Efficacy of screening tests for 11-nor-delta-9-tetrahydrocannabinol -carboxylic acid detection in urine

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Abstract

Context: Cannabis-abused drivers in Thailand were lower prevalence than alcoholic and amphetamine addicts and police officers were often disregarded. Thus, determination of cannabis metabolites by Thai screening test kits was most important driver inspection. However, missing of interpretation on results of cannabis screening test kits were commonly happening, which according by different of immunoassay application and/or procedure. Urine storage condition was affected to screening results and also may affect to results of confirmatory test by gas chromatography/mass spectrometry (GC/MS). **Aims:** To compare efficacy of test kits for 11-nor-delta-9-tetrahydrocannabinol-carboxylic acid (THC-COOH) detection in urine and studied on urine storage condition effects on THC-COOH detection using different test kits and GC/MS for screening and confirmatory method, respectively. **Materials and Methods:** Each standard THC-COOH solution was dissolved in pooled-urine (10–200 ng/ml), which was applied to three commercial test kits. The calculation of sensitivity, specificity, positive predictive value, negative predictive value, and efficiency of test was done. Each pooled-urine containing with THC-COOH (50 ng/ml) was stored in different temperatures and duration prior analysis by test kits and GC/MS. **Results and Discussion:** Three commercial test kits were gave positive results at 50–200 ng/ml of THC-COOH. No false positive result was appeared. THC-COOH in pooled-urine was detected at 25, 4, and −20°C during 1, 7, and 14 days for all test kits. THC-COOH in storage urine at room temperature (25°C) and longest period (14 days) was able to detect by GC-MS, however, in low concentration. **Conclusions:** Research finding was providing useful information for forensic laboratory where perform THC-COOH detection for both screening and confirmatory tests.

Key words: 11-nor-delta-9-carboxytetrahydrocannabinol, cannabinoids, cannabis, immunoassay, screening tests, gas chromatography/mass spectrometry

INTRODUCTION

Cannabis (*Cannabis sativa* L.) was psychoactive substance and used as fiber sources and planting for centuries. Cannabis plants are including hemp and marijuana belongs to family Cannabaceae and genus Cannabis.[1] Cannabis is containing the characteristic chemicals named cannabinoids, which were secretary terpenes in resin constituent by glandular trichomes and majority in flowering tops and bracts. D9-tetrahydrocannabinol (THC) cannabidiol is major cannabinoids for the psychoactive constituent predominating in drug strains and the non-psychoactive constituent predominating in fiber strains, respectively.[1-3] Cannabis is the most broadly worldwide planting and illegally used drug. For long decade, marijuana has been used as a psychoactive substance and hemp had used fiber in rope.[4] Referring of marijuana consuming as an intoxicant in the United State can be found in the favorite literature review since 1850s. The US Federal Bureau of Narcotics began to recorded marijuana as harmful and addictive since 1930s. Marijuana was characterized as a Schedule I

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drug, which was high potential of abuse without any medical recognized or purposed by the Controlled Substances Act in 1970.[4] The cannabis addict numbers are estimated to be as high as 224 million worldwide, and prevalence of use has remained stable in recent years.[5] However, the decriminalization and legalization of certain types of marijuana use have been a highly controversial topic in recently years, which due to be effective treatment for many of medical conditions.[6,7] The level of dose can be predicted mainly effects of a single exposure to marijuana (like most animals experience).[8] When smoking marijuana, THC was rapidly absorbed and oral ingestion was slower absorbed and inconsistently rather than smoking, however, it can produces similar pharmacologic effects.[4,8-11] After cannabis ingestion, the beginning of psychoactive effects is uncertain when compared with smoking. Ingestion of fatty foods can be increased rate of THC oral absorption.[10,11] THC is rapidly absorbed after breathing from the lungs into the blood circulation, which is metabolized by liver enzymes or distributed to adipose tissue, the lungs, and spleen depended on its lipophilic condition.[12,13] THC is oxidized mainly into 11-hydroxy-delta-9-THC in liver and then into 11-nor-delta-9-THC-carboxylic acid (THC-COOH).[13,14] THC-COOH is further rapidly conjugated to glucuronic acid by ester bonding and then formed to THC-COOH-glucuronide. THC is slowly released from the body's lipid-storage compartments, thus, plasmatic terminal half-life of THC is estimated at 1–6 h for rarely users and 20–36 h for chronic users.[13,15-17] THC is gradually excretions in majority of acid metabolite forms, and its main eliminate route is through feces (60–80%); the urinary route is minor excretion (20–35%). The major urinary metabolite is excreted THC-COOH-glucuronide. Only a small amount of free THC-COOH is excreted (<4%), along with only traces of THC. The urinary excretion half-life of THC-COOH was approximated at 30 h[14] and to 3–4 days,[16] depended on conditions of cannabis use. In cannabis cigarette smoker, THC and it metabolites in oral fluid (OF) and urine were unable to estimate the concentration of THC and it metabolites due to large variation, however, positive OF and/or urine THC were indicators of recent cannabis exposure.[18]

Passive exposure to cannabis smoke may induce effects on behavior and psychomotor skills, and have legal consequences, including the risk of being falsely considered as a cannabis user. Differentiation of cannabis smoke exposure may come to be concerned especially in occupational circumstance or when driving vehicles, for enable to discriminate between passive cannabis and active cannabis exposures and to limitation of difference to be detected positive following passive exposure.[13] Specific biomarkers in urine, blood, OF, hair, and sebum for identification of passive exposure were reported. Therefore, positive tests were evaluated in all specimens following extremely high passive exposure, some characteristics were observed in each matrix compared to cannabis active use. THC-COOH urinary level should be detected below the positive threshold used to confirm active smoking of cannabis, especially after normalization to creatinine level. Blood THC and THC-COOH determination were an appropriate alternative

way for evaluate passive exposure as low and very low amounts of THC and THC-COOH contained, respectively. There can be determined in hair, OF, and sweat/sebum emulsion and no THC-COOH should be detected.[13,19,20]

The prevalence of psychoactive drug use among drivers was early reported in developed countries. Most of them attended to high risk drivers, which were involved in a road crash or being suspected of using the substances when driving. On recently literature reviews, common illegal psychoactive substance used among drivers implicated in motor vehicle accidents were cannabis (3.5–27%), cocaine (33%), amphetamine (4.6–14%), opioid (19%), and benzodiazepine (3–12%).[21-24] Synthetic cannabinoid used drivers were more frequently symptoms of nervous impairment, such as confusion, disorientation, and incoherent, slurred speech than drivers who used marijuana.[25] Synthetic cannabinoids were also associated to cause of death, which was confirmed as coronary arterial thrombosis in combination with synthetic cannabinoid use and accident was frequent manner of death.[26]

In Thailand, the prevalence of psychoactive drug and alcohol use among 1635 motor vehicle drivers had been investigated from five geographical areas during 2005–2006.[24] Positive urine samples were 158 (9.7%) for psychoactive drug analysis and the three top of most frequently detected licit drugs were antihistamines (2.0%), sedative cough suppressant (0.7%), and benzodiazepines (0.2%). Illicit drugs detected included amphetamine (1.8%), cannabis (1.1%), mitragynine (Kratom) (0.9%), and morphine (0.1%).[24] According to previous report,[24] we were concerned about cannabis abuse drivers, which lower percent of prevalence than amphetamine, were often disregarded by police officers rather than alcoholic or amphetamine addicted drivers that were more frequent finding. Moreover, suspect cannabis abuse drivers who seize and collected urine for determine cannabis metabolites by screening test. At this point, Thai screening test kits were most important to judged drivers, who are cannabis abuse or not. Missing of result interpretation on cannabis screening test kits may be occurred by different of immunochromatographic application and/or procedure, i.e. card and strip tests. Urine sample storage condition, such as, temperature and duration period may affect to results of screening tests and also may affect to results of confirmatory test or gas chromatography/mass spectrometry (GC/MS). Aims of this research were to compare efficacy of test kits for THC-COOH detection in urine and studied on storage conditions of urine specimen, which were affect to THC-COOH detection using test kits and GC/MS method.

MATERIALS AND METHODS

Instrumentation and Reagents

Instrumentation consisted of an HP 6890 GC with an autosampler (Agilent, Waldbronn, Germany), HP 5973 MS (Agilent), and HP-5 MS capillary column (3 m x 0.25-mm

i.d., 0.25 μm film thickness, Agilent). All solvents and reagents were analytical grade. Methanol, acetone, hexane, ethyl acetate, dichloromethane, isooctane, acetic acid, tetrabutylammonium hydroxide, and dimethyl sulfoxide were purchased from Merck (Darmstadt, Germany), methyl iodide and methyl tert-butyl ether (MTBE) from Sigma-Aldrich (Deisenhofen, Germany) and THC-COOH (standard solution) as well as deuterated compounds from Cerilliant (Austin, TX). Cannabinoid immunoassays or test kits for urine included two of card tests (JSP Pharmaceutical Manufactory, Thailand; Bioline: BRIA LAB, Thailand) and one of strip test (Bioline: BRIA LAB, Thailand).

Urine Collection and Sample Preparation

Ten healthy volunteers (five female and five male) participated in this study. None had a history of cannabis use nor came in contact with hashish, marijuana, or cannabis smoke within the last month before the study. The research program had to pass the approval of Board of Human Research Ethics Committees and all volunteers gave written consent. 100 mL of urine sample was taken from each volunteer (Total volume for all volunteers approximate 1 liter of urine). Pooled-urine sample was refrigerated and transferred to the laboratory for standard sample preparation. THC-COOH standard solution was dissolved in pooled-urine at concentration = 10, 30, 50, 100, and 200 ng/ml, respectively. All THC-COOH standard solution was detected by three test kits and samples contained with 50 ng/ml of THC-COOH was stored at 25, 4, and −20°C during 1, 7, and 14 days prior analysis by screening test kits and GC/MS, respectively.

THC-COOH Detection by Test Kit

Each standard THC-COOH (10, 30, 50, 100, and 200 ng/ml) dissolved in pooled-urine was applied to test kits including two of card tests and one of strip test according by instruction of manufacturers. All of each samples were run in 5-time for each test kit. The appearance of one magenta colored band at control line, two magenta colored bands at control and test lines, and no appearance of magenta colored band at control line were interpreted as positive, negative, and invalid results, respectively. The calculation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of test kits were done by following formulae: % sensitivity = (TP/TP + FN)× 100; % specificity = (TN/TN + FP)× 100; %PPV = (TP/TP + FP)× 100; %NPV = (TP/TP + FP)× 100; % efficiency = (TP + TN/TP + FP + TN + FN)× 100: TP = number of true positive; TN = number of true negative; FP = number of false positive; and FN = number of false negative.

Effects of Storage Condition for THC-COOH Detection

We were selected samples, which contained 50 ng/ml of THC-COOH dissolved in pooled-urine as cutoff point of test kits, were stored different temperatures including 25, 4, and −20°C, which referred to room, refrigerate, and deep freeze temperatures, and different duration including 1, 7, and 14 days prior analysis by screening test kits and GC/MS, respectively. Each 50 ng/ml of THC-COOH sample was applied to each test kits according by instruction of manufacturers same as previous study.

Each urine sample was prepared before injection by GC/MS, which was extracted THC-COOH by liquid-liquid extraction (MTBE:dichloromethane:ethyl acetate, 30:30:40 v/v) and then derivatized with methyl iodide.[20,27] The organic layer was separated, and the solvent evaporated at 30°C in a slight nitrogen stream. Dry residue was dissolved in 20 μL of methanol and then injected to GC/MS system; a HP-5 MS capillary column was used. The carrier gas was He (constant flow: 1 mL/min), the injection volume 1 μL (splitless injection), the injector temperature 250°C, and the transfer line temperature 280°C. The oven temperature program was 2 min isothermally at 60°C, 40°C/min to 170°C, 8°C/min to 270°C, 7.75 min isothermally at 270°C, 30°C/min to 300°C, and 5 min isothermally at 300°C. EI ionization (70 eV) was used, ion source temperature 230°C, and quadrupole temperature 150°C. The following ions (methyl derivative of THCCOOH) were measured in selected ion monitoring (SIM) mode (dwell time per ion: 30 ms): *m/z* 313-target, 341, 357, 372 for THC-COOH (Rt: 18.5 min). Ion ratios and retention time were used as identification criteria for THC-COOH.[20,27] For quantification, the peak areas of the ions specified as "target" were used. Quantification was based on peak-area ratios relative to the respective internal standard. Each sample at same storage condition was extracted and analyzed in triplicate and results were represented as mean ± SD of concentration.[19,20,27]

RESULTS AND DISCUSSION

THC-COOH added pooled-urine samples were positive results [Figure 1] after applied to three test kits, however, there were gave positive results only at concentration 50–200 ng/ml. Negative results [Figure 2] were appeared on concentration of THC-COOH was lower than 50 ng/ml, which were implied that the detection limits of all test kits were cutoff at 50 ng/ml as can be met to manufacturers informed. No false positive result was appeared for all negative control, which applied to three test kits; however, false negative results were happened when concentration of THC-COOH contained samples was lower than 50 ng/ml. Thus, all test kits were unable to detect THC-COOH in lower concentration, which may affected to sensitivity and NPV of all test kits, therefore, specificity and PPV of all test kits were still high as shown in Table 1. We can suggested that Thai test kits had high specificity and sensitivity at cutoff point of test (50 ng/ml). THC-COOH detection in pooled-urine samples (50 ng/ml) were detected at 25, 4, and −20°C during 1, 7, and 14 days by all test kits, which may implied

Table 1: Evaluation of three test kits presented as sensitivity, specificity, PPV, NPV, and efficacy

Parameter/test kit	Card test kit I (%)	Card test kit II (%)	Strip test kit (%)
Sensitivity	60	60	60
Specificity	100	100	100
PPV	100	100	100
NPV	66.7	66.7	66.7
Efficacy	88.3	88.3	88.3

PPV: Positive predictive value, NPV: Negative predictive value

Table 2: Concentration THC-COOH (ng/ml) in different storage condition after detected by GC/MS

Day/ temperature	THC-COOH concentration (ng/ml)		
	25°C	4°C	−20°C
1	2.74±0.13	-	-
7	1.64±0.25	1.96±0.45	3.72±0.53
14	1.56±0.17	1.83±0.34	3.15±0.28

THC-COOH: 11-nor-delta-9-tetrahydrocannabinol-carboxylic acid, GC\MS: Gas chromatography/mass spectrometry

that no effects of storage condition on THC-COOH detection of test kits. However, amount of THC-COOH detection by GC-MS was affected by different urine samples, which was stored at higher temperature (room temperature) and longer period (14 days) by decreased of THC-COOH concentration [Table 2]. Liquid-liquid extraction for sample preparation was also affected to amount of THC-COOH by lower yield of extract, hint, there was limitation of our study that should be prepare urine sample by solid-phase extraction, however, this method was expensive and unable to develop in common laboratory.

These results are comprehensively demonstrate the important role that initial immunoassay screening tests play in determining a presumptive positive followed by a secondary confirmatory method. Immunoassays employing a cutoff concentration of 50 ng/mL, [19,20,24] whereas initial tests with a 20 ng/mL cutoff concentration produced multiple positive results.[24] This is important because some private non-regulated drug testing programs utilize lower initial screening cutoffs (e.g., 20 ng/mL), such as non-smokers exposed to secondhand cannabis smoker,[19,20,24] which was contrasted to our study shown negative test result in concentration of THC-COOH lower than 50 ng/mL. Due to cannabis exposure was excreted THCCOOH in urine primarily as a glucuronide conjugate along with small amounts of free metabolite.[23] The differences of immunoassay are response to specimens containing ≥15 ng/mL of THC-COOH, which were likely due to differences in cross-reactivity with the glucuronide conjugate of THC-COOH.[24] Our study was simulating urine specimen by added THC-COOH to pooled-urine from non-exposure people, thus, in actually of exposure specimen, cannabinoids in urine should be also glucuronide conjugate of THC-COOH and implied cutoff may be lower
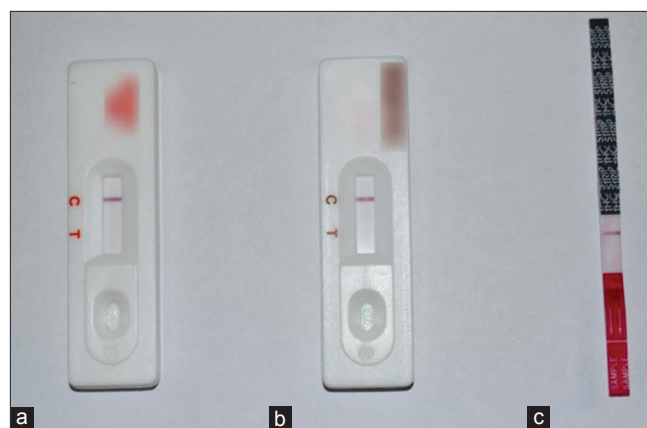

Figure 1: One of magenta colored band at control line interpreted as positive results for gas chromatography/mass spectrometry detection (50 ng/ml) in urine by (a) bioline (card), (b) JSP (card), (c) bioline (strip) screening tests
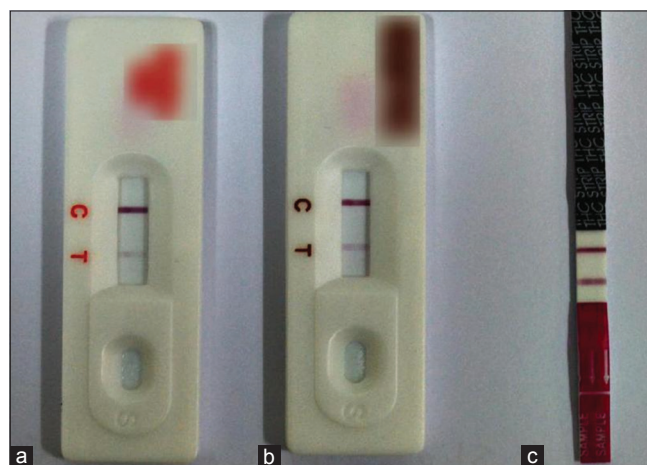

Figure 2: Two magenta colored bands at control and test lines interpreted as negative results for 11-nor-delta-9-tetrahydrocannabinol-carboxylic acid detection (negative control) in urine by (a) bioline (card), (b) JSP (card), (c) bioline (strip) screening tests

than 50 ng/mL as previous reports. However, lack of insert information regarding immunoassay cross-reactivity with the glucuronide conjugate at a 20 ng/mL cutoff concentration in test kit packages. No effects on screening of THC-COOH when urine samples were stored at different temperatures and periods. This results was support that Thai screening tests of cannabinoids, which can useful for police or government

staffs for test urine from suspect person at on site of seize and at station, which take time longer prior perform urine test.[28] In case of GC/MS method, poor results were presented due to low concentration of THC-COOH detection, which may be limitation of our study that uses liquid-liquid extraction for GC/MS rather than use of solid-phase extraction. However, the results of THC-COOH detection by GC/MS were suggested that urine samples for confirmatory test such as GC/MS were need to keep in deep freeze and THC-COOH in urine sample can be detect within 14 days or 2 weeks. Stability of THC and its derivatives in whole blood sample was extended the storage period at −20°C within 5 months by adding preservative mixtures (fluoride citrate and fluoride oxalate) and reducing agents such as ascorbic acid, sodium metabisulfite, and glutathione.[29] Thus, improving of urine storage conditions by adding preservative mixtures and reducing agents can be prolonged THC-COOH and its derivatives in urine for GC/MS analysis and there was interesting topic for further study. In conclusion, our finding may provide useful information for police and/or forensic staffs, as well as, medical technician who perform THC-COOH detection both screening and confirmatory tests.

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