabilities. (Oneil Bhalala, 2003). HPLC assay was used to estimate the cotinine and nicotine levels (Behera Digambar, Uppal Rajan, Majumdar Sidharath, 2004)

Two rapid and popular methods—capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) have been compared for analysis of cotinine in human urine. Cotinine was analyzed in less than 7 min, with detection limits of 5 and 3.2 ng mL) for CE and HPLC, respectively. The performance of the methods was evaluated in terms of sensitivity, specificity, precision, accuracy, and limits of detection and quantification. Calibration plots were linear in the range 50–4,000 ng mL), at least, and mean recoveries were satisfactory

for both techniques. The methods were successfully used for quantification of cotinine in urine (Piotr Kowalski et al. 2007).

New cholesterol-modified adsorbents have been obtained by chemical modification of silica gel of different porosity with cholesterol ligands. Although recovery by this extraction procedure were optimum over a relatively broad range of sample pH (3.1–8.0), analytical conditions such as sample loading, washing and elution conditions, concentration of cotinine to be extracted, and the type of adsorbent used for extraction were found to affect the efficiency of the procedure and had to be controlled for optimum recovery. When these conditions were controlled, recovery of cotinine from spiked human urine was reproducible and depended on compound ionization. Quantitative analysis of cotinine was performed by reversed-phase high-performance liquid chromatography with UV detection (T. Welerowicz, K. Sliwka and B. Buszewki, 2007).

Determination of nicotine in antismoking pharmaceutical product was developed using the simple differential pulse polarography which is simple, reliable and fast. (Atle Hannisdal, et al.2007). The significant advantage of this method is that comprises a liquid-liquid extraction step or simple dilution and filtration step before analysis in the polarography vessel. It is a method of choice for quantitative determination of nicotine in gum, patches and tablets complementary to the chromatographic which is commonly used. It is also shown that a glassy carbon electrode with a mercury film can also be used for the determination of nicotine in antismoking pharmaceutical products.

OBJECTIVES

I. To determine electroanalytical properties of cotinine

II. To detect the presence of cotinine in urine sample of the smoker and non smoker.

III. Study the efficiency of voltammetry technique in determination cotinine.

IV. Quantitative and qualitative measurements of cotinine by voltammetry technique

MATERIALS AND METHODS

1.0. MATERIALS

1.1. Sample source

Smoker and non - smoker urine taken from volunteer student of Universiti Sains Malaysia Kubang Kerian. The urine was put in the sterile plastics bottles and seal and also store in the fridge at -8°C. Only 10 samples were used in this experiment.

1.2. Chemicals and Reagents

Boric Acid (Merck), Orthophosphoric Acid (Merck), Glacial Acetic Acid (Merck), Sodium Hydroxide 2M (Merck), 32% Acid Hydrocholic (Merck), Cotinine - approx 98% (Sigma).

1.3. Apparatus

Voltammetric cell, volumetric flask (1000mL, 100mL, 25mL), Glass pipette (5mL, 10mL), micropipette (20uL, 200uL, and 1mL), beaker (50mL, 100mL). pH meter.

2.0. PREPARATION OF STANDARD AND SAMPLE

2.1. Standard

Cotinine standard solution: 5 ppm. Weight 0.5mg of solid cotinine. Dissolved it and top up with Deionized Water until 100mL. Store in the fridge and renew it

frequently. Then prepare the 1ppm and 100ppb standard solution by diluting the 5 ppm cotinine standard solution.

2.2. Sample

Filter the urine sample using Whatman No.41 filter paper. Dilute urine sample – 1 ml urine in 25 ml deionized water. The sample is ready for analysis.

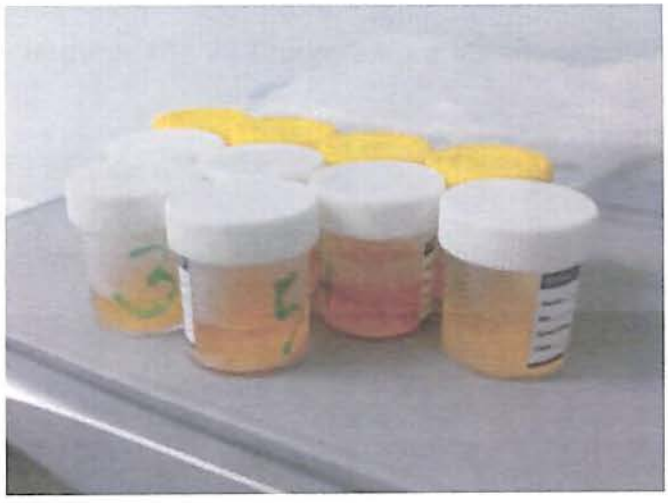


Figure 2: Human Urine Sample

3.0. INSTRUMENTATION

Voltammetry Analyser – VA 757 with VA stand consists of the Multimode Mercury Electrode (MME) as shown in figures 3, 4 and 5. The electrode system consists of:

i. Working Electrode: Hanging Mercury Drop Electrode

ii. Reference Electrode: Ag/AgCl/KCl

iii. Auxillary Electrode: Platinum

Figure 3: Methrom 757 VA Computerance Voltammetric Analyzer

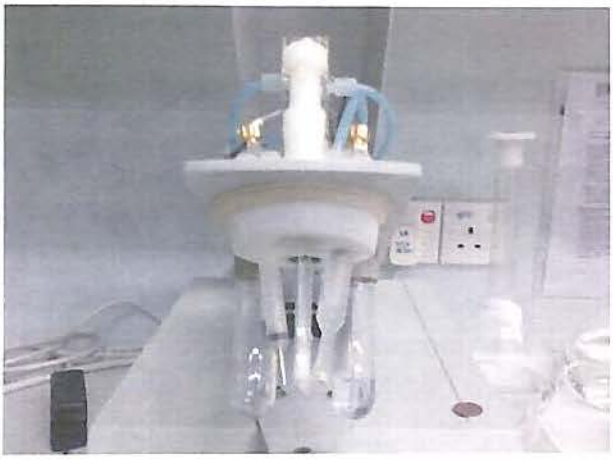


Figure 4: Electrode at Voltametry

Figure 5: Electrode and voltametric cell